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ORIGINAL ARTICLE

Wilms' tumor protein 1: an early target of progestin regulation in T-47D breast cancer cells that modulates proliferation and differentiation

CE Caldon[1,2], CSL Lee[1], RL Sutherland[1,2] and EA Musgrove[1,2]

[1]*Cancer Research Program, Garvan Institute of Medical Research, Sydney, NSW, Australia and* [2]*St Vincent's Clinical School, Faculty of Medicine, University of NSW, NSW, Australia*

Progesterone regulates the proliferation and differentiation of normal mammary epithelium. In breast cancer cells, progesterone and its synthetic analogs, progestins, induce long-term growth inhibition and differentiation. However, the mechanisms responsible are not fully understood. When T-47D breast cancer cells were treated with the synthetic progestin ORG 2058 (16α-ethoxy-21-hydroxy-19-norpregn-4-en-3,20-dione), all isoforms of Wilms' tumor protein 1 (Wt1) mRNA and protein were rapidly downregulated. We reasoned that the decrease in Wt1 levels may contribute to the long-term antiproliferative and differentiative effects of progestins as proliferation and differentiation are known end points of Wt1 action. Consistent with this idea, Wt1 small interfering RNA led to a decrease in S phase and cyclin D1 levels, and increased Oil-Red-O staining, indicating increased lipogenesis. Conversely, overexpression of Wt1 attenuated the decrease in S phase induced by ORG 2058 at 48–96 h. This was accompanied by the sustained expression of cyclin D1 despite progestin treatment, and increased levels of retinoblastoma (Rb) phosphorylation at sites targeted by cyclin D1-Cdk4 (Ser249/Thr252). Wt1 overexpression also attenuated the ORG 2058-mediated increase in fatty acid synthase levels and reduced lipogenesis. Thus, Wt1 downregulation was sufficient to mimic the effects of progestin and was necessary for complete progestin-mediated proliferative arrest and subsequent differentiation. Furthermore, Wt1 overexpression modulated the effects of progestins but not anti-estrogens or androgens. These results indicate that Wt1 is an important early target of progestins that regulates both proliferation and differentiation in breast cancer cells.
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Correspondence: Associate Professor EA Musgrove, Cancer Research Program, Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst, NSW 2010, Australia.
E-mail: e.musgrove@garvan.org.au
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Introduction

Wilms' tumor protein 1 (Wt1) is a transcription factor important in development and cancer pathogenesis. During development, Wt1 is essential for normal organogenesis of the kidney, gonads, bone marrow, iris and vasculature (Scharnhorst *et al*., 2001). Known targets of Wt1 include genes in differentiation, cell cycle and apoptosis pathways (Lee and Haber, 2001).

Wt1 has generally been recognized as a tumor suppressor due to its role in the pediatric kidney cancer, Wilms' Tumor and other genetic diseases (Lee and Haber, 2001). However, Wt1 is overexpressed in a wide variety of cancers, including leukemia, ovarian cancer, malignant mesothelioma, neural tumors and renal carcinoma (Oji *et al*., 1999; Nakatsuka *et al*., 2006). There are conflicting reports on the role of Wt1 in breast cancer. Initially, Silberstein *et al*. (1997) reported that Wt1 is expressed in normal tissue, but only in two of 21 cancers, and hence is a potential tumor suppressor in breast cancer. By contrast, other groups have found that Wt1 is not expressed in normal mammary cells (Loeb *et al*., 2001), but is overexpressed in 30–75% of breast cancers (Loeb *et al*., 2001; Miyoshi *et al*., 2002; Nakatsuka *et al*., 2006), and that Wt1 overexpression is correlated with poor prognosis (Miyoshi *et al*., 2002). In support of a role for Wt1 as a putative breast oncogene, overexpression of Wt1 enhances proliferation and its downregulation suppresses proliferation in breast cancer cells (Oji *et al*., 1999; Zapata-Benavides *et al*., 2002; Han *et al*., 2004; Tuna *et al*., 2005).

Wt1 has four main isoforms, resulting from two alternative splicing events involving Exon 5 and the end of Exon 9 (the KTS motif). The +KTS and –KTS isoforms are thought to be functionally distinct (Scharnhorst *et al*., 2001). In the kidney, they occur in a defined ratio that is different during development and in adulthood (Haber *et al*., 1991). Isoform ratios may also be altered in cancer: for example, the relative +Exon 5/−KTS (+/−) levels are decreased in sporadic kidney tumors (Baudry *et al*., 2000).

We hypothesized that Wt1 may be regulated by progestins in breast cancer cells, based on evidence for the regulation of Wt1 by other steroid hormones in breast cancer cells (Zapata-Benavides *et al*., 2002), regulation of Wt1 by progesterone in endometrial stromal cells (Anthony *et al*., 2003) and the implication

of Wt1 as a target of Pax2 during progesterone-mediated mammary gland development (Silberstein *et al*., 2002).

During mammary gland development, progesterone regulates a complex array of events including cell proliferation, differentiation and apoptosis (Sutherland *et al*., 1998). Synthetic analogs of progesterone, progestins, are widely used as components of hormone-replacement therapy and contraceptives, and they may either exacerbate or protect against tumor formation, depending on the formulation, context and dose. The potentially deleterious effects of progestins were recently re-emphasized in the Women's Health Initiative Study on hormone-replacement therapy, which documented an increased risk of breast cancer for post-menopausal women treated with progestin and estrogen rather than estrogen alone (Writing Group for the Women's Health Initiative, 2002).

Progestins have a biphasic effect on the proliferation of breast cancer cells in culture, initially stimulating G_1 cells to enter S phase, but the predominant effect is long-term growth inhibition and differentiation (Musgrove *et al*., 1991; Kester *et al*., 1997; Sutherland *et al*., 1998), including lipogenesis (Chambon *et al*., 1989). Progestin treatment inhibits cell cycle progression by decreasing cyclin D1 and cyclin E and the activity of their associated cyclin-dependent kinases (CDKs) (Musgrove *et al*., 1998, 2001), decreasing c-Myc and modulating CDK complex formation through increases in the CDK inhibitors $p18^{INK4C}$, $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ (Groshong *et al*., 1997; Sutherland *et al*., 1998; Swarbrick *et al*., 2000). The downstream effects of progestins on CDK complex formation and activity that lead to growth arrest have thus been well characterized. However, the molecular intermediaries between progestins, the cell cycle machinery and the induction of differentiation have not yet been fully defined. Here we identify a new role for Wt1 as one such intermediary, highlighting its potential oncogenic ability through overexpression disrupting normal control of these fundamental processes.

Results

Expression of Wt1 isoforms and Pax2 in a panel of breast cancer cell lines

Owing to conflicting previous reports of the expression of Wt1 in breast cancer cell lines (compare Loeb *et al*. (2001) and Zapata-Benavides *et al*. (2002)), we initially examined the expression of Wt1 mRNA and protein in a panel of normal breast epithelial cell strains, immortalized breast epithelial cell lines and both estrogen receptor (ER)-positive and -negative breast cancer cell lines.

Wt1 mRNA and protein were not detectable or present at trace levels (relative expression of 0.04 or less) in both finite lifespan mammary epithelial strains (HMEC 184 and HMEC 219-4) and immortalized breast epithelial cell lines (HMEC 184-A1 and HMEC 184-B4) (Figures 1a and b). MCF10A immortalized breast epithelial cells had low levels of Wt1 protein. However, Wt1 was expressed at detectable levels in 10 of 17 breast cancer cell lines, with the highest expression occurring in the ER-positive cell lines T-47D, MDA-MB-468 and MDA-MB-134 (Figures 1a and b).

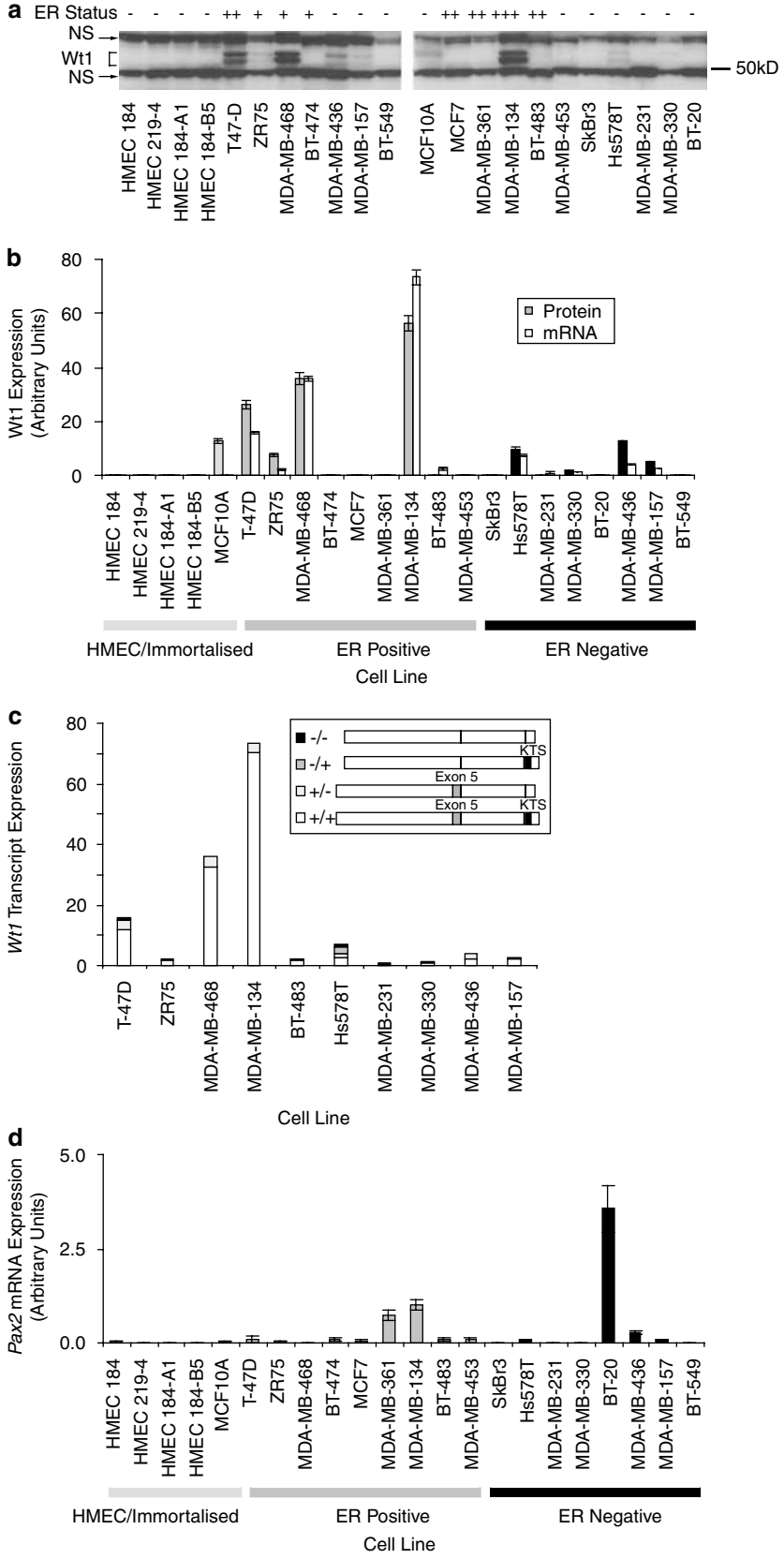
The expression of the four major transcripts of *Wt1* was also examined across the panel of cell lines. The *Wt1* Exon 5+/KTS+ (+/+) transcript was identified as the major transcript in all cell lines, with an average ratio of transcript expression of 7.0 (+/+):1.3 (+/−):1.4 (−/+):0.3 (−/−) across the Wt1-expressing cell lines (Figure 1c). T-47D cells have an average ratio of transcripts of 7.7 (+/+):1.8 (+/−):0.5 (−/+):0.1 (−/−). Despite the predominance of one transcript, the Wt1 protein appears as a doublet, likely reflecting the low levels of other transcripts, post-translational modification and/or use of alternative initiation sites (Scharnhorst *et al*., 2001).

We also examined the expression of the developmental gene *Pax2*, which has been reported to be overexpressed in breast cancer, and has a possible regulatory nexus with Wt1 in progesterone action in the mammary gland (Silberstein *et al*., 2002). However, expression of *Pax2* was rare and not related to ER positivity or Wt1 expression (Figure 1d), arguing against Pax2 as a major upstream regulator of Wt1 in breast cancer cells.

Wt1 is an early target of progestin regulation

The high levels of Wt1 expression in several ER-positive cell lines, and previous publications indicating that Wt1 is steroid-regulated, led us to hypothesize that Wt1 is responsive to progestins in breast cancer cells. In initial experiments, we observed a striking downregulation of Wt1 following treatment with the synthetic progestin ORG 2058 (16α-ethoxy-21-hydroxy-19-norpregn-4-en-3,20-dione). We then measured Wt1 mRNA and protein expression in T-47D human breast cancer cells over 24 h following treatment with ORG 2058. Wt1 levels decreased rapidly after progestin treatment, within 2 h for RNA and 4 h for protein (Figures 2a and b), and continued to decrease through to 24 h, when the protein was barely detectable. Wt1 protein remained undetectable until at least 96 h (Figure 2a). By contrast, the level of cyclin D1 protein, which is one of the major conduits for progestin-mediated cell cycle effects (Musgrove *et al*., 2001), rose at 4–8 h, and then declined to below control levels at 48 h (74% of control levels) continuing through to 72 and 96 h (52% of control levels; Figures 2a and c). This is consistent with previous data demonstrating a biphasic change in cyclin D1 expression preceding the biphasic change in S phase fraction (Musgrove *et al*., 1993, 1998; Groshong *et al*., 1997).

During the course of these studies, progestin regulation of *Wt1* mRNA was confirmed by microarray studies (Bray *et al*., 2005; Graham *et al*., 2005), which additionally showed that *Wt1* is regulated by a range of synthetic progestins (Bray *et al*., 2005). It has also been reported that the isoforms of Wt1 are differentially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BT-549
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MCF7
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MDA-MB-134
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regulated by progesterone in endometrial cells (Anthony *et al*., 2003). However, the ratio of transcripts did not vary significantly in T-47D cells following 8 or 24 h ORG 2058 treatment (average ratio of 8.3 (+/+):1.5 (+/−):0.2 (−/+):0.1 (−/−) in ethanol-treated cells vs 8.9 (+/+):1.0 (+/−):0.1 (−/+):0.0 (−/−) in ORG 2058-treated cells).

siRNA-mediated decrease in Wt1 expression reduces S phase and cyclin D1 expression

The data presented in Figure 2 indicate that Wt1 is markedly downregulated by progestin treatment, and that this substantially precedes downregulation of cyclin D1. Progesterone and progestins regulate a large number of end points in breast epithelial cells, including proliferation, cell–cell interactions, apoptosis and lipogenesis (see Introduction). As Wt1 is a transcription factor with known cell cycle targets, we hypothesized that the early downregulation of Wt1 by progestins may contribute to the growth inhibitory cell cycle effects of progestins. To test this idea, T-47D cells were treated with Wt1 siRNA to mimic the decrease in Wt1 induced by progestin treatment.

From a panel of four chemically synthesized siRNAs, two were selected that significantly decreased Wt1 expression within 24 h. Total *Wt1* mRNA levels were reduced by ~73%, and protein by ~61%, 48 h after siRNA transfection (Figures 3a and b). Treatment of T-47D cells with either Wt1 siRNA decreased S phase by a third, 48 h after siRNA treatment, measured by propidium iodide and 5-bromodeoxyuridine (BrdU) incorporation (Figure 3c). Similar changes were also observed at 24 and 72 h post siRNA treatment (data not shown). By comparison, ORG 2058 elicited a 91% decrease in Wt1 protein levels by 48 h and a 65% decrease in S phase (Figures 3a and b).

Wt1 has been previously reported to have direct or indirect effects on end points that are also regulated by progestins, including c-Myc (Han *et al*., 2004), cyclin D1 (Zapata-Benavides *et al*., 2002), cyclin E (Loeb *et al*., 2002), $p21^{Waf1/Cip1}$ (Englert *et al*., 1997) and Cdk2 activity (Kudoh *et al*., 1995). Investigation of the effect of Wt1 siRNA on these end points identified downregulation

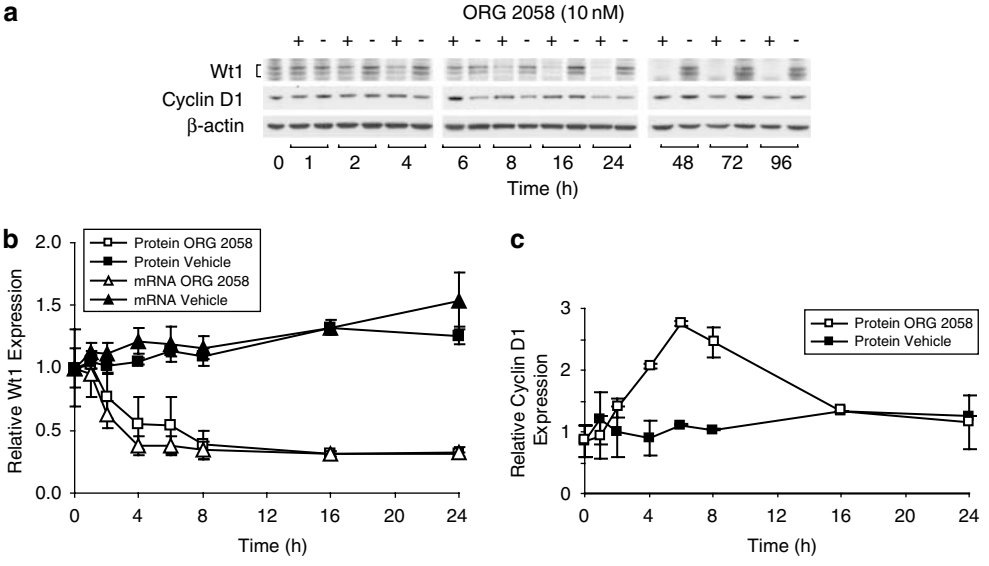

Figure 2 Wt1 is rapidly downregulated in T-47D cells following treatment with progestin (ORG 2058). T-47D cells were treated with 10 nM ORG 2058 or vehicle control and matched protein and RNA lysates were collected at 0, 1, 2, 4, 6, 8 and 24 h. Further protein lysates were collected at 48, 72 and 96 h. Data are representative of two experiments. (**a**) Wt1 and cyclin D1 protein were detected by western blotting. β-Actin was used as a loading control. (**b**) *Wt1* total mRNA was measured by qPCR using *RPLPO* as an endogenous control. Wt1 protein was quantitated by densitometry, and corrected for loading using β-actin. (**c**) Cyclin D1 protein was quantitated by densitometry, and corrected for loading using β-actin. □, Wt1 or D1 protein/ORG 2058; ■, Wt1 or D1 protein/vehicle control; △, Wt1 mRNA/ORG 2058; ▲, Wt1 mRNA/vehicle control.

Figure 1 Wt1 is not expressed in the majority of immortalized or finite lifespan breast cell lines, but is expressed in some breast cancer cell lines. (**a**) Wt1 protein expression in cell lines was analysed by western blotting, and equivalent loading confirmed by Ponceau Red staining of the membrane as well as the expression of nonspecific bands (NS). Estrogen receptor (ER) status of cell lines is as marked (+ to + + +: ER positive; and −: ER negative). (**b**) Western blots of protein samples were quantitated by densitometry. *Wt1* total mRNA was measured by qPCR using *GAPDH* as an endogenous control. *Wt1*-expressing cell lines had an average C_t of 29 cycles. Error bars are s.e.m. of triplicate measurements. (**c**) *Wt1* transcript levels were measured by qPCR using *GAPDH* as an endogenous control. Inset: Representation of Wt1 isoforms. (**d**) Relative levels of *Pax2* transcripts in cell lines were measured by qPCR, using *RPLPO* as an endogenous control. *Pax2*-expressing cell lines had an average C_t of 39 cycles. Error bars are s.e.m. of triplicate measurements.

of cyclin D1 as a response common to ORG 2058 treatment and Wt1 siRNA (Figures 3a and d), and of similar magnitude. Quantitative real-time PCR analysis (qPCR) demonstrated that *cyclin D1* transcripts were also decreased by Wt1 siRNA (Figure 3e). There was a concomitant decrease in the phosphorylation of retinoblastoma (Rb) on the cyclin D1/Cdk4-specific sites Threonine 249/Serine 252 (pRb (249/252)) (Figures 3a and d), similar to the decrease observed with ORG 2058 treatment. By contrast, cyclin E and Cdk2 levels remained constant after Wt1 siRNA treatment, as did $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ levels and cyclin E-Cdk2 activity (Figure 3a). Similarly, Wt1 siRNA did not cause any decrease in the known progestin target c-Myc (not shown). Thus in this context, decreased Wt1 expression appears to be associated with a decrease in S phase by specific downregulation of cyclin D1 and the activity of its associated CDKs.

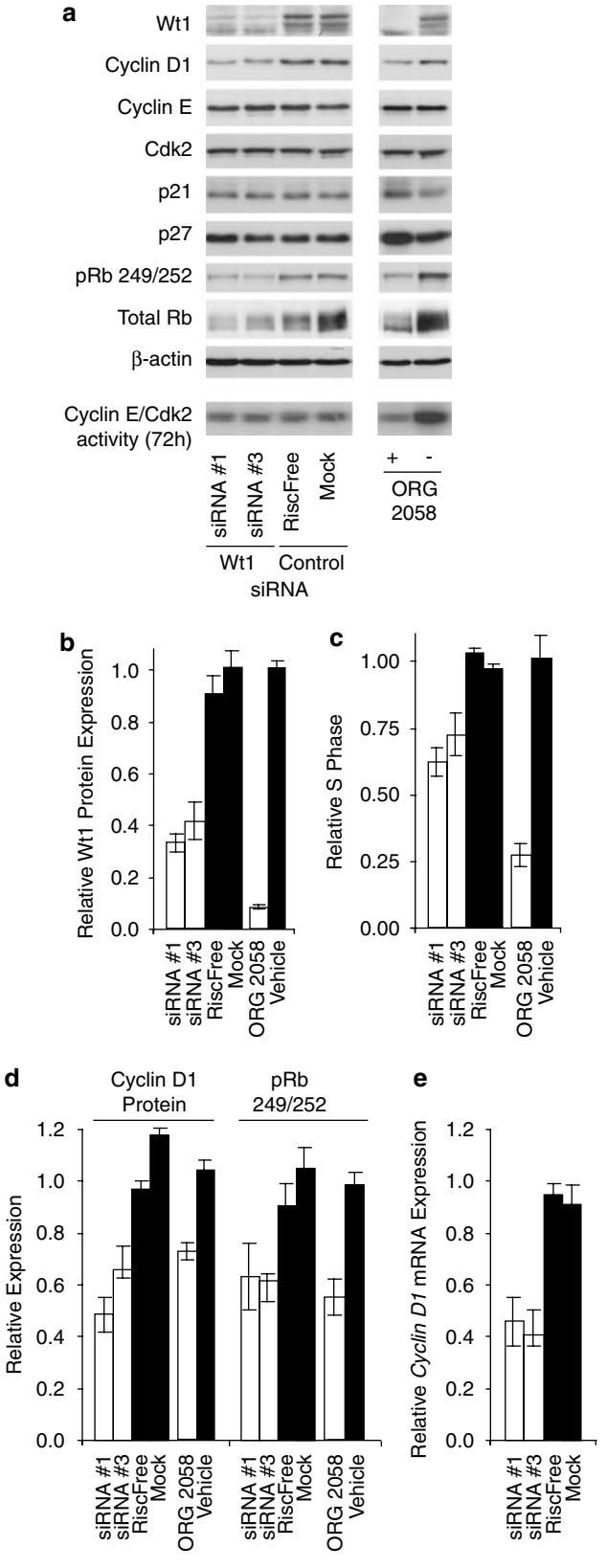

Figure 3 Wt1 siRNA treatment downregulates S phase and cyclin D1. T-47D cells were transfected with Wt1 and control siRNAs and lysates collected 24, 48 and 72 h post transfection. The 48 h time point is shown unless otherwise indicated. Controls are RISC-Free siRNA control (RiscFree) and a mock transfection (Mock). Data are pooled from three independent experiments, except where indicated. Error bars represent s.e.m. (**a**) Wt1 and a panel of cell cycle proteins were detected by immunoblotting 48 h after transfection with Wt1 or control siRNA. For comparison, parallel untransfected cultures were treated with 10 nM ORG 2058 or vehicle control. β-Actin was used as a loading control. The kinase activity of cyclin E immunoprecipitates towards Histone H1 substrate was detected at 72 h post transfection. Data are representative of up to three independent experiments. **(b)** Wt1 protein levels were quantitated by densitometry and normalized for loading using β-actin. (**c**) S phase was determined by quantitating BrdU-positive cells or DNA histograms using flow cytometry. (**d**) Protein levels were quantitated by densitometry. Cyclin D1 levels were normalized for loading using β-actin, and pRb (249/252) normalized to total Rb. (**e**) *Cyclin D1* mRNA levels 72 h post treatment were measured by qPCR using *RPLPO* as an endogenous control.

siRNA-mediated decrease in Wt1 expression increases lipogenesis of T-47D cells

Progestins increase lipid biosynthesis in T-47D cells (Chambon *et al*., 1989), and Wt1 overexpression downregulates lipid metabolism pathways in HEK293 cells (Rae *et al*., 2004). We investigated whether the downregulation of Wt1 by siRNA would induce the accumulation of lipid, using Oil-Red-O, a stain specific to lipids found in intracytoplasmic fat droplets (Ramírez-Zacarías *et al*., 1992). T-47D cells cultured for 5 days in the presence of ORG 2058 showed a marked increase in lipid accumulation in all cells (Figure 4a). Cells treated with Wt1 siRNA in parallel also showed patches of intense lipogenesis, while siRNA control cultures displayed only sparse lipid accumulation (Figure 4a). Lipid accumulation was not observed in every Wt1-siRNA-treated cell, likely reflecting cell-to-cell variation in siRNA uptake. However, T-47D cells treated with Wt1 siRNA for 4 days showed a modest, but statistically significant, increase in levels of *fatty acid synthetase* (*FAS*) mRNA ($P<0.02$; Figure 4b). FAS is an important enzyme in lipogenesis and an indicator of activation of the lipid synthesis pathway in these cells (Horton *et al*., 2002), as well as being a known target of progestins (Chambon *et al*., 1989). We identified siRNA

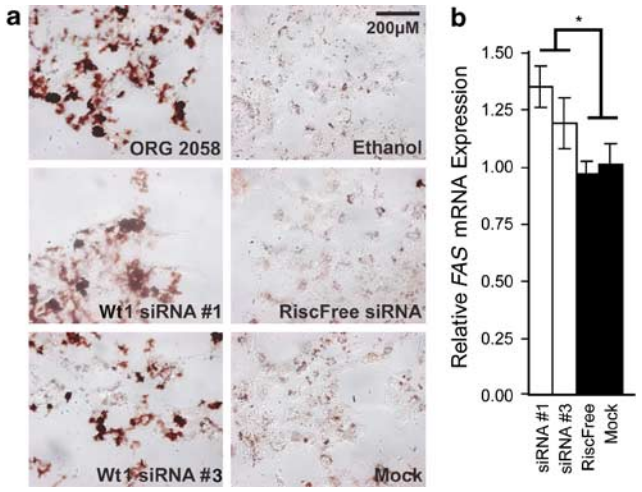

Figure 4 Wt1 siRNA treatment increases lipogenesis. T-47D cells were transfected with Wt1 siRNAs, control siRNA (RISC-free) or mock-transfected for 5 days. For comparison, parallel untransfected cultures were treated with 10 nM ORG 2058 or vehicle control. (**a**) Cells were fixed and stained with Oil-Red-O to identify accumulation of intracellular lipid droplets, and imaged using a phase contrast microscope. Data are from a representative experiment, one of three giving similar results. (**b**) *FAS* mRNA was measured by qPCR using *RPLPO* as an endogenous control. Error bars represent s.e.m., *Represents $P<0.02$.

#1 as the more effective siRNA to ablate Wt1 (Figure 3b), and this was also more effective at inducing *FAS* mRNA ($P<0.001$). Together, these data identify an increase in lipid levels that is associated with decreased Wt1 expression in T-47D cells, implying that the downregulation of Wt1 is sufficient to both induce differentiation and inhibit proliferation.

Progestin-mediated growth arrest is attenuated by overexpression of Wt1

As decreased Wt1 levels mimicked several long-term effects of progestin treatment, we asked whether a decrease in Wt1 was necessary for the growth inhibition and differentiation induced by progestin, by stably overexpressing the major transcript of Wt1 in breast cancer cells, Wt1 (+/+). When a T-47D cell line overexpressing Wt1 (+/+) and a vector control cell line were treated with ORG 2058 or ethanol vehicle over 24–96 h, the overexpressing cells showed an attenuated decrease in S phase (Figure 5a). This corresponded to an overall increase in proliferation of the Wt1-overexpressing population: the cell number of the vector population remained stable after 48–72 h ORG 2058 treatment, while the Wt1-overexpressing population continued to increase in cell number until 72 h post progestin treatment (Figure 5b). In parallel, a decrease in cyclin D1 and pRb (249/252) was observed in response to progestin treatment of vector control cells, which corresponded to the decrease in S phase (Figures 5c and d). By contrast, in Wt1-overexpressing cells, cyclin D1 protein and mRNA did not decrease in response to progestin treatment (Figures 5c and d). The levels of pRb (249/252) in overexpressing cells, while decreased by 58% with addition of progestin, did not undergo the 82% decrease seen in the vector cells treated with progestin (Figures 5c and d). In order to confirm that these observations were not the result of clonal variation, we also tested key end points in pooled populations expressing Wt1. Again, Wt1 expression prevented the progestin-mediated downregulation of cyclin D1 protein (Figure 5c). This coincided with attenuation of the decrease in S phase with progestin treatment; T-47D pcDNA pools decreased to 39% of control S phase following progestin treatment, whereas T-47D Wt1 (+/+) pools only decreased to 56% of control S phase following progestin treatment. Overexpression of Wt1 thus appears to protect cyclin D1 from progestin-induced downregulation, resulting in higher levels of cyclin D1/Cdk4-mediated phosphorylation of Rb, and attenuated downregulation of S phase.

Wt1 overexpression reduces progestin-induced lipogenesis

Wt1-overexpressing cells did not undergo the morphological changes associated with progestin treatment, that is increased surface area and formation of long extracellular processes (Figure 6a). This led us to infer that Wt1-overexpressing cells may not be undergoing the same differentiative processes, including accumulation of lipids. Although the Wt1-overexpressing cells had a twofold higher basal level of Oil-Red-O staining, the vector control cells still accumulated 15% greater absolute levels of Oil-Red-O than Wt1-overexpressing cells after 5 days ORG 2058 treatment. Overall, the vector cells showed an average 10.8-fold increase in lipid accumulation with progestin treatment, compared with a 4.6-fold increase in lipid accumulation in Wt1-overexpressing cells ($P<0.03$, Figure 6b). The Wt1-overexpressing pooled population displayed a moderate attenuation in the accumulation of lipid after ORG 2058 treatment compared with the vector control population (Figure 6b). The more modest effect likely results from the lower level of Wt1 expression compared with the clonal population (see Figure 5c). We corroborated the decrease in lipogenesis in Wt1-overexpressing cells by analysing the mRNA expression of *FAS*. The induction of *FAS* mRNA following ORG 2058 treatment was reduced by ~50% in Wt1-overexpressing cells ($P<0.001$, Figure 6b). Thus, Wt1 overexpression attenuated both the antiproliferative and differentiative effects of progestin treatment.

Wt1 specifically modulates progestin effects on proliferation and lipogenesis, and is not a general effector for steroid hormone action

Wt1 is responsive to multiple steroid hormones in mammary epithelial cells and other steroid-responsive cells (Zaia *et al*., 2001; Zapata-Benavides *et al*., 2002; Anthony *et al*., 2003). This raised the question of whether Wt1 is a general effector of steroid hormone action, or whether the effects that we have identified are specifically associated with progestins. We therefore treated Wt1-overexpressing and vector control T-47D cells with a range of steroid hormones and antagonists,

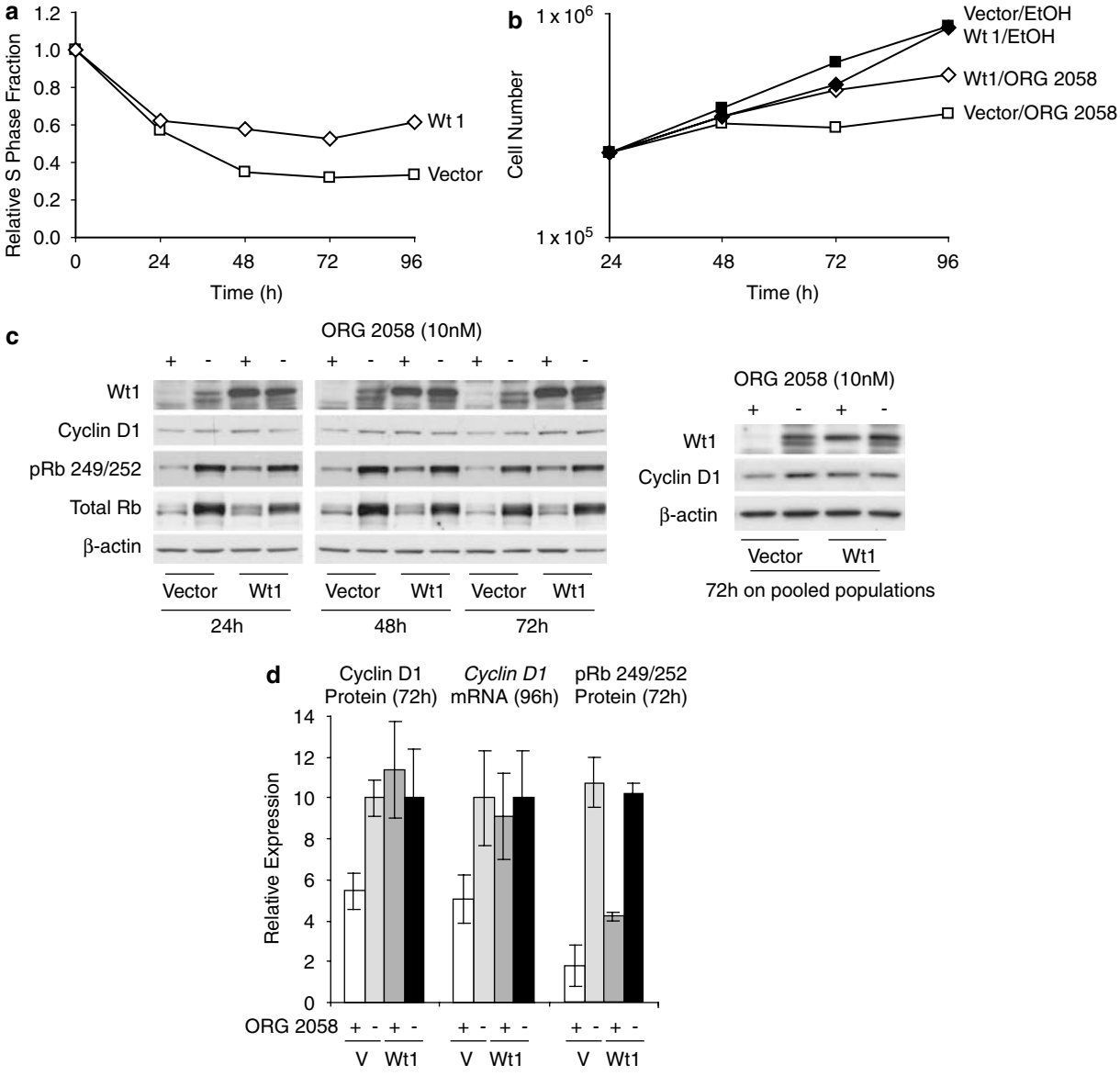

Figure 5 Progestin inhibition of proliferation and cyclin D1 expression is attenuated in T-47D cells overexpressing Wt1. Clonal T-47D cell lines constitutively expressing Wt1 or vector control (pcDNA) were treated with progestin (10 nM ORG 2058) or vehicle (ethanol). Cells were plated 2 days before treatment and harvested following 24, 48, 72 and 96 h of treatment. Data are representative of three separate experiments with similar results. Where indicated, pools of T-47D cells constitutively expressing Wt1 or vector control (pcDNA) were used, and these were harvested after 72 h of treatment. Data are representative of two separate experiments with similar results. (**a**) Cells were harvested for DNA analysis by flow cytometry. The S phase fraction is presented graphically relative to the value in vehicle-treated cultures of each cell line. One representative experiment is shown. (**b**) Cell number was determined by hemacytometer cell counting. The data presented are the means of triplicate cell counts in a representative experiment. Error bars representing s.e.m. are smaller than the symbol. The same representative experiment as in (**a**) is shown. (**c**) Cells were harvested for western blotting, and probed for Wt1, cyclin D1, pRb (249/252), Total Rb and β-actin. (**d**) Protein levels were quantitated by densitometry. Cyclin D1 levels were normalized for loading using β-actin, and pRb (249/252) normalized to total Rb. *Cyclin D1* mRNA levels were measured by qPCR using *RPLPO* as an endogenous control. Error bars represent s.e.m.

and then determined whether Wt1 overexpression attenuated the effect of these hormones on either growth arrest or lipid metabolism.

Wt1-overexpressing cells and vector control cells were treated for 48 h with ORG 2058 (10 nM), the estrogen receptor antagonists 4-hydroxy-tamoxifen (OH-Tam, 1 μM) and ICI 182780 (7α-[9-(4,4,5,5,5- pentafluoropentylsulfinyl) nonyl] estra-1,3,5,(10)-triene-3,17β-diol) (100 nM), the androgen/progestin R1881 (methyltrienolone, 17β-hydroxy-17α-methyl-estra-4,9,11-trien-3-one) (10 nM), the androgen dihydroxytestosterone (5α-androstan-17β-ol-3-one, DHT, 100 nM) and the vehicle control ethanol. In vector control T-47D cells, ORG 2058, OH-Tam, ICI 182780 and R1881 caused a significant

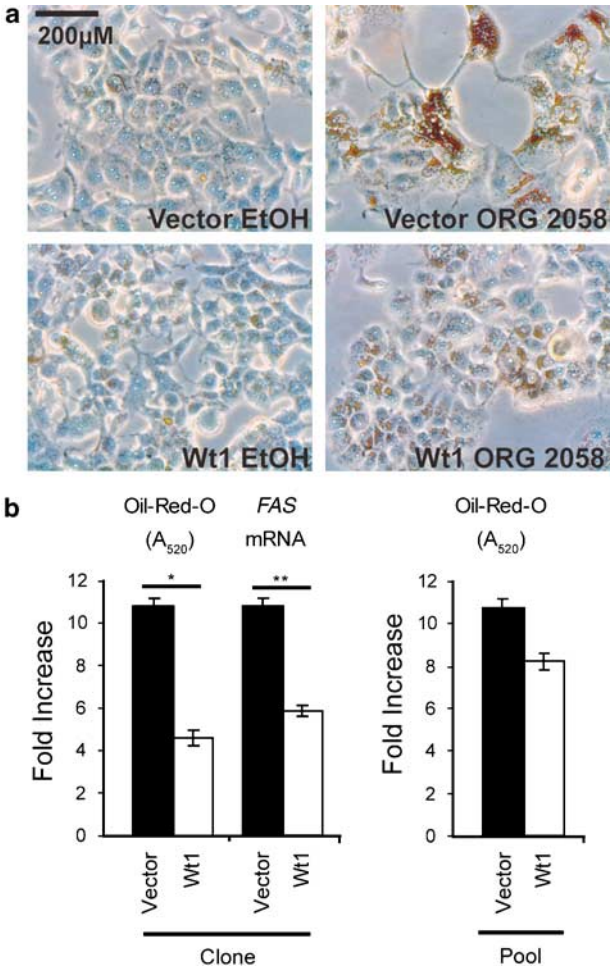

Figure 6 Wt1 overexpression attenuates progestin-mediated lipogenesis. T-47D cells constitutively expressing Wt1 or vector control (pcDNA) were treated with progestin (10 nM ORG 2058) or vehicle control for 5 days. Data are representative of three separate experiments for clonal populations, or two separate experiments for pooled populations. Error bars represent s.e.m. (**a**) Cells were fixed and stained with Oil-Red-O to identify accumulation of intracellular lipid droplets, and imaged using a phase contrast microscope. Data from clonal populations are shown. (**b**) Lipid accumulation was quantitated by spectrophotometric analysis of Oil-Red-O stain. *Indicates $P<0.03$. *FAS* mRNA was measured by qPCR using *RPLPO* as an endogenous control. **Represents $P<0.001$.

decrease in S phase (Figure 7a). With Wt1 overexpression, the decrease in S phase was attenuated in the presence of ORG 2058 and R1881, but not OH-Tam or ICI 182780 (Figure 7a). As R1881 acts both as a progestin and an androgen (Ormandy *et al*., 1992), and the androgen DHT had no effect on S phase in control cells, the ability of Wt1 overexpression to attenuate growth arrest in the presence of R1881 is presumably due to the action of R1881 as a progestin. We further examined the effect of these steroid hormones/antagonists on cyclin D1 expression after 5 days of treatment. ORG 2058, OH-Tam, ICI 182780 and R1881 caused a decrease in cyclin D1 protein and mRNA in vector control cells, however only OH-Tam and ICI 182780 caused a decrease in cyclin D1 protein and mRNA levels in Wt1-overexpressing cells (Figures 7b and c). Thus, the effect of Wt1 on steroid/antagonist-induced growth arrest appears to be specific to progestins, and does not extend to anti-estrogens such as OH-Tam or ICI 182780.

To examine the action of Wt1 in the context of steroid/antagonist-induced lipogenesis, we treated T-47D cells expressing Wt1 and corresponding vector control cells with the same range of steroid hormones and antagonists for 5 days, and then either stained cells with Oil-Red-O for lipid analysis or collected cells for analysis by qPCR. Both ORG 2058 and the androgen/progestin R1881 induced significant accumulation of both lipid and *FAS* mRNA in the control cells (Figures 7d and e). Androgens have been reported to induce lipogenesis in T-47D cells, albeit not to the same extent as progestins (Chambon *et al*., 1989), and accordingly we only observed modest effects of DHT on lipid production or *FAS* mRNA in the vector control cells. ICI 182780 and OH-Tam also either induced marginal lipid and *FAS* mRNA accumulation or had no effect (Figures 7d and e). By contrast, T-47D cells overexpressing Wt1 showed attenuated accumulation of lipid by Oil-Red-O staining and also significantly lower expression of *FAS* mRNA when the cells were treated with either ORG 2058 or R1881 (Figures 7d and e), but not DHT or ICI 182780. Thus, the ability of Wt1 to modulate lipogenesis induced by steroid hormones appears to be confined to the steroids/antagonists with progestin activity and does not encompass androgen-mediated lipogenesis.

Discussion

Progesterone plays an important role in controlling proliferation during mammary development and carcinogenesis. The key downstream effectors of progestin-mediated growth inhibition, such as cyclin D1, have been well characterized. However, little is known about how progestins coordinate cell cycle regulation with their differentiative effects, for example lipogenesis. We have identified that Wt1 can specifically modulate progestin effects on both proliferation and differentiation, suggesting a role for Wt1 in coordinating these processes.

Wt1 is an early target for downregulation by ORG 2058, with decreased *Wt1* mRNA apparent within 2 h. Although increased Wt1 expression can increase the proliferation of breast cancer cells (Oji *et al*., 1999; Zapata-Benavides *et al*., 2002; Tuna *et al*., 2005), unlike other pro-proliferative molecules such as cyclin D1, cyclin E and c-Myc, Wt1 did not undergo a brief increase in expression followed by long-term decrease in expression after ORG 2058 treatment. This implies a specific role for regulation of Wt1 in the long-term progestin inhibition of proliferation, rather than in generalized regulation of cell cycle progression following progestin treatment. This conclusion is supported by the lack of effect of Wt1 overexpression on growth

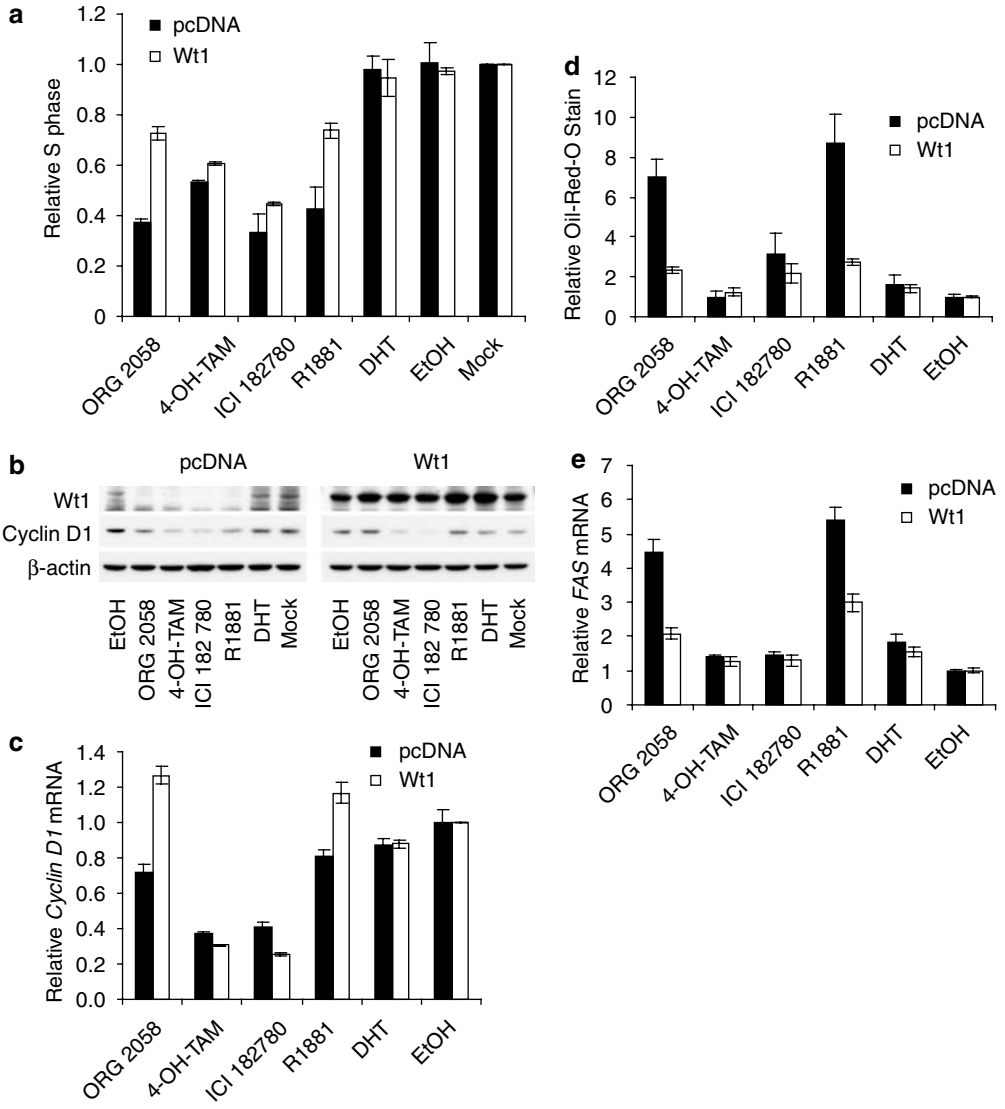

Figure 7 Wt1 specifically modulates the activity of progestins on proliferation and lipogenesis. T-47D cells constitutively expressing Wt1 or vector control (pcDNA) were treated with a range of steroid hormones/antagonists (ORG 2058, 10 nM; 4-OH-tamoxifen (OH-Tam), 1 μM; ICI 182780, 100 nM; R1881, 10 nM; dihydroxytestosterone (DHT), 100 nM, vehicle control (EtOH) or left untreated (Mock)). Data are either pooled or from a representative experiment, one of two giving similar results. Error bars represent s.e.m. (**a**) Cells were treated for 48 h, and harvested for DNA analysis by flow cytometry. The S phase fraction is presented graphically relative to the value in mock-treated cultures of each cell line. (**b**) Cells were harvested for western blotting, and probed for Wt1, cyclin D1 and β-actin. (**c** and **e**) RNA was harvested 5 days following steroid hormone/antagonist treatment, and *cyclin D1* mRNA (**c**) and *FAS* mRNA (**e**) analysed by qPCR using *RPLPO* as an endogenous control. (**d**) Lipid accumulation was quantitated by spectrophotometric analysis of Oil-Red-O stain.

inhibition following anti-estrogen treatment. Our analysis showed that a decrease in Wt1 levels in T-47D cells led to a decrease in cyclin D1 levels and cyclin D1/Cdk4-specific Rb phosphorylation, mimicking responses that are central to progestin inhibition of proliferation (Musgrove *et al*., 1998, 2001). Although Wt1 siRNA and ORG 2058 treatment had effects on cyclin D1 expression that were similar in magnitude, decreased Wt1 expression did not affect the S phase fraction to the same extent as ORG 2058 treatment (Figure 3), and had no effect on cyclin E/Cdk2 expression or activity. Thus, Wt1 downregulation led to regulation of some, but not all, of the multiple known effectors of progestin-mediated growth inhibition, likely accounting for the less-pronounced effects on S phase fraction compared with ORG 2058.

The overexpression of Wt1 resulted in maintenance of endogenous levels of cyclin D1 after ORG 2058 treatment and provided partial protection from progestin-mediated growth inhibition. Cyclin D1 overexpression in T-47D cells confers resistance to progestin-induced growth arrest (Musgrove *et al*., 2001), implicating maintenance of cyclin D1 levels as the mechanism for the reduced progestin sensitivity after Wt1 overexpression. The failure of cyclin D1 maintenance to completely rescue proliferation in the

experiments presented here indicates that endogenous levels of cyclin D1 may not be sufficient to maintain proliferation in the face of ORG 2058 effects on other G_1 CDK complexes. These include cyclin E/Cdk2, which decreases in activity after ORG 2058 treatment even when cyclin D1 is overexpressed (Musgrove *et al*., 2001), and cyclin D3/Cdk4, another known progestin target and potential mediator of growth inhibition (Musgrove *et al*., 1998). Overall, it appears that the effect of Wt1 siRNA and overexpression on cyclin D1 is sufficient to have a significant impact on proliferation, although it is not sufficient to wholly recapitulate or block the effects of progestin treatment.

Several other steroid hormones and antagonists, including anti-estrogens, also cause growth arrest by mechanisms that involve the downregulation of cyclin D1 (Watts *et al*., 1995; Hui *et al*., 2002). As Wt1 levels can be regulated by multiple steroid hormones/antagonists (Zaia *et al*., 2001; Zapata-Benavides *et al*., 2002; Anthony *et al*., 2003), this raised the question of whether Wt1 is a general effector of steroid action on cyclin D1. The action of Wt1, however, was specific to modulation of progestin activity on cyclin D1 and proliferation, as we observed no effect of Wt1 overexpression on the action of anti-estrogens on either S phase or cyclin D1 levels (Figure 7).

The potential role of Wt1 as a positive regulator of cyclin D1 levels and activity in breast cancer cell lines has been suggested in other studies (Zapata-Benavides *et al*., 2002; Tuna *et al*., 2005). Wt1 may prevent the downregulation of cyclin D1 by binding directly to its promoter region: the *cyclin D1* promoter contains Sp-1/Egr1 sites which play a role in its regulation by steroid hormones and mitogens (Castro-Rivera *et al*., 2001), and Wt1 is known to bind to Sp1 and Egr1-like sites in its role as a transcription factor (Lee *et al*., 1999). As the action of Wt1 on cyclin D1 is confined to progestins, it is likely that it modulates a progestin-specific action on the *cyclin D1* promoter. Curiously, Wt1 can also act as a repressor of cyclin D1 and related CDK activities in mouse embryonic fibroblasts (3T3 cells) and monkey kidney fibroblasts (Kudoh *et al*., 1995). This is consistent with the profound tissue specificity exhibited by Wt1, as Wt1 acts to growth arrest cells in certain tissues such as kidney, but promotes proliferation in others such as the retina and myeloid cells (Englert *et al*., 1997; Scharnhorst *et al*., 2001; Wagner *et al*., 2002).

Wt1 also has an important role in regulating lipogenesis in T-47D cells. Reduction in Wt1 levels by siRNA was sufficient to induce lipogenesis and *FAS* mRNA expression, while, conversely, the overexpression of Wt1 inhibited the levels of lipogenic enzymes and lipid accumulation due to progestin treatment by approximately 50%. As progestins directly target a number of regulators of lipogenesis and lipogenic enzymes such as S14, FAS and sterol response element binding protein (SREBP)-1c (Martel *et al*., 2006), it is notable that the downregulation of Wt1 alone was able to stimulate lipogenesis without these supporting progestin-mediated effects. Wt1 overexpression does not, however, attenuate androgen-induced lipogenesis, implying that Wt1 affects progestin-specific control of lipid synthesis.

Two possible mechanisms for the effects of Wt1 on progestin-induced lipogenesis have been identified. First, Wt1 binds to and inhibits SREBPs (Rae *et al*., 2004). As SREBPs transcriptionally regulate genes involved in cholesterol and fatty acid synthesis, this provides a mechanism whereby Wt1 can directly regulate lipogenesis. The same authors observed that the upregulation of Wt1 repressed, and downregulation of Wt1 enhanced, the levels of lipogenic enzymes, consistent with our observations (Rae *et al*., 2004). A second potential mechanism is through the modulation of cyclin D1. Cyclin D1 expression inhibits adipocyte maturation and hence lipid accumulation (Fu *et al*., 2004), and cyclin D1 antisense oligonucleotides induce lipogenic genes in the mammary epithelium *in vivo* (Sakamaki *et al*., 2006). Wt1 may therefore have a coordinating role in progestin regulation of lipogenesis: by simultaneously inhibiting SREBPs and downregulating cyclin D1, Wt1 may synchronize the regulation of these proteins with the direct regulation of other lipogenic proteins by progestin. Importantly, by simultaneously modulating both cyclin D1 and lipogenesis, Wt1 acts to coordinate growth inhibition and differentiation in breast cancer cells, analogous to its role in the developing retina and leukemia cells (Scharnhorst *et al*., 2001; Wagner *et al*., 2002, 2005). The overexpression of Wt1 in breast cancer cells disrupts the coregulation of lipogenic responses and growth inhibition with other targets of progestin, resulting in a less-differentiated and more rapidly proliferating population.

There is an ongoing debate as to whether Wt1 acts as a tumor suppressor or oncogene in carcinogenesis (Loeb and Sukumar, 2002). In this context, it is of significance that the transcript distribution of Wt1 varies considerably between tissues: only 7% of Wt1 transcripts are KTS negative in breast cancer cell lines (Figure 1), whereas in human kidney development, where Wt1 can act as a tumor suppressor in Wilms' tumor, 22% of the transcripts are KTS negative (Haber *et al*., 1991). In mammary epithelial cells, the Wt1 (+/+) isoform promoted a transformed phenotype, whereas overexpression of the Wt1 (−/−) isoform decreased proliferation (Burwell *et al*., 2007). Thus, it is likely that the high relative levels of Wt1 (+KTS) isoforms in breast cancer cell lines contribute to the pro-proliferative effects of Wt1 in breast cancer cells, and consequently to a role as a potential breast oncogene. This does not, however, exclude a role for the other isoforms, as indicated by the recent observation that overexpression of the Wt1 (−/−) isoform may increase motility and invasion in ZR-75 and SKBr3 breast cancer cells (Jomgeow *et al*., 2006). More commonly however, overexpression of the Wt1 (−/−) isoform has been observed to have tumor suppressor effects in mammary epithelial cells (Reizner *et al*., 2005; Burwell *et al*., 2007).

There is now accumulating evidence supporting Wt1 (+/+) as a breast oncogene involved in modulating endocrine responses. First, we have identified high Wt1

expression, especially the Wt1 (+/+) isoform, in a subset of breast cancer cell lines, but not in non-transformed mammary epithelial cells. Nakatsuka *et al*. (2006) have identified Wt1 upregulation in a significant proportion of solid tumors that originate from steroid hormone-regulated tissues, and Wt1 is also regulated by steroid hormones in *in vitro* studies of prostate, breast and endometrial cells (Zaia *et al*., 2001; Zapata-Benavides *et al*., 2002; Anthony *et al*., 2003). As Wt1 is able to provide partial resistance to progestins in breast cancer cells, at least in part via the breast oncogene cyclin D1, the overexpression of Wt1 (+/+) may disrupt the endocrine response in mammary epithelial cells, and contribute to excess proliferation and failure to differentiate during breast oncogenesis.

Materials and methods

Cell lines and cell culture

T-47D cells were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum and insulin (10 μg/ml), except as noted in the text. T-47D (7–2), a clonal T-47D cell line with steroid sensitivity matching that of the parental T-47D cells, has been previously described (Musgrove *et al*., 1994). Wt1-overexpressing cell lines were derived by transfection of pcDNA 3.1 and pcDNA Wt1 (+/+) (generously provided by Dr C Roberts, Oregon Health and Science University, Portland, OR, USA) into T-47D (7–2) cells by Lipofectamine 2000 (Invitrogen, Mt Waverley, Vic., Australia). Pools or clones of stably transfected cells were selected by G418 treatment (400 μg/ml) and Wt1 expression was confirmed by western blotting.

A panel of normal breast epithelial cell strains, immortalized breast epithelial cell lines and breast cancer cell lines were obtained and cultured as previously described (Musgrove *et al*., 1995; Sweeney *et al*., 1998). MCF-10A cells were maintained in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (Invitrogen) supplemented with 5% (v/v) horse serum (Invitrogen), 20 ng/ml human recombinant epidermal growth factor (BioScientific, Gymea, NSW, Australia), 0.5 μg/ml hydrocortisone (Sigma, Castle Hill, NSW, Australia), 100 ng/ml cholera toxin (Sigma), 10 μg/ml bovine insulin (Sigma), 50 U/ml penicillin G (Invitrogen) and 50 μg/ml streptomycin sulfate (Invitrogen). Matched protein and cytoplasmic RNA were harvested from early passage cells proliferating exponentially.

Steroid hormones and antagonists were each dissolved in ethanol at 1000-fold final concentration and added to cells in exponential growth. The following steroid hormones/antagonists were used: ORG 2058 (Amersham Biosciences, Rydelmere, NSW, Australia), R1881 (Sigma), DHT (Sigma), 4-OH-Tamoxifen (trans-4(1-(2-(dimethylamino) ethoxy) phenyl)-2-phenyl-1-butenyl) phenol, Sigma) and ICI 182780 (a kind gift of Dr Alan Wakeling, Astra-Zeneca Pharmaceuticals, Alderly Park, Cheshire, UK). Control cultures received ethanol vehicle to the same final concentration.

RNA extraction and quantitation of mRNA expression

Total RNA or cytoplasmic RNA was extracted using the RNeasy Minikit (Qiagen, Doncaster, Vic., Australia) as described in the manufacturer's instructions. Reverse transcription was performed as instructed on 1 μg of RNA using the Reverse Transcription System (Promega, Annandale, NSW, Australia), with the following amendments. Each reaction was incubated for 60 min at 42°C, before inactivating at 95°C for 5 min. cDNA was diluted to 500 μl with H_2O and stored at −20°C.

qPCR was performed on an Applied Biosystems ABI Prism 7900HT. All reagents and specific Gene Expression Assays were purchased from Applied Biosystems (Scoresby, Vic., Australia). Gene Expression Assays were *cyclin D1* – Hs00277039_m1, *estrogen receptor 1 alpha* – Hs00174860_m1, *FAS* – Hs00188012_m1, *Pax2* – Hs00240858_m1, *Wt1* (all isoforms) – Hs00240913_m1, *Wt1* E5 exon – Hs01104296_m1, *Wt1* KTS exon – Hs01103755_m1, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) – 4326317E and *human large ribosomal protein RPLPO* – 4326314E. For each reaction, 4.5 μl of diluted cDNA was mixed with 5 μl TaqMan Universal PCR Master Mix and 0.5 μl of the Gene Expression Assay. Each reaction was pipetted in triplicate using the EpiMotion 5070 (Eppendorf, North Ryde, NSW, Australia), before amplifying using standard Gene Expression Assay conditions. For *Pax2* analysis, cycle number was extended to 45 cycles to detect rare transcripts. Standard curves of each mRNA were created using serial dilutions of T-47D cDNA, except for *Pax2* analysis, where MDA-MB-134 cDNA was used. Relative expression was determined using the C_t method with the *RPLPO* or *GAPDH* levels as internal controls.

The ratio of Wt1 splice variants was calculated essentially as described in Siehl *et al*. (2004). It was assumed that splicing of the E5 and KTS exons was independent, and the relative C_t values were adjusted for reaction efficiency.

siRNA knockdown of Wt1 in T-47D cells

siRNAs (Wt1 siRNA1 (D-009101-01-0050), Wt1 siRNA3 (D-009101-03-0050) and siCONTROL RISC-Free siRNA (D-001220-01-20)) were purchased from Dharmacon (Lafayette, CO, USA) and transfected at 20 nM using Lipofectamine 2000. Transfection with fluorescinated siRNAs (siGLO RISC-Free siRNA D-001600-01-20) showed that >98% of target cells were transfected.

Western blot analysis and kinase assays

Cell lysates were collected as described previously (Swarbrick *et al*., 2000). Protein lysates (10–30 μg) were separated using NuPage polyacrylamide gels (Invitrogen) and transferred to PVDF membranes. The membranes were incubated with the following primary antibodies: Wt1 (sc-192) and cyclin E (HE12) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); cyclin D1 (DCS6, Novocastra, Newcastle-upon-Tyne, UK); $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ (C24420 and K25020, respectively, BD Transduction Laboratories, Lexington, KY, USA); total Rb (554136, BD Pharmingen, North Ryde, NSW, Australia); pRb-P-Ser249-Thr252 (CA1007, Oncogene Research Products, San Diego, CA, USA) and β-actin (AC-15, Sigma). The secondary antibodies were horseradish peroxidase-conjugated sheep anti-mouse or donkey anti-rabbit (Amersham) and specific proteins were visualized by chemiluminescence (Perkin-Elmer, Rowville, Vic., Australia).

The activity of cyclin E immunoprecipitates (C-19, Santa Cruz Biotechnology) from whole-cell extracts toward a histone H1 substrate was determined as previously described (Swarbrick *et al*., 2000).

Proliferation assays

Proliferation was assessed by flow cytometry using either propidium iodide or BrdU/propidium iodide staining, as previously described (Swarbrick *et al*., 2000; Musgrove *et al*., 2001), or by hemacytometer cell counts.

Oil-Red-O staining

Lipid metabolism was assessed by Oil-Red-O staining of intracytoplasmic lipid droplets, essentially as described (Chambon *et al*., 1989). Oil-Red-O uptake was quantitated by extracting the stain with 100% isopropanol, and measuring spectrophotometrically at 510 nm as previously described (Ramírez-Zacarías *et al*., 1992). Data were normalized using cell counts of cell cultures grown in parallel. Oil-Red-O-stained cells were imaged by rinsing thoroughly with H_2O prior to photography on a phase contrast microscope.

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