ARTICLES

Hypomethylation-linked activation of *PAX2* mediates tamoxifen-stimulated endometrial carcinogenesis

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Tamoxifen, a selective oestrogen receptor modulator, has been used in the treatment of all stages of hormone-responsive breast cancer. However, tamoxifen shows partial oestrogenic activity in the uterus and its use has been associated with an increased incidence of endometrial cancer. The molecular explanation for these observations is not known. Here we show that tamoxifen and oestrogen have distinct but overlapping target gene profiles. Among the overlapping target genes, we identify a paired-box gene, *PAX2*, that is crucially involved in cell proliferation and carcinogenesis in the endometrium. Our experiments show that *PAX2* is activated by oestrogen and tamoxifen in endometrial carcinomas but not in normal endometrium, and that this activation is associated with cancer-linked hypomethylation of the *PAX2* promoter.

The triphenylethylene derivative tamoxifen has been used as the treatment of choice for all stages of hormone-responsive breast cancer and it can prevent breast cancer in high-risk women[1]. However, tamoxifen shows partial oestrogenic effects in other target tissues[1,2]. These partial oestrogenic actions produce beneficial effects on bones and the cardiovascular system in postmenopausal women but are also associated with an increased incidence of endometrial cancer[1,2]. Endometrial carcinoma is the most common gynaecological malignancy in which oestrogen has been identified as a classic aetiological factor[3,4]; clinically, most endometrial cancers are type I oestrogen-dependent endometrioid adenocarcinomas[5].

Despite well-established epidemiological evidence that oestrogen is involved in endometrial carcinogenesis and the well-documented association of tamoxifen with increased incidence of endometrial cancer, the molecular basis underlying this association is not understood. Oestrogen exerts its biological activities by binding to oestrogen receptors (ERs), ERα and ERβ, which function as ligand-dependent transcription factors and regulate target gene transcription. It is thought, on the basis of crystal structures of the hormone-binding domains of ERs bound to ligand[6,7], that tamoxifen acts as an ER antagonist by binding to ERs and by inducing a conformational change that blocks the interaction of ERs with coactivator proteins in the mammary gland. This molecular mechanism is not compatible, however, with the partial oestrogenic activity of tamoxifen in the uterus. Relevant to this, we have shown that tamoxifen-bound ER can interact with a p160 family of coactivators[8], suggesting that tamoxifen may be actively involved in gene regulation in the endometrium.

Genomic action of tamoxifen in endometrium

To understand the genomic basis for the partial agonistic activity of tamoxifen in the endometrium, we first investigated, using Affymetrix human genome arrays, the gene expression profile of endometrial epithelial cells (EECs) immunomagnetically purified from type I endometrioid carcinoma samples (cancerous EECs or cEECs) under treatment with oestrogen and tamoxifen. cEECs from stage I and stage II endometrioid carcinoma samples were pooled separately and cultured. Pilot experiments were done first to determine the optimal duration of treatment, and we found that a 3-h treatment of cEECs with 17β-oestradiol (E2) yielded the greatest coverage (about 75%) of currently known ER target genes[9–12]. Thus, we chose a 3-h treatment for subsequent experiments.

We grew cEECs in the absence of oestrogen and then treated them with E2 or tamoxifen for 3 h or left them untreated (control). Each treatment was done in duplicate, and each replicate was analysed in duplicate arrays. Gene array analysis indicated that oestrogen and tamoxifen regulated the transcription of two sets of genes that had an overlapping but distinct pattern (Fig. 1a). There were 97 genes that were regulated by oestrogen and 114 genes that were regulated by tamoxifen. Among these genes, only 35 were regulated (21 upregulated and 14 downregulated) by both oestrogen and tamoxifen (Supplementary Fig. 2); thus, most of the genes were uniquely regulated by either oestrogen or tamoxifen.

Similar experiments were done in EECs that were immunomagnetically purified from samples of normal endometrium that were age-matched to the cancers (normal EECs or nEECs). The comparison of cEECs with nEECs showed an overall increase in gene expression in cEECs: more genes were upregulated in cEECs by oestrogen (42 versus 33) and tamoxifen (51 versus 35), and fewer genes were downregulated in cEECs by oestrogen (55 versus 62) and tamoxifen (63 versus 70). These experiments clearly show that tamoxifen does not function merely by affecting the transcription of oestrogen target genes. Instead, tamoxifen has its own target genes and these include some of those targeted by oestrogen. Therefore, the genomic view of tamoxifen action should include some of the oestrogen target genes as well as the target genes that are unique to tamoxifen (Supplementary Fig. 2).

We verified the results of the gene array experiments by using real-time polymerase chain reaction with reverse transcription (RT–PCR) to analyse the expression of selected genes in both EEC cultures and

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in rat uterine tissues under treatment with oestrogen or tamoxifen. Six genes, *erythroid Kruppel-like factor* (*EKLF*), *protein kinase Cα* (*PKCα*), *paired-box gene 2* (*PAX2*), *human kidney water channel* (*hKID*), *cyclin F* and *retinoic acid receptor β1* (*RARβ1*), were selected for their functional relevance to cell growth and proliferation and to represent different levels of regulation. Of these genes, *EKLF*, *PKCα* and *PAX2* represented genes that were upregulated by both oestrogen and tamoxifen in EEC cells at high, modest and low levels, respectively; *hKID*, *cyclin F* and *RARβ1* represented genes that were downregulated in these cells at high, modest and low levels, respectively. The expression of *EKLF*, *PKCα* and *PAX2* was increased in cEECs by treatment with tamoxifen or oestrogen, whereas that of *hKID*, *cyclin F* and *RARβ1* was decreased with this treatment (Fig. 1b). The expression pattern of these genes measured by real-time RT–PCR was analogous to that obtained in the gene array analysis.

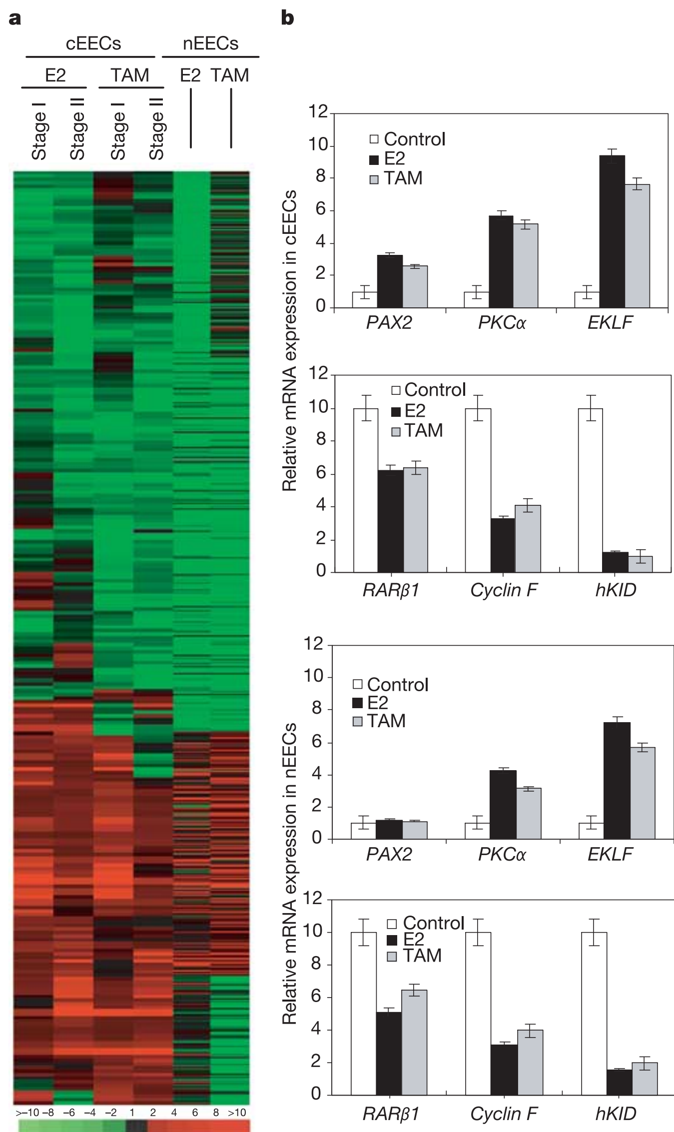

Figure 1 | Genomic view of tamoxifen action. **a**, Clustering of genes regulated by E2 and tamoxifen (TAM) in EECs immunomagnetically purified from stage I and stage II endometrioid carcinoma samples (cEECs) and from samples of normal endometrial epithelium (nEECs). **b**, Real-time RT–PCR verification of the gene array analysis. cEECs and nEECs were untreated (control) or treated with either 100 nM E2 or 5 μM tamoxifen for 3 h. Trizol Reagent was used to extract total RNAs for analysing mRNA expression by real-time RT–PCR. Each bar represents the mean ± s.d. of triplicate experiments.

Similar experiments were done in nEECs. In these cells, the expression patterns of *EKLF*, *PKCα*, *hKID*, *cyclin F* and *RARβ1* were consistent with those observed in cEECs by gene array analysis and real-time RT–PCR analysis, except for *PAX2*, whose expression was not affected in nEECs (Fig. 1b). Similar results were also obtained in rat uterine tissues from ovariectomized adult female CD rats. The proliferative effect of oestrogen and tamoxifen on the endometrium was also confirmed by measuring the wet weight of the treated rat uteri and the thickness of the luminal epithelia, and by immunohistochemical staining of the cell proliferation marker, proliferating cell nuclear antigen, and the epithelial cell marker, Ki-67 (Supplementary Fig. 3).

Molecular effectors of tamoxifen in cancer

Because oestrogen and tamoxifen are both implicated in endometrial carcinogenesis and are both thought to exert their biological activities through binding to ERs, we considered that molecular effectors that mediate the carcinogenic roles of oestrogen and tamoxifen might be among the genes that are commonly regulated by these two molecules. Both gain-of-function and loss-of-function experiments were done on these genes to examine their roles in the proliferation of endometrial carcinoma cells and in the growth stimulation of transplanted endometrial tumours in athymic mice.

First, we reasoned that if the upregulated genes are the effectors of oestrogen and tamoxifen in endometrial cell proliferation, then overexpression of these genes in endometrial carcinoma cells would lead to cell proliferation without stimulation by oestrogen or tamoxifen. Oestrogen-responsive endometrial carcinoma cell lines, ECC-1 (refs 8, 13–15) and Ishikawa[8,16–18], were used in these experiments. These cells were infected individually with retroviruses carrying complementary DNAs for the 21 upregulated genes, and the effect of this overexpression on cell proliferation was analysed by flow cytometry.

Both oestrogen and tamoxifen stimulated the proliferation of ECC-1 and Ishikawa cells infected with retroviruses carrying an empty vector (Fig. 2a). Notably, even without stimulation by oestrogen or tamoxifen, ECC-1 and Ishikawa cells infected with retroviruses carrying *PAX2* also showed significant stimulation of cell proliferation. Cells infected with retroviruses carrying *EKLF* or *PKCα* showed marginal stimulation of cell proliferation, whereas cells infected with retroviruses carrying cDNAs for the remainder of the 21 genes showed minimal effects (data not shown). Overexpression of the genes was confirmed by western blotting when antibodies were available or RT–PCR when antibodies were not available (see Fig. 2b for examples of western blotting for EKLF, PKCα or PAX2). Although the overexpression of these proteins may not represent physiological levels, these experiments indicate that PAX2 could be a key effector in mediating cell proliferation in response to oestrogen and tamoxifen treatment in ECC-1 and Ishikawa cells.

To gain support for this observation, we used retrovirus-delivered short interfering (siRNA) to silence individually the expression of the 21 upregulated genes and then investigated the effect of silencing on cell proliferation by flow cytometry analysis. We reasoned that if a gene functions to mediate the cell proliferative effect of oestrogen and tamoxifen, then a knockdown in the expression of that gene will decrease or diminish the cell proliferative effect of oestrogen or tamoxifen. Whereas silencing of the expression of EKLF or PKCα had marginal effect on the oestrogen- or tamoxifen-stimulated proliferation of ECC-1 and Ishikawa cells, silencing PAX2 expression led to a marked decrease in proliferation (Fig. 2c). The effect of silencing expression of the other genes on the proliferation of ECC-1 cells was minimal (data not shown). In addition, a *PAX2* mutant in which the activation or repression domain was deleted had a dominant-negative effect on the PAX2-mediated proliferation of ECC-1 cells under treatment with oestrogen and tamoxifen, and this effect could be rescued by wild-type PAX2 (Supplementary Fig. 4). Together,

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these findings support a role for PAX2 in mediating the cell proliferative activity of oestrogen and tamoxifen in endometrial cells. The knockdown of gene expression was confirmed by western blotting or RT–PCR, depending on the availability of individual antibodies. Examples of western blotting for EKLF, PAX2 and PKCα are shown in Fig. 2d.

Analogously, gain-of-function and loss-of-function experiments were done with all of the 14 downregulated genes. We reasoned that if downregulation of one of or some of these genes were required for the cell proliferative effect of oestrogen and tamoxifen, then overexpression of these genes would counteract the oestrogen- or tamoxifen-stimulated effect on the proliferation of ECC-1 cells and silencing of the expression of these genes would result in cell proliferation even without stimulation by oestrogen or tamoxifen. However, both gain-of-function and loss-of-function mutation of each of the 14 genes had a minimal effect on oestrogen- or tamoxifen-stimulated cell proliferation (see Supplementary Fig. 5 for examples of experiments with hKID, cyclin F and RARβ1 in ECC-1 cells). These findings suggest that the key factors mediating the cell proliferative effects of oestrogen and tamoxifen are not among the genes that are downregulated by these molecules.

Growth stimulation of ECC-1 tumours by PAX2

To establish further the role of PAX2 in mediating the proliferative effect of oestrogen and tamoxifen and to investigate the possible involvement of PAX2 in endometrial carcinogenesis, we transplanted three types of endometrial tumour developed from ECC-1 cells onto ovariectomized athymic mice (BALB/c; Charles River). The transplanted tumours showed unchanged expression of PAX2 (infected with retroviruses carrying an empty vector), overexpression of PAX2 (infected with retroviruses carrying the *PAX2* gene) or specific knockdown of PAX2 expression (infected with retroviruses carrying an oligonucleotide specific for *PAX2* siRNA).

Tumour growth was monitored in mice that received no treatment or treatment with oestrogen or tamoxifen. In athymic mice that received a transplant of ECC-1 tumours with unchanged PAX2 expression (neither overexpression nor knockdown), both E2 and tamoxifen stimulated tumour growth over 24 weeks: a greater stimulation was observed with E2 treatment, and a lesser effect was observed with tamoxifen treatment (Fig. 3a). In athymic mice that received a transplant of ECC-1 tumours that had PAX2 overexpression, tumour growth was observed even with no treatment, and treatment with oestrogen or tamoxifen further enhanced the ECC-1 tumour growth (Fig. 3b). In athymic mice that received tumour

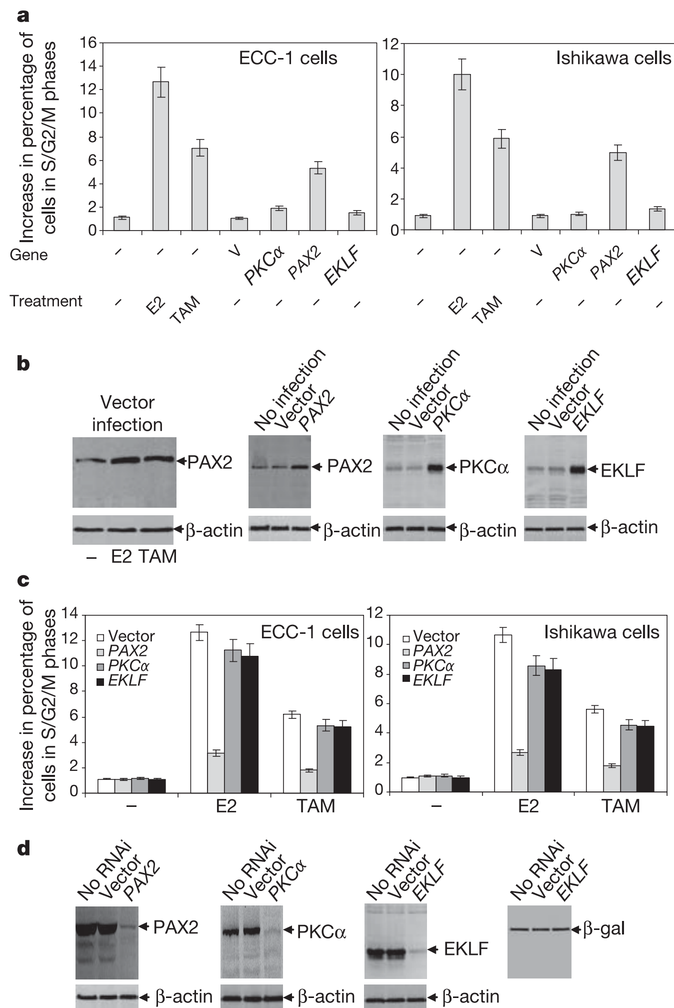

Figure 2 | Effect of *EKLF*, *PKCα* and *PAX2* on the growth of EECs. **a**, **b**, ECC-1 and Ishikawa cells were infected with retroviruses carrying *EKLF*, *PKCα* or *PAX2*. Cell proliferation was analysed by FACS (**a**) and protein expression was examined by western blotting (**b**). **c**, **d**, ECC-1 and Ishikawa cells were infected with retroviruses carrying sequences encoding siRNA specific for *EKLF*, *PKCα* or *PAX2*, and cell proliferation and protein expression were analysed as in **a**, **b**. In **a** and **c**, each bar represents the mean ± s.d. of triplicate experiments.

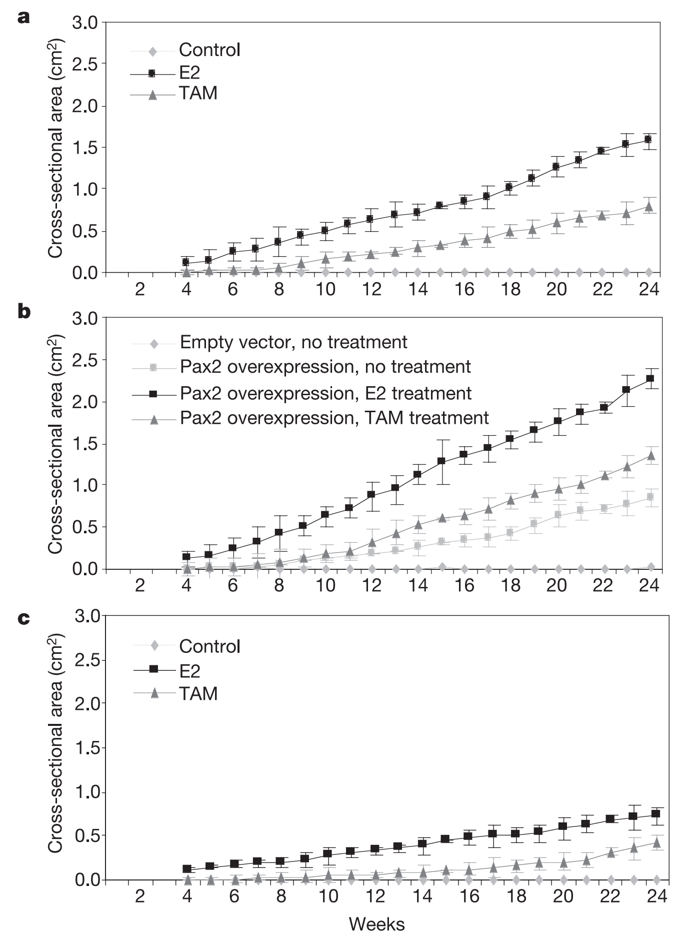

Figure 3 | Effect of PAX2 on the growth of transplanted ECC-1 tumours in nude mice. **a**, No changes in PAX2 expression; **b**, PAX2 overexpression; **c**, PAX2 'knockdown'. ECC-1 cells overexpressing PAX2 or with PAX2-specific knockdown were transplanted onto ovariectomized athymic mice. Tumour growth was monitored after treatment with vehicle, E2 or tamoxifen. ECC-1 cells stably infected by retroviruses carrying an empty vector were used as a control. Tumours were measured weekly with Vernier callipers. The cross-sectional area was calculated using the formula length × width/4 × π; each point represents the mean ± s.d. of triplicate measurements.

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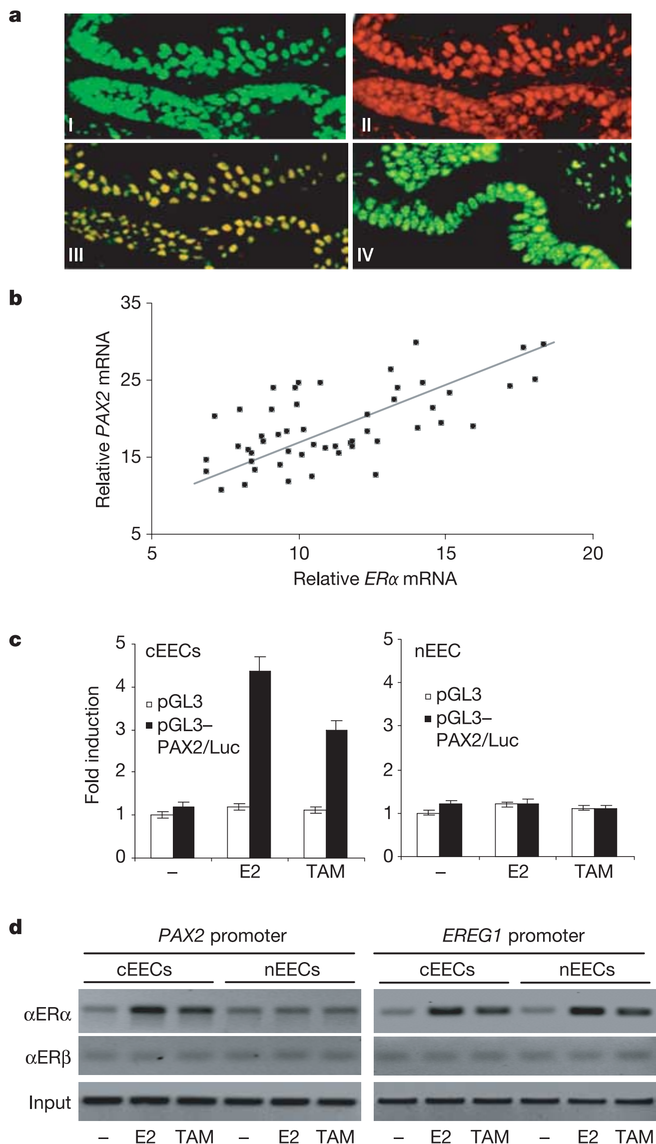

Figure 4 | PAX2 as a downstream target of ERα. **a**, Sections of human endometrial cancer samples were stained for ERα (green, I), PAX2 (red, II) or both (III). Sections adjacent to tumour samples were stained for both ERα and PAX2 (IV). **b**, Real-time RT–PCR analysis of *ERα* and *PAX2* mRNA expression in tumour samples. **c**, Luciferase activity in cEECs (left) and nEECs (right) transfected with a construct encoding luciferase driven by a 2.0-kb upstream regulatory region of *PAX2*. Each bar represents the mean ± s.d. of triplicate experiments. **d**, ChIP analysis of the occupancy of ERs on *PAX2* and *EREG1* promoters.

transplants with PAX2 knockdown, tumour growth stimulation by oestrogen and tamoxifen was greatly attenuated (Fig. 3c).

Similar experiments were done in mice that received transplanted endometrial tumours in which the expression of *EKLF* had been manipulated. Tumour growth in these mice was similar to controls (data not shown). Collectively, these experiments strongly indicate that PAX2 is a key effector of the oestrogen- and tamoxifen-stimulated growth of ECC-1 tumours.

PAX2 as a downstream target of ERα

To support further the observation that *PAX2* is a downstream effector of oestrogen and tamoxifen in stimulating the growth of endometrial cells, we analysed the expression of ERα and PAX2 in the 53 ER-positive endometrioid carcinoma samples used in microarray analysis. The expression patterns of ERα and PAX2 proteins were determined with dual immunofluorescence staining, and the expression levels of *ERα* and *PAX2* messenger RNAs were measured by real-time RT–PCR in these samples. The pattern of *ERα* expression was well correlated with the expression of *PAX2* in endometrial cancer tissue (Fig. 4a). In real-time RT–PCR experiments, the relative level of *ERα* expression was plotted against that of *PAX2* expression (Fig. 4b). Statistical analysis with the SAS system found a Spearman correlation coefficient of 0.71223 ($P < 0.0001$) and a Kendall Taub correlation coefficient of 0.62345 ($P < 0.0001$), further indicating a strong correlation between the expression of *ERα* and the expression of *PAX2* in ER-positive endometrial carcinomas and supporting the idea that *PAX2* is a downstream target of ERα in endometrial cells.

Examination of the *PAX2* promoter did not reveal a typical oestrogen response element. However, the upstream regulatory region of *PAX2* does contain numerous *cis*-elements for binding transcription factors such as Sp1 and NF-κB[19], which are implicated in indirect binding to ER[8,20–23]. To gain support for the notion that *PAX2* is a direct downstream target of ERs, we investigated the effect of oestrogen and tamoxifen stimulation on the activation of luciferase driven by the *PAX2* promoter by using a plasmid construct carrying a ~2.0-kb fragment of the *PAX2* promoter plus the first exon[19]. Both oestrogen and tamoxifen could indeed stimulate luciferase gene expression, but only in cancerous EECs and not in normal EECs (Fig. 4c). Furthermore, chromatin immunoprecipitation (ChIP) assays detected the presence of ERα but not ERβ on the *PAX2* upstream regulatory region after treatment with E2 or tamoxifen in cEECs but not in nEECs (Fig. 4d). The ChIP results were confirmed by real-time PCR quantification (Supplementary Fig. 6). Collectively, these findings support the idea that *PAX2* is a downstream target of oestrogen and tamoxifen in the endometrium.

PAX2 promoter hypomethylation

To understand why *PAX2* is activated in cancerous EECs and cancer cell lines but not in normal endometrium, we examined the methylation status of *PAX2* promoter by methylation-specific PCR in the 53 type I endometrioid carcinoma samples and in the 19 samples from normal endometrium. We found that the *PAX2* promoter was hypermethylated in normal endometrium, but over 75% of the carcinoma samples (40 of 53) showed *PAX2* promoter hypomethylation (Fig. 5a). These results were confirmed by bisulphite DNA sequencing (Fig. 5b). Furthermore, treatment of nEECs with the methyltransferase inhibitor 5-aza-deoxycytidine (5-aza-dC) led to *PAX2* reactivation, ERα recruitment and PAX protein expression (Fig. 5c). Together, these results suggest that the reactivation of *PAX2* expression in cancerous endometrial cells is associated with loss of the methylation mark in the *PAX2* promoter.

Finally, we investigated the possible mechanism underlying the alteration in *PAX2* methylation in cancerous versus normal endometrial cells. It is thought that methylated CpG may be bound by methyl-CpG binding proteins (MeCPs), which in turn are associated with transcription repression complexes containing mSin3A and histone deacetylase (HDAC)[24]. Thus, we examined the association of MeCP2, mSin3A and HDAC1 with the *PAX2* upstream regulatory region in nEECs and cEECs by ChIP. We found that MeCP2, mSin3A and HDAC1 were present in the *PAX2* upstream regulatory region in nEECs but not in cEECs (Fig. 5d), suggesting that loss of the methylation mark in *PAX2* in cEECs is linked to loss of the association of a protein complex containing MeCP2, mSin3A and HDAC1.

Discussion

Biochemical and animal experiments[6,14,16,17,25–33], as well as genetic studies[34–37], strongly suggest that an ER-dependent pathway implicated in gene transcriptional regulation underlies the mechanism of tamoxifen action in the uterus. We have shown not only that tamoxifen regulates gene transcription in endometrial epithelial

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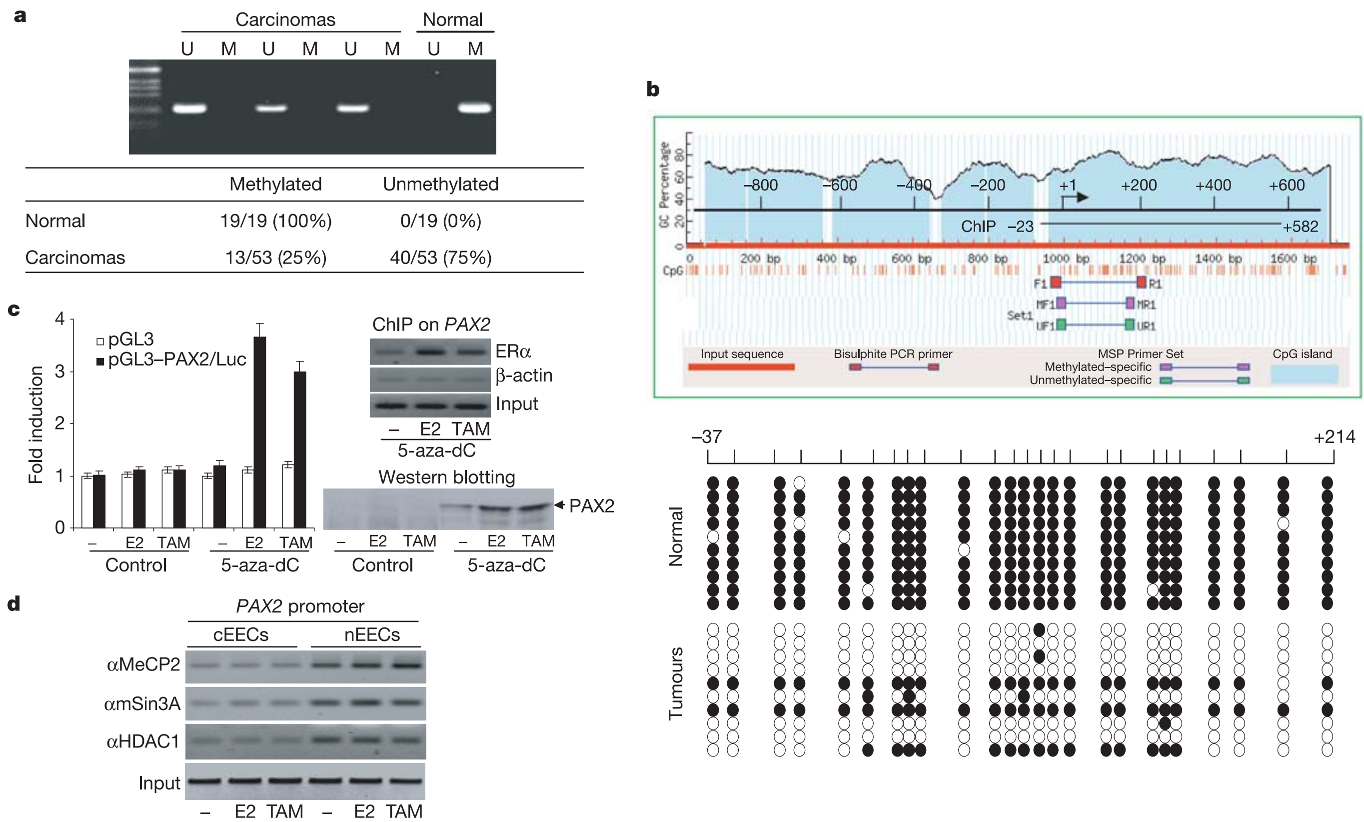

Figure 5 | Cancer-linked hypomethylation of the *PAX2* promoter. **a**, Methylation-specific PCR analysis of the *PAX2* upstream regulatory region in endometrial carcinomas and in normal endometrium. M indicates hypermethylated *PAX2*; U indicates unmethylated *PAX2*. **b**, Results of bisulfite DNA sequencing of the *PAX2* upstream regulatory region. **c**, Normal EECs were treated with 5-aza-2′-deoxycytidine and then transfected with a construct encoding luciferase driven by the upstream regulatory region of *PAX2*. Cells were assayed for luciferase activity, ERα recruitment and PAX2 expression. Each bar represents the mean ± s.d. for triplicate experiments. **d**, MeCP2, mSin3A and HDAC1 are associated with the *PAX2* upstream regulatory region in nEECs but not in cEECs, as measured by ChIP assays.

cells, but also that the genes targeted by tamoxifen are largely different from those targeted by oestrogen. Our experiments indicate that gene transcriptional regulation may dictate the role of tamoxifen in endometrial carcinogenesis. Our observations also indicate that tamoxifen is a compound with distinct genomic activity and is not simply a partial ER agonist as traditionally described.

We have shown that *PAX2* is a common target of oestrogen- and tamoxifen-bound ERα and can promote, both *in vitro* and *in vivo*, the growth of endometrial cancer cells. Typically, *PAX2* expression accompanies high rates of cell division[38–40]. *PAX2* is expressed in Wilms tumour[41], a childhood renal tumour of embryonic origin, and in a high proportion of primary tumours including breast, ovarian, lung, colon, prostate and lymphoma[39,40,42]. Our observation that PAX2 is a crucial effector in mediating the proliferation and growth of endometrial cells and tumours also indicates that PAX2 has a role in carcinogenesis. Furthermore, we found that *PAX2* is silenced in normal endometrium and reactivated in endometrial cancer, and this reactivation is associated with cancer-linked hypomethylation of the *PAX2* promoter. Although hypomethylation was the first epigenetic alteration to be characterized in cancer[43], its role has been under-appreciated for many years in favour of hypermethylation. However, gene reactivation by cancer-linked hypomethylation has been rediscovered[44–47]. Future studies need to focus on delineating the cellular milieu surrounding, and the molecular mechanism underlying, both aberrant methylation patterns.

Identification of tumour-specific molecules that function as targets is crucial for the development of cancer drugs and thus is a chief goal of cancer research. Here, the identification of *PAX2* as a target of oestrogen and tamoxifen that mediates their carcinogenic roles in the uterus may provide useful information for designing safer drugs for the treatment of breast cancer and endometrial cancer. It will be interesting to investigate the mechanism involved in the loss of *PAX2* methylation mark in endometrial carcinomas. Perhaps more relevant to our findings, exactly how PAX2 functions to promote cell proliferation is still not known, and the pathophysiological relevance and the epigenetic regulation of other oestrogen and tamoxifen target genes need to be explored.

METHODS

Immunomagnetic purification of EECs. Minced endometrial tissues were digested in DMEM/F12 medium (Life Technologies) supplemented with 1% fetal bovine serum, 2 mg ml^{-1} of collagenase I (Sigma) and 2 mg ml^{-1} of hyaluronidase (Sigma) at 37 °C for 2 h. Cells were collected by centrifugation, digested with trypsin, resuspended in PBS, 1% bovine serum albumin and 2 mM EDTA, and purified by using an Epithelial Enrich kit (Dynal) in accordance with the manufacturer's recommendations. The pooled EEC pellets were resuspended in phenol-red-free DMEM/F12 medium containing 15 mM HEPES, 2.0 nM L-glutamine and 10% charcoal-dextran-stripped calf serum (Hyclone Labs). The purity of the cell cultures was judged to be >96% by immunohistochemical staining and cell sorting using antisera to cytokeratins 7, 10 and 18 (MN 116; Dako).

Human genome array analyses. Total RNA was extracted with Trizol Reagent (Invitrogen) and processed for use on Affymetrix U95A Human Genome arrays according to the manufacturer's protocol. Arrays were scanned with a GeneArray Scanner (Agilent Technologies). Raw data were collected and analysed by Microarray Suite and Data Mining Tools software (Affymetrix). Experiments were done on two replicate chips for each treatment in each ECC cell pool. We used Mann–Whitney pairwise comparisons to identify genes that were differentially expressed. Genes with concordance exceeding 80.6% were considered significantly different ($P < 0.1$). Gene lists from both comparisons were

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processed by Genespring software (Silicon Genetics) to identify common Affymetrix probe sets in both lists.

Gene overexpression by a retroviral vector system. We prepared recombinant retroviruses expressing all 35 genes by using a Retro-X Universal Packaging System (BD Biosciences) according to the vendor's manual. mRNA sequences representing the 35 genes and information on their open reading frames (ORFs) were obtained from RefSeq (http://www.ncbi.nlm.nih.gov/RefSeq/). The ORFs were individually amplified by PCR and cloned. For infection, cells were incubated with viruses at a multiplicity of infection of 10 for 6 h. Fresh medium was then added and the cultures were continued for another 48 h. To generate PAX2-overexpressing ECC-1 cells for transplantation into nude mice, infected cells were grown in the presence of 400 $\mu g\,ml^{-1}$ of G418 for 3 weeks. Individual drug-resistant clones were collected, pooled and expanded.

Gene silencing by a retroviral siRNA system. Recombinant retroviruses carrying specific oligonucleotides for generating siRNAs to the 35 genes described here were prepared by using a Knockout RNAi system (BD Biosciences) according to the vendor's manual. mRNA sequences and ORF information were obtained from RefSeq. Sequences were masked to remove repetitive sequences with RepeatMasker (http://www.repeatmasker.org/), and vector contamination was masked by searching with NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) against UniVec (http://www.ncbi.nih.gov/VecScreen/UniVec.html). Three unique 19-oligonucleotides for each target were selected as described in the Supplementary Methods. For infection, cells were incubated with viruses at a multiplicity of infection of 10 for 6 h. Fresh medium was then added and the cultures were continued for another 48 h. To generate *PAX2*-silenced ECC-1 cells for transplantation into nude mice, infected cells were grown in the presence of 2 $\mu g\,ml^{-1}$ of puromycin for 3 weeks. Individual drug-resistant clones were collected, pooled and expanded. The oligonucleotide sequences synthesized for RNA interference are given in the Supplementary Information.

Methylation-specific PCR. We analysed 53 type I endometrioid cancer samples and 19 normal endometrial samples. DNA was extracted and denatured with NaOH and treated with sodium bisulphite for 16 h. The following methylation-specific primers for the *PAX2* promoter were used: left M primer, 5′-GGGTTTTTTTCGTCGAAGTTC-3′; right M primer, 5′-ACTAAAACCTCGACTCCCGAT-3′; left U primer, 5′-GGTTTTTTTTGTTGAAGTTTGG-3′; right U primer, 5′-AAAACTAAAACCTCAACTCCCAAT-3′. DNA from peripheral blood lymphocytes of healthy individuals and water blanks were used as negative controls for methylated genes. DNA from peripheral blood lymphocytes treated with *Sss*I methyltransferase (New England Biolabs) was used as a positive control for methylated alleles.

Bisulphite sequencing. Bisulphite sequencing was done in 10 tumour samples and 10 normal tissues. Primer sequences for bisulfite sequencing of the *PAX2* fragment were 5′-GTTTGTAGTTTTTAGAGAGATATATAT-3′ (forward) and 5′-AAATTAACAAAAAAATAACAATCCC-3′ (reverse), which amplify the -37 to $+214$ region upstream of *PAX2*. This area contains 25 CpG sites. Amplified PCR products were purified with a Gel Extraction kit (Qiagen) and ligated into the pCR4–TOPO plasmid vector with a TA-cloning system (Invitrogen). Plasmid-transformed *Escherichia coli* were cultured and plasmid DNA was isolated with QIAprep 96 (Qiagen). Purified plasmid DNA containing the *PAX2* sequence was sequenced with an ABI 377 automated sequencer using BigDye Terminator chemistry (Applied Biosystems) and the M13 reverse primer. Samples that had clones with >50% methylation of CpGs were designated partially methylated. Samples containing clones with >75% methylation of CpGs sites were designated methylation positive. All other samples were designated methylation negative.

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