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PRECLINICAL STUDY

The liver receptor homolog-1 regulates estrogen receptor expression in breast cancer cells

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Abstract Estrogen receptor-α (ER) is expressed in the great majority of breast cancers, and the inhibition of ER action is a key part of breast cancer treatment. The inhibition of ER action is achieved using anti-estrogens, primarily tamoxifen, and with aromatase inhibitors that inhibit estrogen biosynthesis, thereby preventing ER activation. However, resistance to these therapies is common. With the aim of identifying new molecular targets for breast cancer therapy, we have identified the liver receptor homolog-1 (LRH-1) as an estrogen-regulated gene. RNA interference and over-expression studies were used to investigate the role of the LRH-1 in regulating breast cancer growth and to identify the targets of an LRH-1 action. Promoter recruitment was determined using reporter gene and chromatin immunoprecipitation (ChIP) assays. We show that LRH-1 regulates breast cancer cell growth by regulating the ER expression. Reporter gene and in vitro DNA-binding assays identified an LRH-1-binding site in the ER gene promoter, and ChIP assays have demonstrated in vivo binding at this site. We also provide evidence for new LRH-1 variants in breast cancer cells arising from the use of alternative promoters. Previous studies have shown that LRH-1 functions in estrogen biosynthesis by regulating aromatase expression. Our findings extend this by highlighting LRH-1 as a key regulator of the estrogen response in breast cancer cells through the regulation of ER expression. Hence, inhibition of LRH-1 could provide a powerful new approach for the treatment of endocrine-resistant breast cancer.

Keywords Estrogen · Estrogen receptor · Gene regulation · LRH-1

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Introduction

Estrogen plays a critical role in the development and progression of breast cancer. Its actions are mediated by estrogen receptors, with estrogen receptor-α (ER) being expressed in the majority of breast cancers. ER is a member of the nuclear receptor (NR) superfamily of transcription factors, which acts by regulating specific gene expression upon binding estrogen. Inhibition of ER activity is achieved clinically through the use of selective estrogen receptor modulators (SERMs), such as tamoxifen, that compete with estrogen for binding to ER, to inhibit its activity [1, 2].

Aromatase inhibitors act by inhibiting the conversion of androgens into estrogen, and provide an alternative and effective approach for inhibiting ER activity, with newly introduced aromatase inhibitors, such as Anastrozole and Letrozole, already proving to be have greater efficacy than tamoxifen [3, 4].

The regulation of ER gene expression has been subjected to intense study due to its important role in the regulation of breast cancer and in other important physiological processes, such as cardiovascular protection, bone homeostasis, and osteoporosis, and sexual development in males and females [5]. The coding region of the ER gene is located within eight exons spanning 140 kb on chromosome 6q25 [6, 7]. Regulation of an ER gene expression is complex, with transcription being initiated within multiple promoters spanning 150 kb [8]. Several of these promoters show tissue specificity [9], which has further complicated studies to define the transcriptional regulators of ER gene expression.

The liver receptor homolog (LRH-1), like ER, is a member of the NR superfamily. It belongs to the Ftz-f1 or NR5A subfamily that includes steroidogenic factor-1 (SF-1), members of which are characterised by the presence of an extended DNA-binding domain (DBD), the so-called Ftz-F1 box located at the C-terminus of the DBD [10]. Most NRs bind to DNA sequences conforming to the consensus NR-binding sequence, AGGTCA, either as homodimers (e.g., ER) to palindromes of the AGGTCA motif or to direct repeats of the AGGTCA motif as heterodimers with retinoid X receptor-α (RXRα) [11]. By contrast, members of the Ftz-F1 subfamily bind to sequences having a 5′ extension to the NR DNA-binding motif as monomers, with the Ftz-F1 box targeting the 5′-YCA extension to the NR DNA-binding motif [12], where Y is C or T. Until recently classified as orphan receptors, structural studies have shown that LRH-1 and SF-1 bind phosphatidyl inositols, with their binding being required for maximal activity [13–15]. LRH-1 plays important roles in metabolism, being involved in the regulation of reverse cholesterol transport, lipid and cholesterol absorption, bile acid homeostasis, and steroidogenesis [10]. In particular, the LRH-1 has been implicated in the regulation of aromatase (CYP19) expression in the ovary [16]. Interestingly, in adipose tissue from normal women, CYP19 expression is low and mainly originates from the use of promoter I.4. By contrast, in breast cancer adipose tissue, activation of additional CYP19 gene promoters is seen, including importantly, the gonadal PII promoter. In the latter context, LRH-1 regulates CYP19 expression through binding to a response element in the PII promoter [17–20].

In this study, we show that in breast cancer cells, LRH-1 is co-expressed with ER in breast cancer cell lines and that RNAi-mediated LRH-1 knock-down inhibits breast cancer cell growth. We demonstrate that this is due, at least in part, to the regulation of ER expression by LRH-1 through direct binding to the ER gene promoter. These studies show, for the first time, that ER is an LRH-1 target gene, a finding that is potentially relevant for the development of new therapies for breast cancer.

Materials and methods

Cell lines

COS-1, MCF-7, T47D, ZR75-1, BT474, and MDA-MB-231 cells were routinely cultured in DMEM containing 10% FCS. For estrogen-depletion experiments, the cells were transferred to DMEM lacking phenol red and containing 5% dextran-coated charcoal-stripped FCS for 72 h, as described previously [21]. 17β-Estradiol (estrogen), 4-hydroxytamoxifen, and ICI 182, 780 were prepared in ethanol and added to the medium at a final concentration of 10 nM (estrogen) or 100 nM (4-hydroxytamoxifen, ICI 182, 780). Compounds 5A, 5B, and 5L, which have previously been described [22], were prepared in DMSO and added to a final concentration of 10 μM.

Plasmids

The renilla luciferase reporter gene was RLTK (Promega, UK). LRH-1 and SHP expression plasmids were pCI-LRH-1, pCDM8-hSHP, and the LRH-1; the firefly luciferase reporter gene was SF-1-luc (gifts from Dr. Donald McDonnell and Dr. David Moore) [14]. The F342W/I416W LRH-1 mutant was generated by site-directed mutagenesis of pCI-LRH-1, using primers having the sequences 5′-CAAGCACGAAAAGCTGAGCACCTGGGGGCTTATGTGCAAAA-3′ and 5′-ACTGGGCAACAAGTGGACTATTCCATATGGGCATCACAAGCCGG-3′. The PII-516 aromatase reporter gene was kindly provided by Drs. Colin Clyne and Evan Simpson. ERP-Luc was generated by amplifying the genomic region covering the ER gene promoter A to LRHRE3 (−5993/−117) using high fidelity *pfu* DNA polymerase (Fermentas, UK) with DNA prepared from the human ER gene Bacterial Artificial Chromosome clone RP3-443C4 (Geneservice Ltd, UK). The PCR product was cloned into pJET (Fermentas, UK) (pJET-ERP) and re-cloned into the pGL3-Basic luciferase reporter vector (Invitrogen), following digestion with *Bgl*II restriction enzyme. The three putative LRH-1 response elements in this region, LRHRE1-3, were mutated by site-directed mutagenesis (Quickchange; Stratagene), changing the LRH-1-binding site to a *Bam*HI restriction site in each case (details available on request).

Transfections

MCF-7 and BT474 cells were transiently transfected with 100 ng of SHP or 50 ng of LRH-1, and the total RNA and protein lysates were prepared 48 h later, as previously described [21]. For reporter gene assays, COS-1 cells were transiently transfected in 96-well plates, using FuGene HD (Roche), according to manufacturer's protocols. The cells were transfected with 25 ng of the pGL3-Promoter-based reporter plasmids or 100 ng of the pGL3-Basic, SF-1 Luc, and RLTK, together with 50 ng of pCI-LRH-1. Cells were lysed 24 h following transfection and firefly and renilla luciferase activities were determined using the Dual Glo system (Promega, UK). A similar method was used for assaying ERP-luc and PII-516 aromatase reporter genes in COS-1 or MCF-7 cells.

siRNA

Cells were transfected using Lipofectamine RNAiMAX, according to manufacturer's methods (Invitrogen). RNA and protein were prepared 48 h following transfection. Cell number was estimated using the sulphorhodamine B (SRB) growth assay, as described previously [23]. siRNA LRH-1 On-TargetPlus Smartpool (Dharmacon) comprised siRNAs having the sequences: 5′-CAUAAUGGGCUAUUCAUA U-3′ (#1), 5′-AGAGAAAUUUGGACAGCUA-3′ (#2), 5′-GGAGUGAGCUCUUAAUCCU-3′ (#3) and 5′-GAAGC CAUGUCUCAGGUGA-3′ (#4). siGenome non-targeting siRNA (Dharmacon) had sequences 5′-UAAGGCUAUG AAGAGAUAC-3′, 5′-AUGUAUUGGCCUGUAUUAG-3′, 5′-AUGAACGUGAAUUGCUCAA-3′, 5′-UGGUUUACA UGUCGACUAA-3′.

RT-PCR

Total RNA was collected, and real-time RT-PCR was performed as previously described [23]. Real-time RT-PCR was carried out using Taqman Gene Expression Assays (Applied Biosystems, UK) for ER (ESR1; Hs00174860_m1), CTD (Hs00157201_m1), LRH-1 (Hs00187067_m1), pS2 (TFF1; Hs00170216_m1), SHP (Hs00222677_m1), Dax1 (Hs00230864_m1), SF-1 (Hs00610436_m1) and GAPDH (Hs99999905_m1) on an ABI 7900HT machine.

Western blotting

Cells were cultured and protein lysates prepared as described previously [21]. Antibodies used were anti-ERα (Novacastra Laboratories), and anti-LRH-1 (Perseus Proteomics). SHP, cathepsin D, and β-actin antibodies were purchased from Abcam.

ChIP

Chromatin immunoprecipitation (ChIP) was performed as previously described [23]. Antibody used was LRH-1, and primers for real-time PCR were: LRHRE1 Fwd 5′-CTAGCCCAAGTGAACCGAGA-3′, LRHRE1 Rev 5′-ACCTCAGGTCACGAACCAAA-3′. For normalization, qPCR was performed for the previously described control region for the c-myc gene [24], using oligonucleotides having the sequences: c-myc Fwd 5′-GCCAGTCCAAC CGGCTTATG-3′, c-myc Rev 5′-GGTTCTCCCAAGCAG GAGCA-3′.

In vitro DNA-binding assay

Binding affinities for LRH-1 to LRHRE1 sequences were obtained using a fluorescence polarization assay, following expression and purification of the LRH-1 amino acid residues 79–184 as a MBP fusion protein in *E. coli*, as described previously [12]. Carboxyfluorescin (FAM)-labeled duplex oligonucleotides based on the LRHRE1 sequence 5′-AATT**GCCAAAGCTT**TGGT-3′ and the CYP7A1 LRH-1-binding site described previously [12], (Integrated DNA Technologies, USA), were used. 10 nM of FAM-labeled oligonucleotide was mixed with MBP–LRH-1 DBD at varying concentrations, and polarization was measured in milli-polarization units (mP). The experiments were performed on a Panvera Beacon 2000, with an excitation wavelength of 495 nm, and an emission wavelength of 520 nm. The results were analyzed by SigmaPlot to generate binding data and dissociation constants.

Results

LRH-1 regulates the growth of breast cancer cells

As a strategy to identify key estrogen-regulated genes in breast cancer cells, we modified the estrogen-responsive and ER-positive MCF-7 breast cancer cell line, to conditionally express a transcriptionally repressive ER, PLZF-ER, composed of the PLZF transcriptional repressor fused to the ER DNA and ligand-binding domains [21]. The resulting line grows in an estrogen-dependent manner in the absence of PLZF-ER expression, but induction of PLZF-ER expression blocks its growth. Gene-expression microarray analysis carried out using this line, identified 1,627 genes which showed >1.5-fold regulation by estrogen within 16 h. Of these genes, 149 were repressed by PLZF-ER, suggesting that these genes are important for estrogen-regulated growth of MCF-7 cells (Buluwela et al., in preparation). One of the 149 genes whose expression was repressed by PLZF-ER in MCF-7 cells was LRH-1.

Estrogen treatment of MCF-7 cells showed a four-fold increase in the LRH-1 expression (Fig. 1a), confirming the microarray findings; no stimulation of LRH-1 expression was observed when the cells were treated with the anti-estrogens 4-hydroxytamoxifen or ICI182, 780. Further, LRH-1 expression was considerably higher in ER-positive, compared to ER-negative lines (Fig. 1b), suggestive of an association between the ER expression and the LRH-1 expression in breast cancer cells.

Analysis of the ER-binding and PolII occupancy data, generated from ChIP-chip and ChIP-seq profiling of global ER and PolII binding following treatment of estrogen-depleted MCF-7 cells with estrogen for 1 h [24, 25], showed ER binding to a region 10.5 kb upstream from the LRH-1 exon 1. Real-time PCR of ChIP following estrogen treatment of MCF-7 cells confirmed that estrogen stimulates ER binding to this region of the LRH-1 promoter (Fig. 1d).

Several LRH-1 splice variants have been described [10, 26], including hLRH-1 and LRH-1v2 both of which lack exon 2 encoded amino acids 22–67 (Fig. 2b). In performing immunoblotting for the LRH-1, we noted that many of the commercially available antibodies, whilst detecting the transfected LRH-1 did not detect the LRH-1 in breast cancer lines. As these antibodies were directed to the LRH-1 N-terminus, we wondered whether this was due to the predominance of exon 2-deleted LRH-1 variants in these lines. 5′-Rapid amplification of complementary DNA ends (5′-RACE) for defining 5′ ends of the LRH-1 transcripts in MCF-7 cells identified three main forms (Fig. 2a), the well-described LRH-1 variant 1 (v1) encoding a polypeptide of 541 amino acids, and two new variants, which we named variant 4 (v4) and variant 5 (v5) to distinguish them

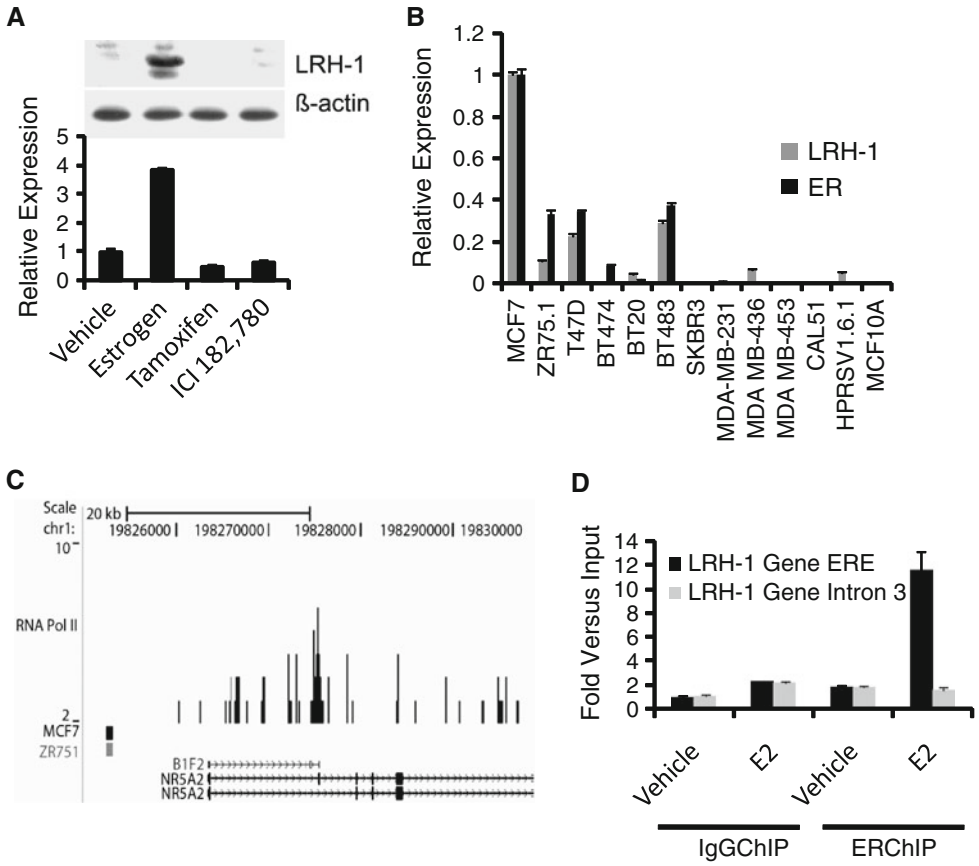

Fig. 1 LRH-1 is an estrogen-regulated gene that functions in breast cancer cell growth. **a** Hormone-depleted MCF-7 cells were stimulated with vehicle, estrogen, tamoxifen or ICI182780 and LRH-1 mRNA levels were assessed by real-time RT-PCR (*lower panel*). Protein lysates prepared following stimulation of hormone-depleted MCF-7 cells were immunoblotted (*upper panel*). **b** The LRH-1 and ER mRNA levels were determined by real-time RT-PCR analysis of total RNA. **c** Shown is the LRH-1 locus with the positions of ER-binding regions (*black box*) and RNA PolII occupancy for MCF-7 cells treated with estrogen for 1 h. Also shown is the region of ER binding for ZR75-1. Shown are the ER and PolII occupancy representations, generated by uploading the ChIP-Seq datasets to the UCSC genome browser gateway (http://genome.ucsc.edu). **d** ChIP was performed using mouse immunoglobulins (IgG) or an ER antibody, and quantitative PCR was carried out for the ER-binding site identified in (**c**). PCR for a region in intron 3 served as a negative control for ER binding. The results of three independent replicates are shown

from the previously described LRH-1 variants. v4 and v5 would be expected to encode polypeptides of 501 and 482 amino acids, respectively. Interestingly, LRH-1-v4 transcription is initiated in intron 1 of the LRH-1 gene, extending the previously identified exon 2 further 5′ into intron 1 (named exon 2a) (Fig. 2c; Supplementary Fig. 1). The mapped 5′ end of LRH-1-v5 defined an exon in intron 2 (here, named exon 2b). Hence, v4 and v5 define transcripts from the previously undescribed, alternative LRH-1 gene promoters. Blast searches of the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) identified one EST clone, TRACH3028697 (GenBank number: DC418114.1; the 5′ end of which precisely matched the 5′ end determined by 5′-RACE. Also identified was an EST clone that contained the sequences present in v4 and extended the 5′ untranslated region by a further 87 bp (Fig. 2b). No corroborating evidence for v5 was found in EST databases.

Expression analysis using isoform-specific real-time RT-PCR showed that v4 and v5 are highly estrogen-regulated, whereas variants hLRH-1, v1, and v2 expression were weakly stimulated by estrogen in MCF-7 cells (Fig. 3a). Note that using these primer sets, we did not distinguish between hLRH-1, v1, and v2. Furthermore, RT-PCR and immunoblotting showed that v4 is the predominant form of LRH-1 expressed in breast cancer cell lines (Fig. 3b, c). However, extensive reporter gene analysis did not reveal a significant difference between LRH-1 v1, and v4 (data not shown; Fig. 3d), despite the fact that v4 lacks the N-terminal 40 amino acids present in v1.

RNA interference (RNAi) used to determine whether LRH-1 is an important mediator of estrogen-stimulated growth of MCF-7 cells, showed that LRH-1 down-regulation using a pool of four small interfering RNAs (siRNA) potently inhibited MCF-7 growth (Fig. 4a). The individual siRNAs all inhibited MCF-7 growth, with the least growth inhibition being observed for siRNA #1, the siRNA that gave the smallest reduction in LRH-1 expression (Supplementary Figs. 2, 4b, f). Transfection with the LRH-1 siRNAs also inhibited the growth of the LRH-1 positive ZR-75-1 and T47D cells, but growth of the LRH-1-negative cell lines, BT474 and MDA-MB-231, was unaffected by the LRH-1 siRNA (Supplementary Fig. 3a).

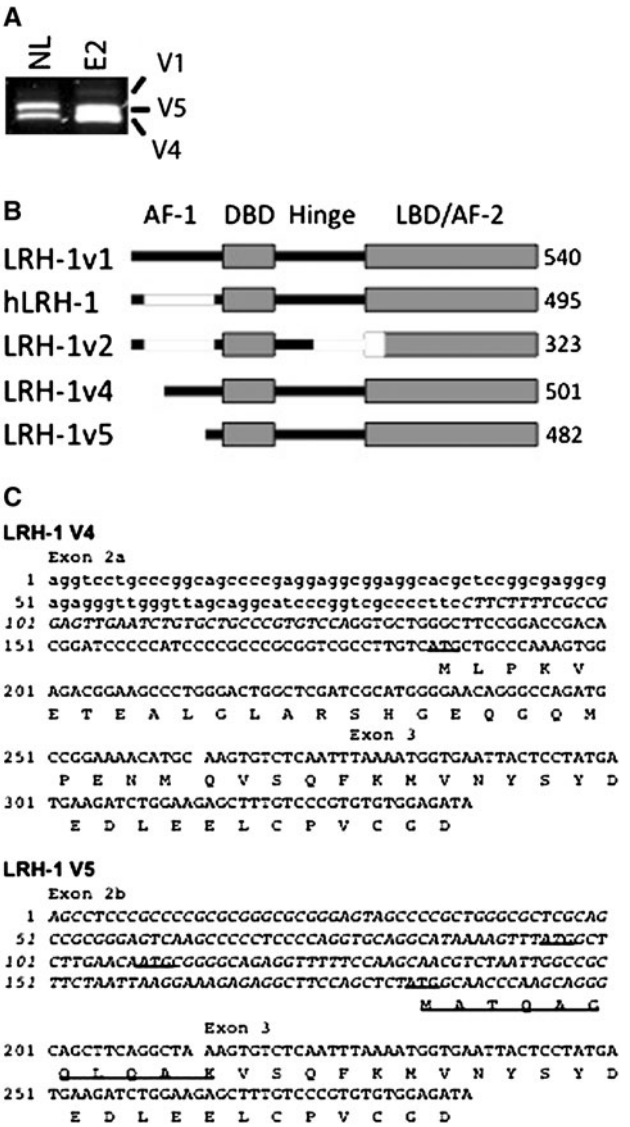

Fig. 2 Identification of new LRH-1 variants in breast cancer cells. **a** Products of 5′-RACE are shown for RNA prepared from MCF-7 cells treated with estrogen (E2) or vehicle (NL) for 8 h. The products were cloned and sequenced to assign their identities, as marked. **b** Shown is a schematic representation of the LRH-1 variants, including variants v4 and v5 identified here. **c** Sequences of LRH-1 variants v4 and v5. The DNA sequences in italics represent the 5′ untranslated regions present in the 5′-RACE cloning products. The sequences in lower case shown for v4 are also present in an EST (UTERU3011183) (http://www.ncbi.nlm.nih.gov/). The underlined amino acids in v5 are the residues arising from the newly described exon (exon 2b)

LRH-1 regulates ER expression in breast cancer cells

Determination of the expression of estrogen-regulated genes showed that LRH-1 knockdown reduced expression of the pS2 and cathepsin D (CTD) genes (Fig. 4c, d), prompting us to determine whether the growth inhibitory effects of the LRH-1 knockdown could be due to the LRH-1 regulation of ER expression. Indeed, ER mRNA and protein were reduced following siRNA for LRH-1 (Fig. 4e, f). Inhibition of ER expression following LRH-1 knockdown was also observed in T47D and ZR75-1 cells (Supplementary Fig. 3b, c).

In order to confirm these findings, we investigated whether synthetic LRH-1 activating compounds could stimulate MCF-7 cell growth. A number of substituted *cis*-bicyclo[3.3.0]-oct-2-enes have been identified as small molecule agonists of LRH-1 and SF-1 [22]. As there was no detectable expression of SF-1 in MCF-7 and BT-474

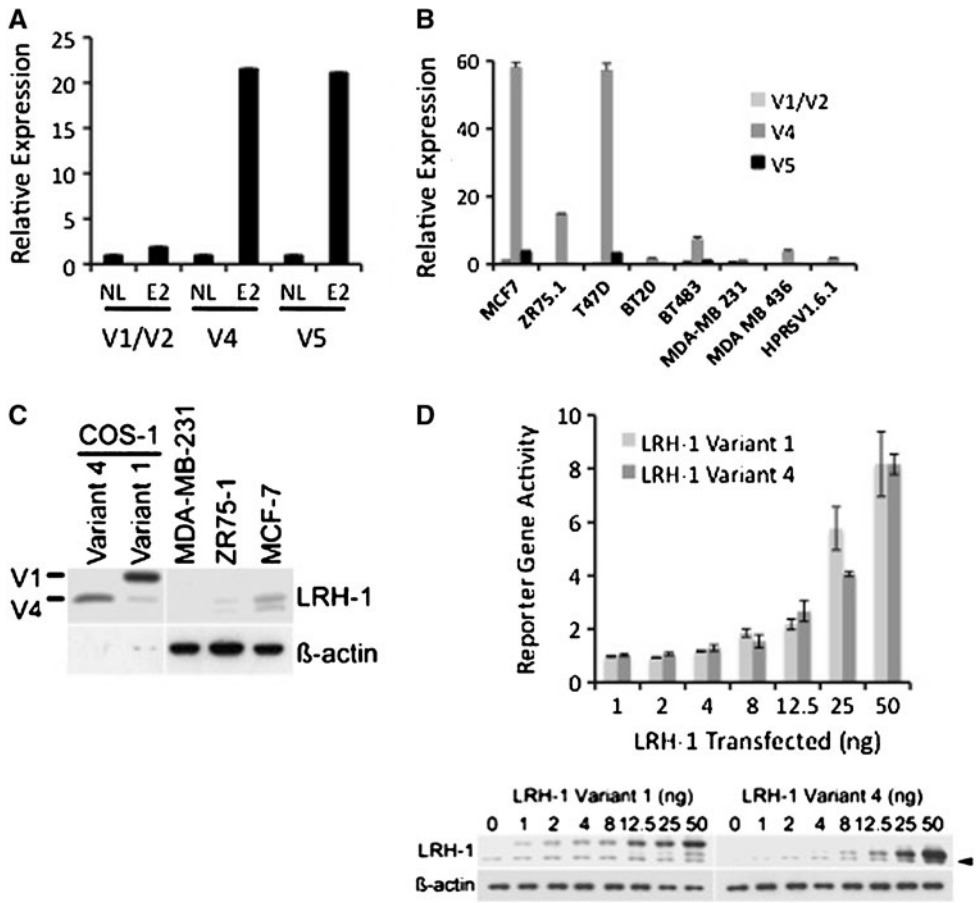

Fig. 3 LRH-1 variant 4 is the major form of the LRH-1 in breast cancer cells. **a** LRH-1 variant mRNA levels in MCF-7 cells were determined by real-time RT-PCR using primers that specifically amplify v4 or v5 sequences. Primers for v1/v2 amplify the LRH-1 variants 1 and 2, as well as hLRH-1. The results for three independent RNA samples are shown, with in each case expression level in the absence of ligand (NL) being taken as 1 and expression level in the presence of E2 shown relative to that. **b** Expression of the LRH-1 variants in breast cancer cell lines is shown. Expression of v1/v2 in MCF-7 was taken as 1 and all other values are shown relative to this. **c** Protein lysates prepared from breast cancer lines and COS-1 cells transfected with LRH-1 v1 or v4 were immunoblotted for LRH-1 and β-actin. **d** COS-1 cells were co-transfected with an LRH-1-responsive luciferase reporter gene (SF-1-luc) and increasing amounts of LRH-1 v1 or v4. Reporter gene activities are shown relative to the vehicle control. Protein lysates prepared from these lysates were immunoblotted for LRH-1 to determine relative expression of the variants (*lower panel*)

cells (Supplementary Fig. 4), we determined the effect of three of these compounds (5A, 5B, and 5L) on cell growth. MCF-7 cell growth was stimulated, whereas growth of the ER-positive, but LRH-1-negative BT-474 cells, was unaffected (Fig. 5a). As seen with a reporter gene assay, these compounds stimulated the activity of an LRH-1 responsive luciferase reporter gene (Fig. 5b), and treatment with the compounds increased the ER levels in MCF-7 cells (Fig. 5c). Moreover, LRH-1 transfection stimulated ER expression in MCF-7 and BT474 cells (Fig. 5d, e), whereas transfection of the small heterodimer partner (SHP), a NR that acts as a co-repressor for LRH-1 [27], reduced ER expression in MCF-7 cells (Fig. 5f, g). Further, LRH-1 transfection stimulated the growth of MCF-7 cells, while SHP inhibited MCF-7 growth (Fig. 5h).

LRH-1 regulates ER expression by direct recruitment to the ER gene promoter

Expression of the human ER gene is initiated at multiple promoters spanning 150 kb [8]. RT-PCR showed that in the lines examined, the majority of ER expression is initiated at promoters A, B, and C, with some expression from promoter F (Fig. 5a; Supplemental Fig. 5). Analysis of the region encoding ER gene promoters A through F revealed the presence of 11 sequences conforming to the LRH-1 consensus binding site (YCAAGGYCR [28]), with the closest of these sites being located less than 6 kb 5′ to promoters A/B (Fig. 6a). In addition, our analysis highlighted an extended palindrome centered around a *Hin*DIII restriction enzyme site (5′-CCAAAGCTTTGG-3′) which

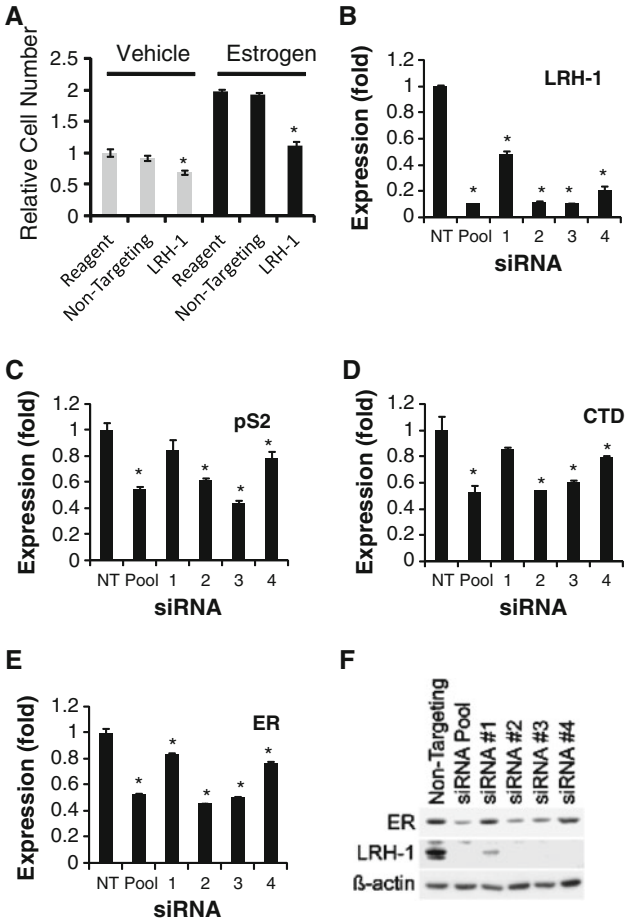

Fig. 4 Targeted knockdown of LRH-1 inhibits Estrogen Receptor (ER) expression. **a** MCF-7 cells grown in estrogen-depleted medium were transfected with the LRH-1 siRNA, treated with vehicle or estrogen and cell number estimated after 4 days using the sulphorhodamine B (SRB) assay. Growth is shown relative to the reagent alone control for three experiments. **b–e** siRNA pool for LRH-1 or individual siRNAs were transfected into MCF-7 cells, RNA and protein was prepared after 48 h. LRH-1 (**b**), pS2 (**c**) cathepsin D (CTD) (**d**) and ER (**e**) mRNA levels were determined by real-time RT-PCR analysis. Shown are the means and s.e.m. for three experiments. *Asterisks* show statistically significant difference (unpaired *t*-test, $P < 0.05$) from the reagent or non-targeting controls. **f** Immunoblotting was performed following preparation of protein lysates from MCF-7 cells transfected with siRNAs for LRH-1

encoded a sequence (underlined) similar to that identified in the mouse SEBP gene as an LRH-1-binding site [29], 912 bp 5′ to the promoter A start site (named LRHRE1).

As promoters A and B constitute the major promoters utilized in MCF-7 cells, we generated an ER promoter luciferase reporter gene (ERP-luc) encoding a 6.0 kb region comprising promoters A through D and containing two putative LRH-1-binding sites (LRHRE2, and LHRRE3) that conform to the LRH-1 consensus binding site sequence, as well as LRHRE1. The ERP-luc reporter was activated by LRH-1 in MCF-7 cells (Fig. 6b). Structural studies have shown that the LRH-1 ligand-binding domain (LBD) has phospholipids bound [13–15]. An LRH-1 mutant (LRH-1-F342W/I416W), which is impaired for phospholipid binding and shows reduced transcriptional activity [14] also showed reduced activation of ERP-luc. Mutation of LRHRE2 and LRHRE3 had modest effects on LRH-1 regulation of the reporter gene, with a considerably larger reduction in reporter gene activity being observed if LRHRE1 was mutated (Fig. 6c), suggesting that LRH-1 binds to the LRHRE1 sequence.

As the LRHRE1 sequence does not conform to the LRH-1 consensus binding site, LRH-1 binding to this sequence was determined using a fluorescence polarization assay using *E. coli*-expressed LRH-1 DBD and FAM-labeled oligonucleotides. The K_d of LRH-1 binding to LRHRE1 (126 nM) was similar to that obtained for the LRH-1 site in the Cyp7A1 gene promoter (200 nM) (Fig. 6d; Supplemental Fig. 5).

ChIP analysis of MCF-7 cell lysates with an LRH-1 antibody demonstrated LRH-1 recruitment to the region of the ER promoter containing the LRHRE1 sequence (Fig. 6e). Taken together, these findings indicate that LRH-1 is recruited to the LRHRE1 sequence in the ER gene promoter, and that this sequence is important for LRH-1 regulation of ER expression.

Discussion

LRH-1 is an estrogen-regulated gene in breast cancer cells

Estrogens play critical roles in the initiation and progression of human breast cancer, as well as other gynaecological cancers. Estrogen actions are mediated by ER which acts primarily as a transcription factor that, upon binding estrogen, regulates the expression of a large number of estrogen-responsive genes [30], causing, in the case of breast cancer cells, inhibition of apoptosis and promotion of proliferation. Defining the key estrogen-regulated genes in cancer cells would provide important insights into the mechanisms by which estrogen/ER promotes breast cancer growth. Using an engineered MCF-7 cell line that conditionally expresses a dominant-negative form of ER in which the PLZF transcriptional repressor was fused to ER [21], we identified LRH-1 as a gene whose expression was repressed upon PLZF-ER expression. RT-PCR analysis and immunoblotting confirmed estrogen-regulation of LRH-1, as has previously been described [31]. As also described previously, siRNA-mediated LRH-1 silencing potently inhibited the estrogen-stimulated growth of MCF-7 cells. Moreover, we observed inhibition of the growth of other ER/LRH-1-positive breast cancer cell lines (ZR75-1 and T47D).

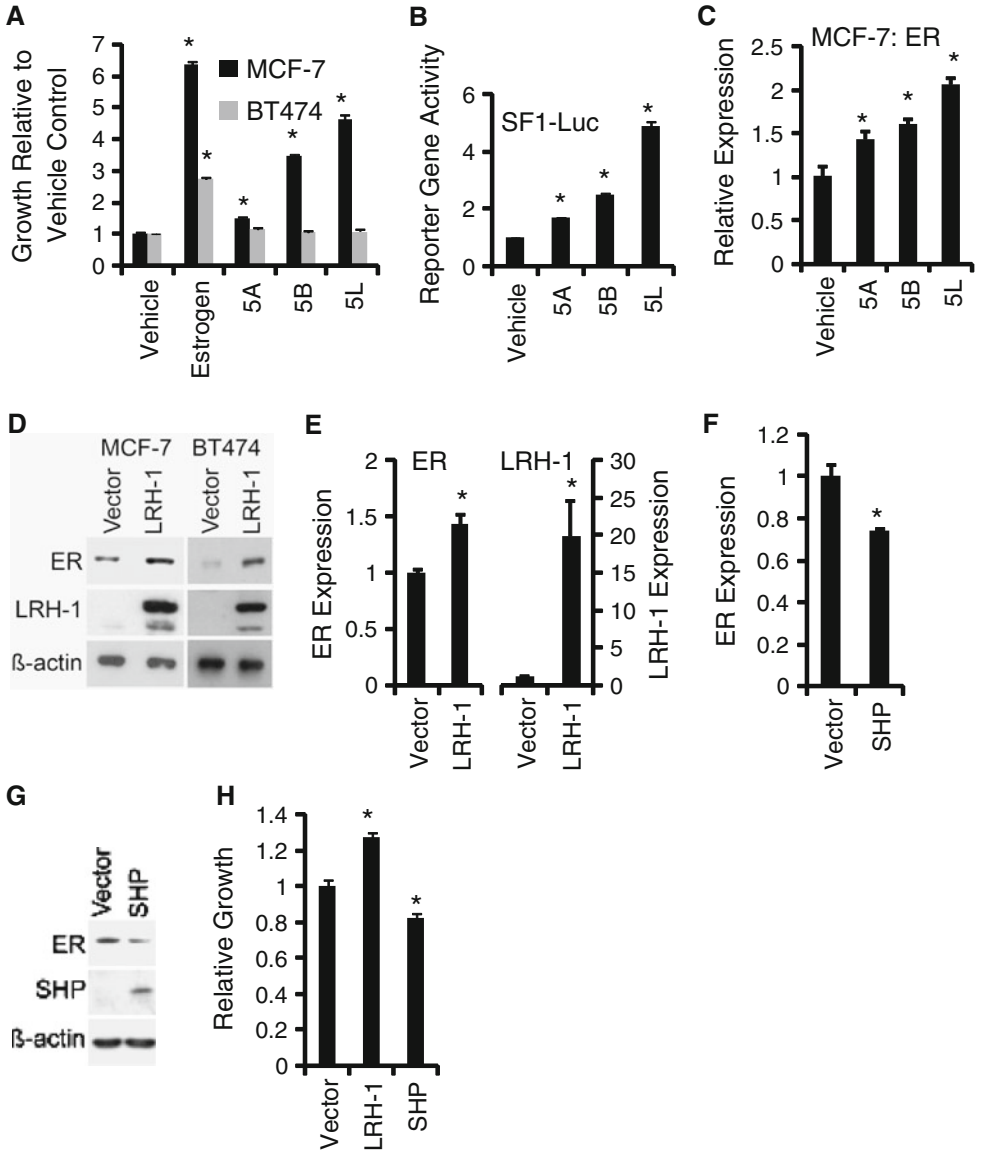

Fig. 5 LRH-1 stimulates Estrogen Receptor (ER) expression in breast cancer cells. **a** Hormone-depleted MCF-7 and BT-474 cells were stimulated with a vehicle, estrogen or LRH-1 activator, over a 12-day period. Shown is growth at day 12, relative to the vehicle control, as measured using the SRB assay. **b** COS-1 cells transfected with an LRH-1 responsive luciferase reporter gene (SF-1-luc) were stimulated with vehicle (DMSO) or the LRH-1 activators 5A, 5B and 5L. Reporter gene activities are shown relative to the vehicle control. **c** RNA was prepared from MCF-7 cells treated with compounds 5A, 5B and 5L for 8 h. ER mRNA levels were determined by real-time RT-PCR. **d**, **g** Protein lysates prepared from MCF-7 cells following transfection with LRH-1 (**d**) or SHP (**g**) were immunoblotted. **e**, **f** Real-time RT-PCR was performed using RNA prepared from MCF-7 cells transfected with LRH-1 (**e**) or SHP (**f**). **h** MCF-7 cells were transiently transfected with LRH-1 or SHP and cell numbers estimated after 4 days using the SRB assay. Growth is shown relative to the vector control. All graphical results are shown as the means and s.e.m. relative to the vehicle or vector controls, of at least three replicates. *Asterisks* denote statistically significant difference ($P < 0.05$) relative to the appropriate vehicle or vector control, determined using the unpaired *t*-test

The study by Annicotte et al. [31] previously identified an estrogen response element 2.3 kb upstream of the LRH-1 exon 1. ChIP based global analysis of ER-binding sites did not, however, detect significant binding to this region in MCF-7 and ZR75-1 cells. Instead, binding to a region 10.5 kb upstream of exon 1 was seen in both cell lines. This region contains the sequence 5′-AGGaCAcacTG ACCT-3′ (starting at chromosome 1 bp 198,252,853 in the NCBI hg18 human genome release, in which exon 1 of the LRH-1 gene is at bp 198,263,393 (http://genome.ucsc.edu)), which conforms well to the consensus ERE sequence (AGGTC AnnnTGACCT) [32]. ChIP analysis confirmed estrogen-stimulated ER recruitment to this site, indicating that this site is involved in ER regulation of LRH-1 expression.

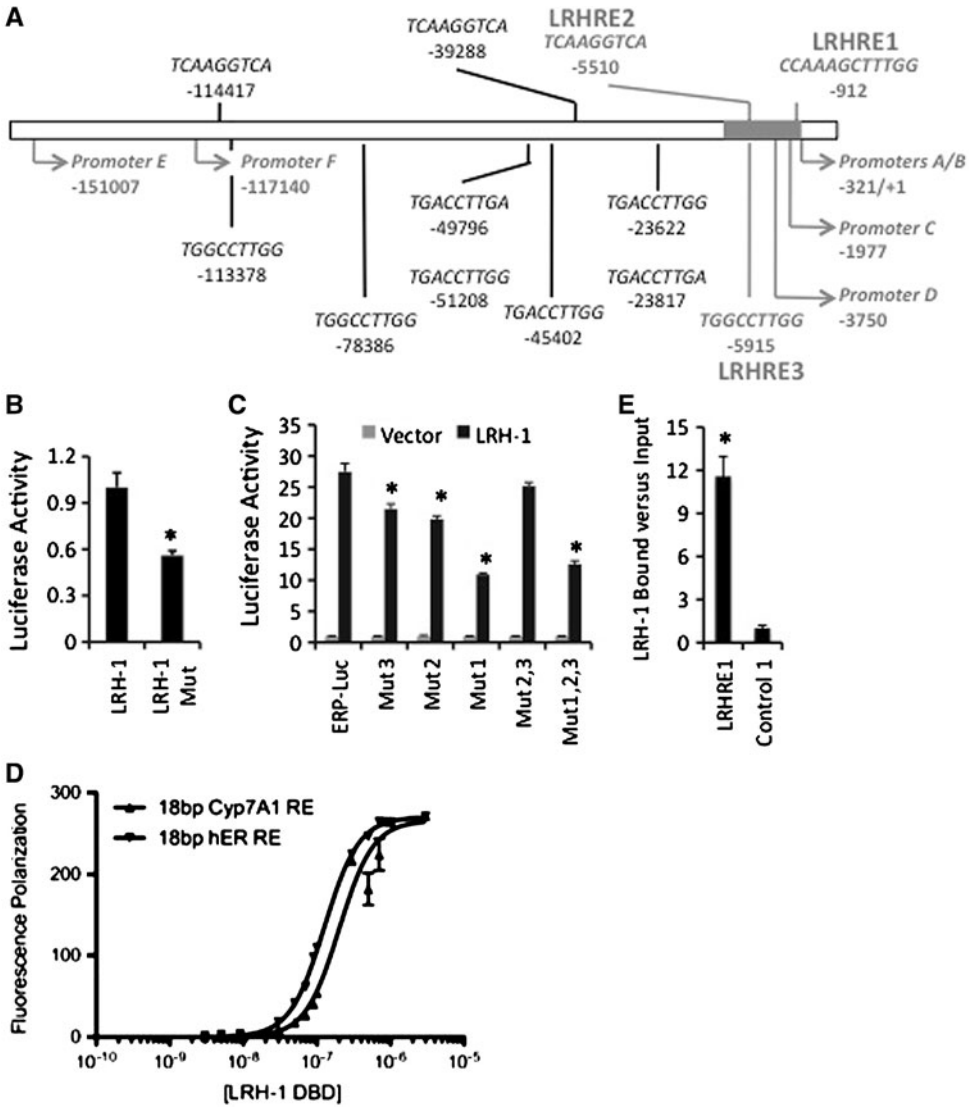

Fig. 6 LRH-1 binds to a specific site within the ER gene promoter. **a** Shown is a schematic representation of the ER gene, with the positions of ER promoters A through F highlighted. Transcription start site positions shown are as previously described [9]. Sequences that are similar to the consensus LRH-1-binding site are shown, *either above or below the rectangle* with the sequences shown *below the rectangle* denote sites on the lower DNA strand, whilst those *above the rectangle* represent sites on the upper DNA strand. The region of the ER gene used to generate the ER promoter luciferase reporter gene (ERP-luc) is shaded. **b** MCF-7 cells were transfected with ERP-luc, together with the LRH-1 or the LRH-1-F342W/I416W mutant. **c** Reporter gene activities were determined for MCF-7 cells transfected with ERP-luc, or mutants in which LRHRE1-3 were mutated. All reporter gene activities are shown as the means and s.e.m., for three replicates, relative to the vector control. **d** DNA binding to LRHRE1 and the Cyp7A1 LRH-1 response element was measured by changes in fluorescence polarization for the LRH-1 DBD. The results of at least three replicates are shown. **e** ChIP was performed using lysates prepared from MCF-7 cells, and real-time PCR for three replicates using primers for a control region mapping to the c-myc locus and the ER promoter encompassing LRHRE1, are shown. In all cases *asterisks* show statistically significant differences ($P < 0.05$), determined using the unpaired *t*-test, between sample 1 and the other samples, or relative to the control samples

A new LRH-1 variant is the predominant form of LRH-1 in breast cancer cells

LRH-1 transcript mapping identified two new LRH-1 variants, both of which are expressed from distinct new promoters, one initiating within intron 1 and extending exon 2 127 bp 5′ (v4), and the second initiating in intron 2 and lacking exon 1 and 2 sequences (v5). Both of these variants are estrogen regulated, but v5 expression is low in breast cancer cells. LRH-1-v1 was the only other variant whose expression was observed by 5′-RACE, but it was also expressed at low levels, with v4 being the major form of LRH-1 in breast cancer cells, which was confirmed by immunoblotting. Forms of LRH-1 corresponding to v4, as well as v1 are seen in the mouse, chicken (Supplementary Fig. 1b, c), and rat, while v4 corresponds to LRH-1

transcripts predicted to come from the xenopus and zebrafish LRH-1 genes. Furthermore, analysis of the ChIP-chip for RNA polymerase II (PolII) [25] showed that at the LRH-1 locus, the strongest binding of PolII is centered on exon 2 sequences (see Fig. 2c), which indicates that in MCF-7 cells the major transcription initiation sites for the LRH-1 gene are centered around exon 2. Finally, real-time RT-PCR using RNA from a panel of human tissues showed that v4 is expressed more widely than the other variants. Indeed, we did not detect expression of the other variants in the absence of v4 expression. Taken together, these findings provide strong evidence for the in vivo importance of LRH-1-v4. A previous study examining LRH-1 expression immunohistochemically in invasive ductal carcinoma showed that 43% of breast tumours are LRH-1 positive [33], expression being negatively associated with clinical stage and histological status and positively associated with steroid receptor status. As this study was carried out using an antibody directed to amino acids 2–33 of LRH-1-v1 (absent in v4), it is possible that the LRH-1 positivity was underestimated, highlighting the need for re-evaluation of LRH-1 in breast cancer, the lack of currently ascribed differences in the activities of LRH-1 variants 1 and 4 notwithstanding. As the antibodies available to us have proved to be unsuitable for immunohistochemistry, we are currently developing new antibodies to address this issue.

LRH-1 regulates estrogen receptor expression in breast cancer cells

LRH-1 knockdown inhibits breast cancer cell growth, as previously described [31] and confirmed here. Importantly, LRH-1 knockdown reduced ER expression, LRH-1 overexpression or stimulation of its activity by synthetic agonists increased ER levels, whilst the LRH-1 repressor SHP reduced ER expression. Reporter gene studies and mutational analysis showed that three sites contribute to the LRH-1 regulation of the ER reporter gene, with an LRH-1-binding site, named LRHRE1 (5′-AATTGCCAAAGCT TTGGT-3′) having a sequence similar to LRH-1-binding site in the mouse SEBP gene (5′-CCCAAAGGCTT-3′) [29], being the most important site for the LRH-1 regulation of ER expression. Finally, ChIP confirmed binding of LRH-1 to the ER gene in a region encompassing this site. Taken together, these results show that LRH-1 regulates ER-positive breast cancer cell growth through a mechanism involving regulation of ER gene expression through direct recruitment to the ER promoter.

Previous study has highlighted a positive regulatory loop in which ER and GATA-3 reciprocally regulate each other in breast cancer cells [34], and provides a possible explanation for the co-expression of ER and GATA-3 in breast cancers (see [34, 35]). Other estrogen-regulated genes have been shown to be important for ER action in breast cancer cells, with FoxA1 being required for the recruitment of ER to the promoters of many estrogen-responsive genes [36, 37]. Our findings show that ER and LRH-1 form a positive cross-regulatory loop in which each transcription factor is required for the expression of the other gene.

The aromatase (CYP19) gene is transcribed from a number of different promoters, with expression from the different promoters being highly tissue selective. The aromatase promoter II (PII) is used in gonadal tissues, and its regulation by SF-1 has been demonstrated [38–40]. Whilst in normal breast tissue, aromatase is expressed at low levels, with promoter 1.4 being utilized, in tumor-bearing breast tissue, aromatase expression is elevated, with transcription being driven largely through PII promoter [19]. These studies have also indicated that SF-1 is not expressed in breast tumours, whilst LRH-1 is expressed in adipose tissue, with LRH-1 and aromatase co-expression being evident particularly in pre-adipocytes [17]. In this context, LRH-1 regulates aromatase expression thorough recruitment to the PII Promoter [17, 18]. Furthermore, regulation of the PII promoter by LRH-1 occurs synergistically with GATA3 [18]. Interestingly, regulation of ER expression by GATA3 has also previously been described [34]. Our preliminary analysis shows that LRH-1 and GATA3 also act synergistically at the ER promoter (Supplementary Fig. 7). Aromatase expression is additionally dependent on tumor-derived growth factors, particularly prostaglandin E_2, acting through protein kinase A (PKA) to stimulate aromatase PII activity [17, 18]. In agreement with these findings, activity of the aromatase PII reporter gene was strongly increased by PKA, whereas PKA did not similarly potentiate the synergism between LRH-1 and GATA3 at the ER gene promoter (Supplementary Fig. 7). Together, these results indicate that aromatase and ER expression are similarly regulated, albeit in different cell types, indicating that the cell-type specificity may be due to differences in cell signaling pathways, such as those involving PKA (for potential model of aromatase and ER regulation by LRH-1 see Supplementary Fig. 7c).

Of course, differential expression of the LRH-1 variants may also be important in specifying cell-type selectivity for the differential regulation of aromatase and ER genes in different cell types. Furthermore, we did not observe expression of the LRH-1 regulated SHP gene in the breast cancer cell lines; nor was Dax1 expression evident in LRH-1 positive breast cancer lines. As SHP and Dax1 are important regulators of LRH-1 activity [27], their presence or absence may also be important in defining promoter and cell-dependent activities of the LRH-1, as well as responses to other signaling pathways, such as PKA.

In conclusion, our studies show that LRH-1 is a key regulator of estrogen responses in breast cancer, which acts by regulating estrogen synthesis in breast tumour tissue and by regulating ER expression in breast cancer cells. As such, LRH-1 presents as an important target for the development of new therapeutic agents for use in breast cancer treatment.

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