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Oestradiol reduces Liver Receptor Homolog-1 mRNA transcript stability in breast cancer cell lines

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ABSTRACT

The expression of orphan nuclear receptor Liver Receptor Homolog-1 (LRH-1) is elevated in breast cancer and promotes proliferation, migration and invasion *in vitro*. LRH-1 expression is regulated by oestrogen (E_2), with LRH-1 mRNA transcript levels higher in oestrogen receptor α (ERα) positive (ER+) breast cancer cells compared to ER− cells. However, the presence of LRH-1 protein in ER− cells suggests discordance between mRNA transcript levels and protein expression. To understand this, we investigated the impact of mRNA and protein stability in determining LRH-1 protein levels in breast cancer cells.

LRH-1 transcript levels were significantly higher in ER+ *versus* ER− breast cancer cells lines; however LRH-1 protein was expressed at similar levels. We found LRH-1 mRNA and protein was more stable in ER− compared to ER+ cell lines. The tumor-specific LRH-1 variant isoform, LRH-1v4, which is highly responsive to E_2, showed increased mRNA stability in ER− *versus* ER+ cells. In addition, in MCF-7 and T47-D cell lines, LRH-1 total mRNA stability was reduced with E_2 treatment, this effect mediated by ERα.

Our data demonstrates that in ER− cells, increased mRNA and protein stability contribute to the abundant protein expression levels. Expression and immunolocalisation of LRH-1 in ER− cells as well as ER− tumors suggests a possible role in the development of ER− tumors. The modulation of LRH-1 bioactivity may therefore be beneficial as a treatment option in both ER− and ER+ breast cancer.

1. Introduction

Liver Receptor Homolog-1 (LRH-1; NR5A2) is an orphan member of the nuclear receptor superfamily that share substantial structural homology within their DNA and ligand binding domains (for review, see [1,2]). LRH-1 is a key mediator in metabolic pathways involved in bile acid synthesis and cholesterol metabolism *via* regulation of cytochrome P450 7A1 (*CYP7A1*) [2]. LRH-1 also has important roles in steroid hormone production in the ovary and testis, through regulation of genes including cytochrome P450 aromatase (*CYP19A1*), steroidogenic acute regulatory protein (StAR), 3β-hydroxysteroid dehydrogenase (HSD2B2) and inhibin α subunit [2]. More recently, LRH-1 has been demonstrated to play an important role in early embryonic tissue via its ability to replace and regulate the expression of octamer-binding transcription factor 4 (OCT4) [1,3]. LRH-1 promotes cell proliferation in gastric, colon and pancreatic cancers [1,2]. We have demonstrated LRH-1's importance in breast cancer, through activation of P450 aromatase, the enzyme that catalyses the conversion of androgens to oestrogen [4].

17β-Oestradiol (E_2) promotes cell proliferation and breast cancer development by binding to its receptor oestrogen receptor α (ERα) and ERβ. In the breast, LRH-1 is involved in E_2 signaling via various pathways creating a positive feedback loop promoting tumor cell proliferation. Firstly, LRH-1 stimulates transcription of the *CYP19A1* gene, primarily occurring in cancer associated fibroblasts (CAFs), thereby contributing to the supply of mitogenic E_2 that acts in a paracrine manner to promote neighbouring epithelial cell proliferation [4]. Breast tumor cells secrete factors such as prostaglandin E_2 (PGE_2), which directly stimulate LRH-1 expression in tumor-associated stroma, and contribute to LRH-1 mediated activation of the *CYP19A1* [5]. LRH-1 also directly regulates expression of ERα in breast cancer cells *via* the activation of a major ERα promoter used in breast cancer cells [6]. In turn, LRH-1 stimulates expression of ERα target genes, such as GREB-1 and pS2, by binding to ERα response elements in their promoters [7]. Finally, LRH-1 is itself regulated by E_2 via binding of ERα to the LRH-1 promoter [8].

Abbreviations: LRH-1, Liver Receptor Homolog-1; ER, oestrogen receptor; miRNA, micro RNA.

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Therefore, a positive feedback loop is established whereby LRH-1 both regulates, and is regulated by ERα.

Consistent with its role in E_2 signaling and regulation of expression by E_2, LRH-1 transcript is largely confined to ER+ breast cancer cell lines, with ER− lines expressing little or no LRH-1 mRNA [6,8]. Recently, Thiruchelvam et al. identified two novel variants, LRH-1v4 and LRH-1v5 that are highly responsive to E_2 treatment. Despite the relative lack of LRH-1 mRNA in ER− breast cancer cells, a number of observations suggest that LRH-1 has actions in ER− breast cancer tissues and cells that may be physiologically relevant. For example, LRH-1 protein was found to be expressed in a subset of ER− human breast cancer tissue by immunohistochemistry [9]. Additionally, LRH-1 protein was expressed at similar levels in both the ER− MDA-MB-231 cell line and the ER+ MCF-7 line [10]. Knockdown of endogenous LRH-1 inhibited tumor cell migration and invasion in ER− MDA-MB-231 cells and cell proliferation in ER+ MCF-7 cells [7,8,10]. This suggests that expression of LRH-1 in ER− cells may be relevant to breast cancer proliferation and invasion, for example by directly stimulating cell cycle genes [11], or stimulating ERα target genes such as GREB-1 [7].

In the present study we assessed LRH-1 mRNA and protein expression in a panel of ER+ and ER− breast cancer cell lines. LRH-1 protein is expressed at similar levels in all cell lines tested despite strong enrichment of LRH-1 mRNA in ER+ cell lines. Differences in LRH-1 mRNA and protein stability between ER+ and ER− cells were observed. This discrepancy may in part be explained by the negative effect of E_2 treatment on LRH-1 mRNA half-life in ER+ cells. This data demonstrated stable expression of LRH-1 mRNA and protein in ER− breast cancer cell lines, suggesting that LRH-1 may have important effects in hormone independent cancers, as it does in ER+ tumors.

2. Materials and methods

2.1. Cell culture

Human breast cancer cell lines (MCF-7, ZR-75, T47D, MDA-MB-231, MDA-MB-361, MDA-MB-453, MDA-MB-468 and HS578T) were obtained from American Type Culture Collection (ATCC) and grown in the recommended culture media and conditions

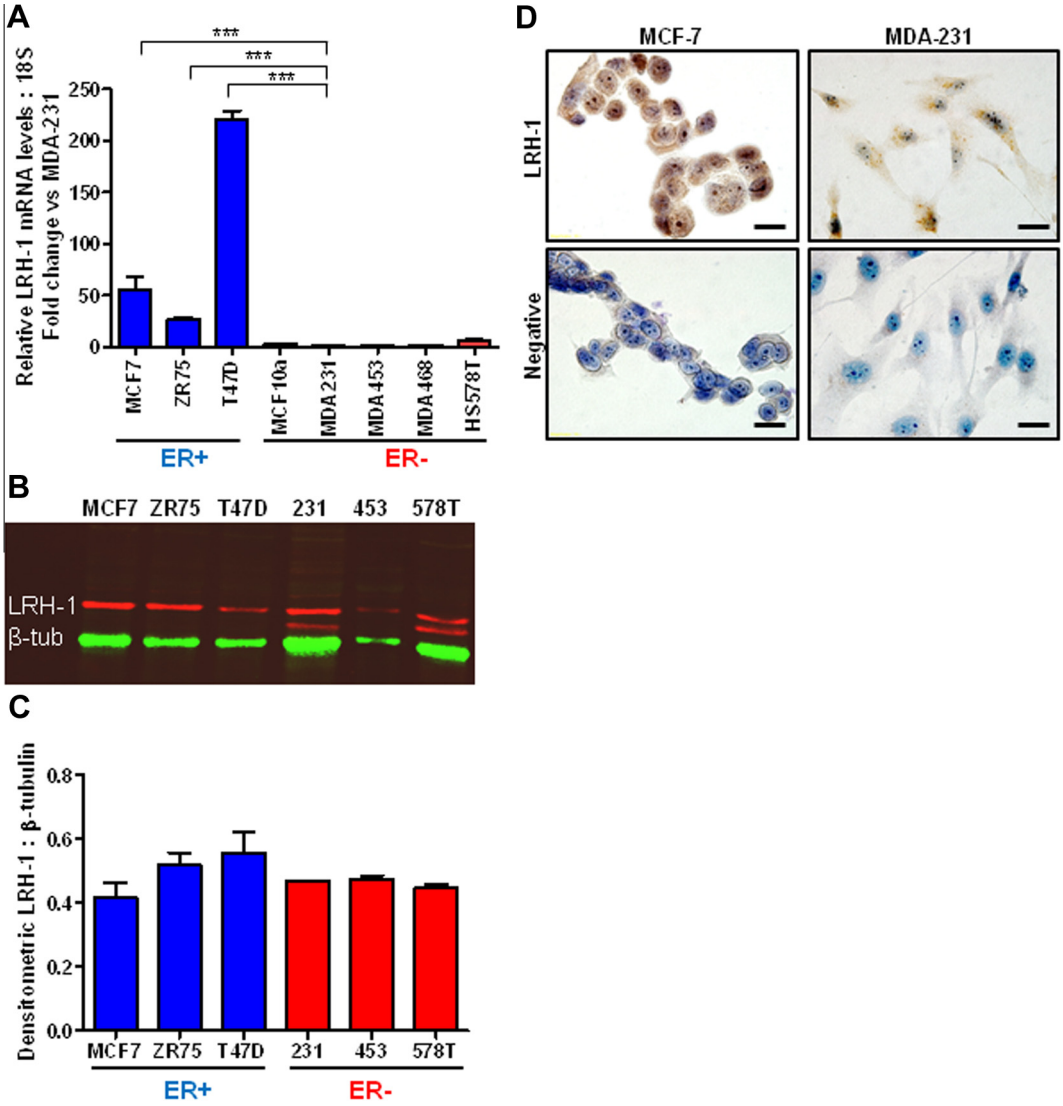

Fig. 1. Discordance in LRH-1 mRNA and protein expression levels in breast cancer cell lines: (A) total mRNA was isolated from a panel of breast cancer cell lines. Levels of expression were determined by Q-PCR and normalized to *18s*. (B) Representative image showing expression of LRH-1 (red) in protein lysates, β-tubulin used as a loading control (green). (C) Densitometry quantification of immunoblot data, (D) LRH-1 immunoreactivity was detected in the nucleus and cytoplasm in MCF-7 and MDA-MB-231 cells. Rabbit IgG was used as a control. Images were taken at 100× magnification. Scale bar, 20 μm. Data shown from independent experiment as mean $n = 3 \pm$ SEM, $^{***}P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

according to ATCC protocols. Cells were incubated for 72 h in medium containing 3% charcoal-stripped serum (CSS) before addition of treatments including 17β-oestradiol (10 nM, Sigma) and/or ICI 182,780 (100 nM, Sigma) for 24 h (ethanol for vehicle control).

2.2. Real time-PCR

Total RNA was prepared using the RNeasy Kit (Qiagen, 74106), treated with DNaseI (Ambion, AM1906), and quantified using a NanoDrop 1000 Spectrophotometer. First strand cDNA synthesis using 1.0 μg total RNA was performed using AMV Reverse Transcriptase (Promega, M5101) primed by random hexamers. PCRs were carried out using Taqman probes against LRH-1 (Hs00187067_m1, Applied Biosystems) and Taqman Gene Expression Master Mix (Applied Biosystems). RT-PCR for LRH-1 v1, v4, v5 and 18s was analysed using the Power SYBR Green PCR Mix (Applied Biosystems) using the following primers: sense 5′-TCTTTAAAGCACGGACTTACACC-3′ and antisense 5′-TATCTCCACACACGGGACAA-3′ (V1/V2), sense 5′-TCTTTAAAGCACGG ACTTACACC-3′, antisense 5′-TATCTCCACACACGGGACAA-3′ (V4), sense 5′-CCAGGTGCAGGCATAAAAGT-3′ and antisense 5′-TATCTCCACACACGGGACAA-3′ (V5) and, sense 5′-CGG CTA CCA CAT CCA AGG AA-3′, and antisense 5′-GCT GGA ATT ACC GCG GCT (18s) [6]. mRNA transcripts were quantified by ABI Prism 7900-HT Real-time PCR system. Fold changes in expression of LRH-1 was calculated using the ddCt method [12] using 18s as an internal control.

2.3. Actinomycin D treatment for determination of mRNA stability

MCF-7, T47D, MDA-MB-231 and HS578T cells were incubated for 72 h in medium containing 3% charcoal-stripped serum (CSS), and then supplemented with 5 μg/ml Actinomycin D (Sigma, A1410) in phenol red free media. The cells were treated with the indicated substances for various time points up to 24 h. RNA was extracted, cDNA synthesised and RT-PCR performed as described above.

2.4. Determination of protein turnover by Cyclohexamide treatment

MCF-7 and MDA-MB-231 cells were incubated for 72 h in medium containing 3% CSS, and then supplemented with 10 mg/ml Cyclohexamide (Sigma, C4849) in phenol red free media. The cells were treated for 0, 4, and 8 h. Protein was extracted, and western blot was performed as below.

2.5. Western blot analysis

Protein extraction and immunoblots for LRH-1 and β-tubulin were performed as described previously [13]. Briefly protein was extracted from cell lysates and separated on a 10% SDS–PAGE gel. The protein was then transferred to a nitrocellulose membrane (Amersham). After blocking with 5% skim milk, the membrane was incubated with anti-LRH-1 antibody (ab18293, Abcam; H2325, R&D systems) for 16 h at 4 °C. Anti-β-tubulin (mab3408, Millipore) was used as a loading control. Protein bands were visualised using the Odyssey infrared imaging system and Odyssey 3.0 software (Licor Biosciences).

2.6. Immunocytochemistry

Cells were fixed with 100% cold methanol, permeabilized with PBS + 0.1% Triton X-100 (Thermo, 28318) followed by blocking with 0.3% H_2O_2, Avidin/Biotin (Vector, SP-2001). The fixed cells

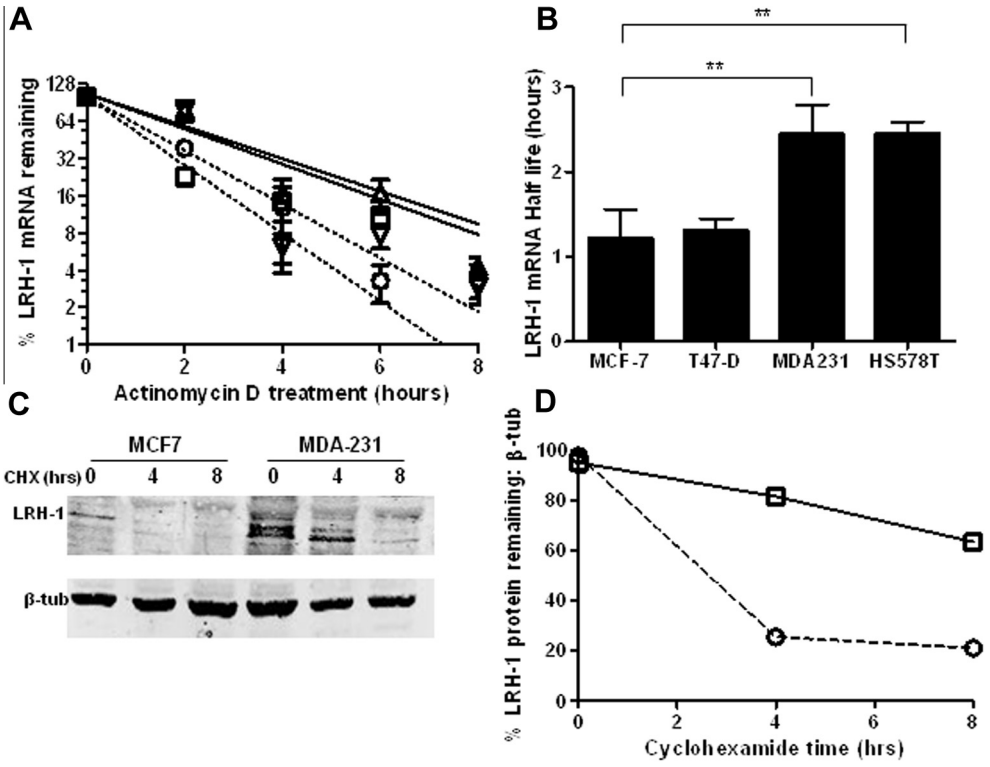

Fig. 2. LRH-1 mRNA and protein stability in breast cancer cell lines: (A) actinomycin-D decay study was performed on ER+ ($n = 2$) and ER− ($n = 2$) cell lines. Each plot is a percentage of the 0 h value on a logarithmic scale. Dotted lines represent ER+, while solid lines represent ER− cells, ○, MCF-7; □, T47-D; △, MDA-MB-231; ▽, HS578T. (B) Half life was interpolated from the decay log curve above and analysed using student *t*-test. (C) Cyclohexamide (10 μg/mL) was used to inhibit de novo protein synthesis. Whole-cell lysates were resolved with SDS–PAGE followed by immunoblotting with αLRH-1, β-tubulin used as a loading control. (D) Densitometry quantification of immunoblot data, dotted line represents MDA-MB-231 and straight line represents MCF-7. ○, MCF-7; □, MDA-MB-231 Data shown from independent experiments as $n = 3 \pm$ SEM, $^{**}P < 0.01$.

were blocked using horse serum (Vector, PK-6101), for 20 min at RT according to the manufacturers protocol. Cells were then incubated with LRH-1 primary antibody (1:200, ab18293 Abcam) at 4 °C O/N. IgG was used as negative control. After washing, the cells were incubated with biotinylated secondary antibody (Vector, PK-6101) for 60 min at RT. The cells were then incubated with ABC reagent (Vector, PK-6101) for 30 min. Finally, 3, 3′-diaminobenzidine (Dako, K3468) staining was used for antibody detection and Myer's Hematoxylin solution (Sigma Aldrich, MHS1) was used for nuclei detection. Staining was photographed with OLYMPUS microscope.

2.7. Statistical analysis

All data are reported as mean ± SE for three or more experiments. Statistical analyses for experiments comparing two groups were performed by two-tailed Student's independent *t* test using GraphPad Prism 5.0 (GraphPad, La Jolla, CA, USA) and a *P* value of < 0.05 was considered statistically significant.

3. Results

3.1. LRH-1. expression in breast cancer cell lines

To confirm that LRH-1 transcript levels positively associate with the ER+ status of breast cancer cell lines, we first quantified total LRH-1 mRNA expression in several ER+ and ER− breast cancer cell lines by RT-PCR (Fig. 1A). Consistent with previous studies [8,14], we observed significantly higher expression of LRH-1 mRNA in ER+ cells compared to ER− cells ($p < 0.0001$) (Fig. 1A). We demonstrate LRH-1 protein levels in a panel of ER− and ER+ cell lines (Fig 1B and C). Two protein bands were detected, at approximately 61 kDa and 56 kDa that correlate to the LRH-1v4 isoform [6]. Interestingly, expression of the 56 kDa isoform was restricted to ER− cells. We also observed a novel predominantly nuclear localisation of LRH-1 in MCF-7 and MDA-MB-231 cells by immuocytochemistry (Fig. 1D).

3.2. LRH-1. transcript and protein stability is reduced in ER+ cells

To address the discrepancy between LRH-1 mRNA and protein expression, we measured the stability of LRH-1 mRNA and protein in ER− and ER+ cell lines. In the presence of the RNA synthesis inhibitor Actinomycin D, LRH-1 total transcript degraded more rapidly in ER+ cells compared to ER− cells (Fig. 2A). The half-life of LRH-1 mRNA in ER− cells was approximately double that in ER+ cells ($p < 0.01$, Fig. 2B). In ER+ breast cancer cell lines, LRH-1 mRNA half-life was 1.2 h in MCF-7 and 1.3 h in T47-D cells. In ER− cell lines, LRH-1 mRNA half-life was 2.5 h in MDA-MB-231 and 2.1 h in HS578T cells. We also determined the degradation rates of LRH-1 protein in ER+ MCF-7 and ER− MDA-MB-231 cell lines by western blot following treatment with a protein synthesis inhibitor, Cyclohexamide (Fig. 2C). LRH-1 protein was significantly more stable in MDA-MB-231 (half-life: 11.69 h) than in MCF-7 (half-life: 3.8 h) cells (Fig. 2D). The LRH-1 isoform v4 (56 kDa) was highly stable after protein synthesis inhibition in MDA-MB-231 cells (Fig. 2C). Collectively, this data suggests that differences in mRNA and protein stability may explain the discrepancy observed between LRH-1 transcript and protein levels across the panel of breast cancer cell lines.

3.3. Expression and stability of LRH-1 transcript variants

Primers specific for LRH-1 isoforms were used to quantify expression levels of previously described LRH-1 variants [6]. We confirmed LRH-1v4 as the predominant form of LRH-1 in ER+ and ER− breast cancer cells lines while little or no LRH-1v1/2 and v5 were detected (Fig. 3A). LRH-1v4 degraded at a more rapid rate in ER+ MCF-7 cells (half life: 1.0 h) compared to MDA-MB-231 cells (half life: 2.2 h) (Fig. 3B).

3.4. Oestradiol reduces LRH-1 transcript stability via ERα

Steroid hormones control the degradation of specific mRNAs, and regulate transcript stability of a variety of genes (including vitellogenin and ERα [15]). To determine the effects of E_2 on LRH-1 total mRNA stability, we treated two ER+ cell lines (MCF-7 and T47D) with E_2 and found significant increase in steady state LRH-1 transcript levels with treatment ($p < 0.001$). Conversely, co-treatment with ERα antagonist ICI 182,780 caused a reduction in mRNA levels ($p < 0.001$, Fig. 4A and B), and total transcript degradation rates were increased (Fig. 4C and D). LRH-1 mRNA half-life was decreased from 1.28 h to 0.47 h in E_2 treated cells ($p < 0.01$, Fig 4E and F) and decrease was inhibited by ICI 182,780 in the presence of E_2 ($p < 0.01$, Fig 4E and F). These findings suggest that the effects of E_2 on LRH-1 mRNA stability are mediated via ERα.

4. Discussion

In the breast, LRH-1 regulates oestrogen production in cancer-associated stroma. In tumor epithelial cells, LRH-1 is involved in

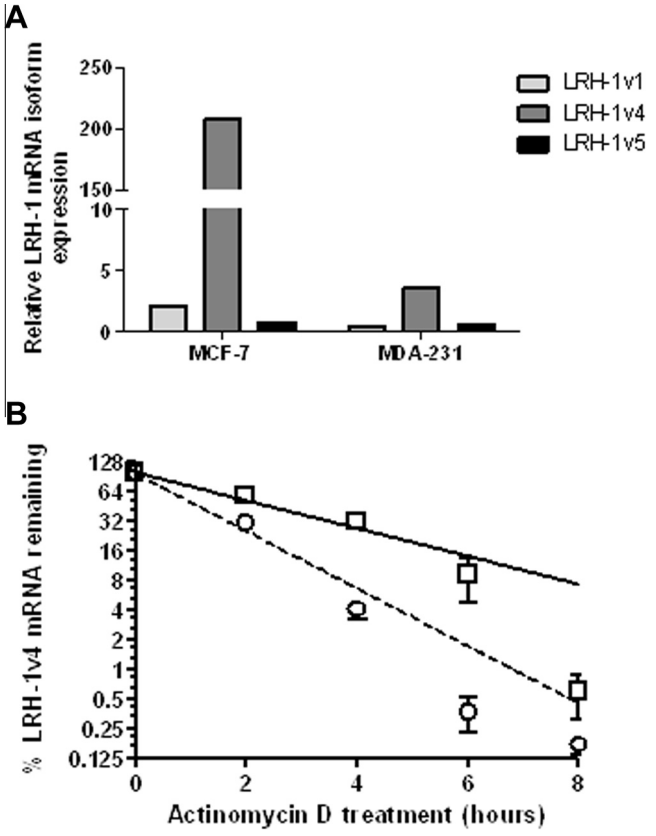

Fig. 3. Expression of LRH-1 isoforms and its stability in breast cancer cell lines. (A) LRH-1 variant mRNA levels were determined by RT-PCR in MCF-7 and MDA-MB-231 cell lines using specific primers that amplify v1, v4 or v5 sequences. (B) Actinomycin-D decay study was performed on MCF-7 and MDA-MB-231 cell lines. Levels of LRH-1v4 were measured at the specified time points. Each plot is a percentage of the 0 h value on a logarithmic scale. Dotted line represents ER+ MCF-7 cells and straight line represents ER− MDA-MB-231 cells. ○, MCF-7, □, MDA-MB-231 Data shown from independent experiments as $n = 3 \pm$ SEM.

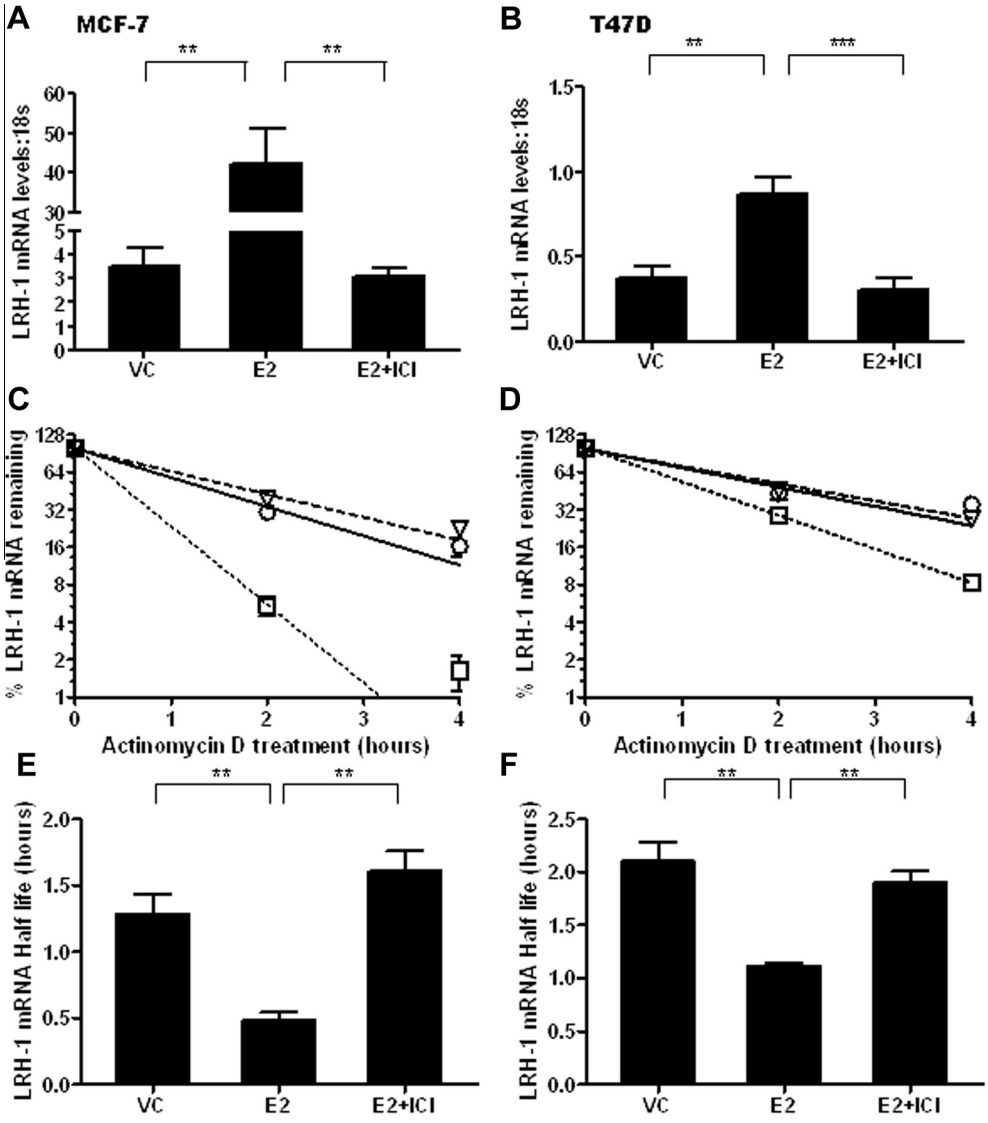

Fig. 4. Oestradiol reduces LRH-1 mRNA stability in MCF-7 (A, C and E) and T47D (B, D and F) cells: Cells were serum starved, then treated with E_2 (10 nM) ± ICI (100 nM) for 24 h. (A and B) Steady state LRH-1 mRNA expression levels were quantified against *18s*. (C and D) Actinomycin-D decay study was then performed. ∇VC, □, E_2; ○, E_2 + ICI. (E and F) Half life was interpolated from decay log curve above. Data shown from independent experiments as $n = 3 \pm$ SEM, $^{**}P < 0.01$, $^{***}P < 0.001$.

ERα-mediated gene transcription, cell proliferation, motility and invasion [7,8,16,17]. Expression of LRH-1 in ER+ breast cancer, and its role in oestrogen synthesis, has driven the search for synthetic antagonists to block its oestrogenic and proliferative effects [18–20]. Previous studies have shown that LRH-1 mRNA expression is restricted to ER+ breast cancer cells. However, apparent expression and activity of LRH-1 in ER− tissues and cells prompted us to investigate LRH-1 expression at the protein level. Here we systematically correlated LRH-1 mRNA and protein expression levels in a panel of ER+ and ER− breast cancer cell lines. Despite large differences in mRNA levels between ER+ and ER− cells, LRH-1 protein is present at similar levels across these cell lines. Interestingly, a ~60 kDa isoform is detected in both ER+ and ER− cells while a ~56 kDa isoform was detected exclusively in the ER− cells; both of which are bioactive. The presence of LRH-1 protein in ER− cells may explain previous studies reporting its functional effects in cancer where siRNA-mediated knockdown of LRH-1 inhibited tumor cell motility and invasion similarly in both ER− MDA-MB-231 and ER+ MCF-7 cells [10]. Immunoblotting indicated similar expression of LRH-1 protein in MCF-7 and MDA-MB-231 cells [10]. In line with clinical immunohistochemistry data, LRH-1 protein is localised in the nucleus of epithelial cells in both ER+ and ER− breast tumor biopsy samples [9].

Tumor cells regulate rapid proliferation by altering the rate of protein synthesis via several mechanisms including gene transcription and changes to mRNA stability. To address the discrepancy observed between LRH-1 mRNA and protein levels, we assessed LRH-1 mRNA transcript and protein stability rates. In ER− cells, mRNA and protein is approximately 2-fold and 3-fold respectively more stable compared to ER+ cells. We identified a stable LRH-1 isoform LRH-1v4 (~56 kDa) in MDA-MB-231. This isoform is likely relevant in breast cancer, and isoform-specific expression studies would clarify its role in ER− tumors.

Our findings contrast with those of a previous report where no change in LRH-1 mRNA stability was measured in MCF-7 treated with E_2 [8]. Along with the two newly discovered variants, our study used primers that detected all five variants, while in the previous study probes measured expression of three transcript variants [6,8]. Our observations are in line with another study identifying LRH-1v4 as the dominant transcript in breast cancer

cells, and its high responsiveness to E_2 [6]. We show that LRH-1v4 transcript is relatively more stable in MDA-MB-231 compared to MCF-7 cells. The increased stability of LRH-1v4 in MDA-MB-231 cells may contribute to the increased translation to protein in MDA-MB-231 cells. We were unable to detect LRH-1v4 in MCF-7 cells, therefore we hypothesise that LRH-1v4 transcript in MCF-7 cells may be highly unstable and possibly modified through post-translation mechanisms. Further studies involving the translation of each transcript isoform may be required to further understand the discordance of LRH-1 transcript and protein in breast cancer cell lines.

Steroid hormones were shown to be the first regulators of mRNA stability and degradation processes [31]. We hypothesised that this may account for the differences in LRH-1 mRNA decay rates observed in ER+ and ER− breast cancer cells. The stability of a large number of mRNA transcripts encoding for proteins such as vitellogenin, ApoVLDII, ovalbumin, conalbumin, renin, progesterone receptor and ERα is regulated by steroid hormones [15]. The earliest characterised is the stabilization of vitellogenin mRNA by E_2 [21]. Treatment with E_2 decreases ERα mRNA stability from 4 h to 40 min in MCF-7 cells [22]. Here we demonstrate that LRH-1 mRNA stability is reduced, like that of ERα, with E_2 treatment in MCF-7 and T47D cells. Furthermore, treatment with the ERα antagonist compound ICI 182,780 increased LRH-1 mRNA stability, implicating an ERα-regulatory mechanism in MCF-7 and T47D cells.

The expression and turnover of mRNAs is a crucial step in post-transcriptional regulation of protein expression and is regulated by *cis*-acting elements located in the 3′UTR, such as the AU-rich elements (AREs) and microRNAs (miRNAs) [23]. These are bound by protein factors that in turn recruit deadenylation enzymes causing mRNA decay [24]. An example of this is observed in the regulation of vitellogenin mRNA stability via an E_2-regulated *cis*-acting element in the vitellogenin 3′UTR [25]. The analysis of two main LRH-1 transcript variants revealed very long 3′-UTRs and sequence analysis of this region revealed a repetition of an AUUUA motif which plays an important role in mRNA decay kinetics [26,27]. Factors binding to these AUUUA motifs may affect the stability of LRH-1 mRNA differentially in ER− and ER+ cell lines. Interestingly these two different transcripts of LRH-1 (5.2 kb and 3.8 kb) were differentially expressed in normal liver and a hepatoma cell line; 5.2 kb transcript was predominant in normal liver tissue, while the 3.8 kb transcript increased in hepatoma HepG2 cells [26]. The authors proposed this difference in transcripts and their 3′-UTRs lengths may impact mRNA stability and LRH-1 isoform expression levels in the different cell lines. Interestingly, we did observe two LRH-1 isoforms of different sizes in the MDA-MB-231 and MCF-7 cells.

Comparatively little is known regarding the regulation of LRH-1 expression and activity in ER− breast cancer cells. In ER− cells, other additional mechanisms may be involved in the stabilisation of LRH-1 mRNA which is reflective of the high proliferative index and metastatic nature of these cells. Protein factors such as heterogeneous nuclear ribonucleoproteins (hnRNPs) and the HuR proteins, and endonucleases have been documented to affect mRNA stability in tumor cell [28].

In conclusion, we have shown different LRH-1 isoforms are expressed in ER− and ER+ breast cancer cells, with protein levels consistent across a panel of breast cancer cell lines. We found mRNA transcript and protein stability rates were reduced in ER+ cells, which may in part explain the discordant mRNA and protein expression observed. LRH-1 is a novel therapeutic target in ER+ breast cancer [19]. The presence of LRH-1 in ER− cells suggests that targeting its proliferative effects may be beneficial in ER− disease, as it is in ER+ tumors.

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