The orphan nuclear receptor LRH-1 promotes breast cancer motility and invasion

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Abstract

The orphan nuclear receptor liver receptor homologue-1 (LRH-1) has roles in the development, cholesterol and bile acid homeostasis, and steroidogenesis. It also enhances proliferation and cell cycle progression of cancer cells. In breast cancer, LRH-1 expression is associated with invasive breast cancer; positively correlates with ER α status and aromatase activity; and promotes oestrogen-dependent cell proliferation. However, the mechanism of action of LRH-1 in breast cancer epithelial cells is still not clear. By silencing or over-expressing LRH-1 in ER-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cells, we have demonstrated that LRH-1 promotes motility and cell invasiveness. Similar effects were observed in the non-tumourigenic mammary epithelial cell line, MCF-10A. Remodelling of the actin cytoskeleton and E-cadherin cleavage was observed with LRH-1 over-expression, contributing to increased migratory and invasive properties. Additionally, in LRH-1 over-expressing cells, the truncation of the 120 kDa E-cadherin to the inactive 97 kDa form was observed. These post-translational modifications in E-cadherin may be associated with LRH-1-dependent changes to matrix metalloproteinase 9 expression. These findings suggest a new role of LRH-1 in promoting migration and invasion in breast cancer, independent of oestrogen sensitivity. Therefore, LRH-1 may represent a new target for breast cancer therapeutics.

Endocrine-Related Cancer (2010) 17 965–975

Introduction

Liver receptor homologue-1 (LRH-1, NR5A2) belongs to the NR5A orphan nuclear receptor subfamily and has important roles in embryonic and adult tissues. LRH-1 expression in embryonic stem cells (ESC) allows activation of Oct 4, a requirement for early embryonic differentiation of primitive endoderm/mesoderm or trophoblast (Gu *et al.* 2005). Other LRH-1 target genes important for endodermal differentiation include *GATA*, *Nkx*, *HNF3- β* , *HNF4- α* and *HNF1- α* (Pare *et al.* 2001). Consistent with its expression and function in embryonic tissue, the deletion of LRH-1 results in embryonic lethality (Galarneau *et al.* 1996, Gu *et al.* 2005).

In the adult tissue, LRH-1 has known functions in steroidogenesis, cholesterol homeostasis and carcinogenesis (reviewed in Francis *et al.* (2003), Clyne *et al.* (2004), Fayard *et al.* (2004) and Lee & Moore (2008)). LRH-1 has been shown to be a tumour-promoting transcription factor in colon, gastric and breast cancers (Botrugno *et al.* 2004, Schoonjans *et al.* 2005, Miki *et al.* 2006, Wang *et al.* 2008). LRH-1 increases cell proliferation in murine pancreatic LTPA and hepatic FL83B cells through the induction of cyclins, D1 and E1, with co-operative recruitment of β -catenin and LRH-1 to their promoters (Botrugno *et al.* 2004). In a mouse model of azoxymethane-induced colon neoplasia, a reduction in LRH-1 expression resulted

in reduced cancer incidence and better survival rates (Schoonjans *et al.* 2005). LRH-1 regulates intestinal crypt length and crypt cell proliferation through the G1 cyclins and c-Myc (Botrugno *et al.* 2004).

In pre- and post-menopausal breast cancer patients, high LRH-1 protein expression is detected in invasive ductal carcinomas (43%), ductal carcinoma *in situ* (28%) and adipose stromal cells (Annicotte *et al.* 2005, Zhou *et al.* 2005, Miki *et al.* 2006). This aberrant expression of LRH-1 in breast cancer stroma activates aromatase transcription thus increasing local oestrogen production within the tumour microenvironment (Bulun & Simpson 1994, Simpson & Davis 2001, Simpson *et al.* 2001, Zhou *et al.* 2005). This is a particularly relevant mechanism in post-menopausal breast tumours as intra-tumoural oestrogen levels and aromatase activity are high compared to pre-menopausal plasma and tissue levels (Pasqualini *et al.* 1996). *LRH-1* has also been described as an oestrogen target gene and shown to be a downstream effector of oestrogen-mediated cell proliferation (Annicotte *et al.* 2005). Hence, a positive feedback mechanism between LRH-1 and oestrogen production may be a critical component of oestrogen-dependent tumour growth. However, the question remains as to whether LRH-1 has additional roles, aside from oestrogen production in stromal cells, and whether LRH-1 plays a role in oestrogen-independent cancer growth.

LRH-1 is held in a constitutively active structural conformation with a stable fourth helical layer. Hence, its activity is modulated by the interaction with other co-activators and co-repressors (Goodwin *et al.* 2000, Lee & Moore 2002, Xu *et al.* 2004, Ortlund *et al.* 2005). Additionally, the identification of phospholipids (Krylova *et al.* 2005, Ortlund *et al.* 2005, Wang *et al.* 2005b) and sphingosine-1-phosphate (Hadizadeh *et al.* 2008) as LRH-1 agonists suggests that the degree of receptor activation may be further regulated with endogenous ligands. This also raises the possibility of pharmacological modulation of LRH-1 activity as treatment for intestinal, gastric and breast cancers. Since LRH-1 controls oestrogen production in a tissue-specific manner, potential LRH-1 antagonists could allow breast-specific suppression of aromatase activity (Safi *et al.* 2005).

Given the recent demonstrations of LRH-1 expression in breast carcinoma cells, this study tests the hypothesis that LRH-1 directly regulates breast cancer migration and invasion. We have found that LRH-1 directly regulates these processes by actin remodelling and post-translational inactivation of E-cadherin, consistent with the epithelial-mesenchymal transition (EMT). This effect also occurs

independent of oestrogen and in non-cancerous cells such as MCF-10A. Based on these findings, LRH-1 is a potential candidate for the development of new breast cancer treatments.

Materials and methods

Plasmids, cell lines and transfections

Human LRH-1 pcDNA expression construct was generated by cloning into the pCDNA3.1 vector. The SureSilencing short hairpin RNA (shRNA) plasmids (KH05888G, SABiosciences, Frederick, MD, USA) were designed to specifically knockdown the expression of LRH-1 by RNA interference. Cells were transfected with the following plasmids pcDNA (control), pcDNA-LRH-1 (+LRH-1), scrambled shRNA (shcontrol) or shRNA against LRH-1 (shLRH-1). The following cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) MCF-7 (ATCC No. HTB-22), MCF-10A (ATCC No. CRL-10317) and MDA-MB-231 (ATCC No. HTB-26) and were cultured according to the manufacturer's procedures at 37 °C with 5% CO₂. Cells were transfected using electroporation (Nucleofector kit V, Amaxa Biosystems, Lonza, Walkersville, MD, USA), Solution V and the recommended programmes as follows: MCF-7 cells – P-20 (high transfection efficiency) and E-14 (high cell viability and short-term expression); MDA-MB-231 cells – X-013; and MCF-10A cells – T-024. These are described online (<http://www.amaxa.com/research-areas/cancer/>). Highest transfection efficiency is detected at 24–48 h post transfection and this was measured by GFP protein expression as expressed by the plasmid constructs, mRNA quantitation by qPCR and western blot analysis.

RNA extraction and quantitation of mRNA expression

Total RNA was extracted using the RNeasy Minikit (Qiagen) as described in the manufacturer's instructions. Reverse transcription was performed as instructed for 1 µg of RNA using the AMV Reverse Transcription System (Promega). qPCR amplification was performed on the Roche LightCycler System (Roche Diagnostics) using Fast Start Master SYBR Green 1 and specific primer sets (Sigma) as previously described for LRH-1 and 18S (Clyne *et al.* 2002). Primer sequences are as follows: LRH-1 (sense, CTG ATA CTG GAA CTT TTG AA; antisense, CTT CAT TTG GTC ATC AAC CTT); 18S (sense, CGG CTA CCA CAT CCA AGG A; antisense, GCT GGA ATT ACC GCG GCT);

E-cadherin (sense, GGC ACA GAT GGT GTG ATT ACA GTC AAA A; antisense, GTC CCA GGC GTA GAC CAA GAA A) and matrix metalloproteinase 9 (MMP9; sense, CGC TAC CAC CTC GAA CTT TG; antisense, GCC ATT CAC GTC GTC CTT AT). Experimental samples were repeated in triplicates for each transfection and quantified by comparison with a six-point standard curve as previously described (Chand *et al.* 2007).

Wound-healing migration assay

Transfected cells were serum-starved overnight before a scratch wound was created in the mono-confluent cell layer created with a sterile plastic pipette tip. Phase contrast microscopy images of the wound were taken at 0, 6, 12 and 24 h of the assay and gap closure measured using Analysis LS Professional (Soft Imaging Systems GmbH, Münster, Germany). For the accuracy of scratch/wound width, three scratch wounds were made per well and each transfection condition was repeated 3–4 times per experiment. Experiments were repeated at least 4 times.

Cytoskeleton F-actin staining with phalloidin

Transfected cells were allowed to attach to the coverslips before creation of a scratch wound in the confluent monolayer as described above. At 48 h post wound creation, cells were washed twice with PBS, fixed in 3.7% paraformaldehyde in PBS and permeabilised in 0.1% Triton X-100 and incubated with 200 µl of Alexa Fluor 488 phalloidin (Invitrogen) staining solution diluted in 1% BSA for 20 min at room temperature. Coverslips were then incubated with the nuclear stain TO-PRO-3 (Invitrogen) at a 1:200 dilution (PBS) covered for 10 min at room temperature. Following two PBS washes, the coverslips were mounted with fluorescent mounting media (Dako, Glostrup, Denmark) and were allowed to dry before being analysed. Immunofluorescence was visualised using an Olympus FV300 confocal microscope and images were captured and analysed using Olympus FluoView™ software. Actin projections were counted in ten cells from each transfection condition and in three separate transfection experiments. Cell volume was measured using the ImageJ software (<http://rsbweb.nih.gov/ij/>).

Cell invasion assay

Following transfection, cells were serum-starved overnight. Cells were then plated at a density of 0.25×10^5 cell per insert onto the Matrigel Invasion Matrix (BD Biosciences, San Jose, CA, USA). Five per cent FCS

was used as a chemoattractant. Invaded cells were stained with 0.005% Crystal Violet (Sigma) according to the manufacturer's instructions. Cells were counted per well and data were presented. Experiments were repeated in triplicate with each transfection condition performed in triplicate invasion chambers per experiment.

Determination of cell number

To determine the cell number in MCF-10A cells, transfected cells grown on the coverslips were fixed and stained with the nuclear stain, 4',6-diamidino-2-phenylindole (DAPI) nuclei using methods described above. The slides were blinded to remove counting bias. Positively stained nuclei were counted, in two slides per transfection condition, and in total cells from three separate experiments.

Western blot analysis

Cells were lysed in 100 µl lysis buffer (5 mm HEPES, 137 mm NaCl, 1 mm MgCl₂, 1 mm CaCl₂, 10 mm NaF, 2 mm EDTA, 10 mm Na pyrophosphate, 1% Nonidet P-40, 10% glycerol and protease inhibitors (Roche)). Total protein concentration was measured using bicinchoninic acid method (Pierce Biotechnology, Rockford, IL, USA). In total, 50 µg cell lysate separated by SDS-PAGE using 10% SDS-PAGE gels were transferred to nitrocellulose membranes by electroblotting. Membranes were blocked with 5% (w/v) milk protein in Tris-buffered saline containing 0.05% Tween-20. Blots were then incubated with primary antibodies as indicated, washed in Tris-buffered saline containing 0.1% Tween-20 and probed with primary antibodies at a dilution of 1:500 for LRH-1 (Abcam, Cambridge, MA, USA), 1:5000 for E-cadherin (BD Biosciences) and 1:10 000 for β-tubulin (Santa Cruz, Santa Cruz, CA, USA). Secondary IgG-conjugated mouse or rabbit antibodies, and Alexa fluor@ 700 (Invitrogen) were used at a dilution of 1:10 000 to visualise protein bands. Band intensities were quantified using the Odyssey infrared imaging system and Odyssey 3.0 Software (LI-COR Biosciences, Lincoln, NE, USA). Blots shown are representative of a minimum of three separate experiments.

Statistical analysis

All experiments were repeated at least three times. Data are presented as mean $\pm$ s.e.m. ANOVA or Student's independent *t*-test was calculated for experiments using GraphPad Prism 5.0 (GraphPad, La Jolla, CA, USA) and a *P* value of <0.05 was considered statistically significant.

Results

LRH-1 expression mammary epithelial cells lines

To ascertain *LRH-1* mRNA and protein expression in cell lines used in the following study, qPCR and western blot analysis were performed. As previously reported (Annicotte *et al.* 2005), the ER-negative MDA-MB-231 cells expressed lower levels of *LRH-1* mRNA compared to the ER-positive MCF-7 cell line ($P < 0.05$; Supplementary Figure 1a, see section on supplementary data given at the end of this article). *LRH-1* mRNA was also detected, however, at lowest levels, in the non-tumourigenic mammary epithelial cell line, MCF-10A ($P < 0.05$ when compared to expression in MCF-7 cells; Supplementary Figure 1a). However, western blot analysis demonstrated similar protein expression in all the three cell lines (Supplementary Figure 1a). Highest levels of LRH-1 was observed in the MCF-10A cell lines compared to MCF-7 and MDA-MB-231 cells ($P < 0.05$; Supplementary Figure 1b), however, no difference in protein was observed between MCF-7 and MDA-MB-231 cells (Supplementary Figure 1b). Therefore, although ER-negative cell lines express less *LRH-1* mRNA than ER-positive cells, the protein expression is similar in all cells.

LRH-1 influences cell motility through actin remodelling in MCF-7 cells

To analyse the function of LRH-1 in breast cancer epithelial cells, endogenous LRH-1 expression was knocked down in the MCF-7 cell line using shRNA. Cells were transfected with control (shcontrol) or LRH-1-specific shRNAs (shLRH-1), and GFP expression from the tagged expression vectors indicated transfection efficiencies of $\sim 70\%$ (Fig. 1a). Twenty-four hours post transfection, shLRH-1 produced a 50% decrease in *LRH-1* mRNA ($P < 0.001$; Fig. 1b) and immunoblots of whole cell lysate demonstrated knockdown of protein by 61% compared to control levels ($P < 0.001$; Fig. 1c and d).

Cell migration is central to the process of EMT and plays a key role in cancer progression and metastasis. To characterise the effects of LRH-1 in breast cancer cell migration we used the wound-healing assay. LRH-1 knockdown significantly impaired the ability of MCF-7 cells to migrate into the created gap. Differences in wound healing were apparent from 6 h post wound initiation; closure of the gap was 21% in controls and 4% in LRH-1 knockdown cells (Fig. 1e). Following this trend, at 12 and 24 h, gap closure was $\sim 10\%$ at both time points in LRH-1 knockdown

compared to 24 and 30% in control MCF-7 cells at 12 and 24 h ($P < 0.01$ and $P < 0.001$ respectively; Fig. 1e).

Actin re-arrangement into stress fibres and the development of protrusions from the outer cell surface mark the start of migration and are the hallmarks of EMT. To investigate the effect of LRH-1 on the development of lamellipodia and cytoskeletal rearrangements, phalloidin stain was used to visualise the actin cytoskeleton. Suppression of LRH-1 significantly reduced cell surface lamellipodia protrusions compared to controls at 24 h post transfection ($P < 0.01$; Fig. 1f and g). The predominantly cortical actin arrangement in these cells was retained after LRH-1 suppression.

The initiating step in tumour metastasis is the dislodgement of cells from the primary tumour, and their subsequent invasion into adjacent tissues followed by metastasis. LRH-1 silencing caused a 20% decrease in cell invasion ($P < 0.05$; Fig. 1h).

To extend the above observations, LRH-1 was over-expressed in MCF-7 cells and cell motility and actin structures were visualised as before. Transfection with an LRH-1 expression vector resulted in a 12-fold increase in *LRH-1* mRNA expression compared with vector-only-transfected cells ($P = 0.005$; Fig. 2a). LRH-1 protein levels were increased twofold in LRH-1-transfected cells versus vector-only-transfected cells ($P < 0.05$; Fig. 2b). Over-expression of LRH-1 accelerated migration into the created wound within 6 h post wound initiation (Fig. 2c and d). In over-expressing cells, 10, 29 and 31% gap closure was seen at the 6, 12 and 24 h time points while 2, 4 and 10% closure was observed at 6, 12 and 24 h in controls ($P < 0.05$, $P < 0.001$ and $P < 0.001$ respectively; Fig. 2c–e). It is to be noted that control transfections with empty vectors (pcDNA or shRNA control) produced some variation in cell migratory patterns (Figs 1e and 2d). This is reflected in the differences in percentage gap closure in controls.

Appearance of filamentous actin protrusions were seen on the cell surface close to the wound edge in LRH-1 over-expressing cells compared to controls and quantitation of protrusion numbers indicated a twofold increase in LRH-1 over-expressing cells ($P = 0.005$; Fig. 2e). Furthermore, MCF-7 cell invasiveness increased by 40% when LRH-1 was over-expressed ($P < 0.05$; Fig. 2f).

LRH-1 influences cell motility through actin remodelling in MDA-231 cells

LRH-1 is known to stimulate expression of both aromatase (Bouchard *et al.* 2005) and is also an ER α target gene (Annicotte *et al.* 2005) in MCF-7 cells.

Figure 1 (a) Transfection efficiency was examined using GFP-tagged shRNA plasmid against the *LRH-1* gene (shLRH-1) in MCF-7 cells. (b) Real-time quantitation of *LRH-1* mRNA in the knockdown and control transfections demonstrated a 50% decrease in transcript levels. (c) Western blot analysis of whole-cell extracts collected 24 h post transfection with either shLRH-1 or control shRNA plasmids. Densitometric analysis revealed a reduction of LRH-1 expression of 60%, normalised to β -tubulin. (d) Wound-healing assay ($\times 4$ magnification) at 6, 24 and 48 h post wound initiation and (e) quantitation of migration following knockdown versus controls. (f) Cell morphology at the wound edge ($\times 100$ magnification), stained for actin fibres with phalloidin (green) and nucleus with ToPro (blue) show actin projections (white arrows) that were reduced at the wound edge in LRH-1 knockdown cells (g). (h) Invasion assays show reduction in invasiveness in LRH-1 knockdown cells. Data are presented as mean $\pm$ S.E.M., $n=3$ separate experiments, * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ versus controls.

To determine whether LRH-1-dependent cell migration occurs independently of oestrogen, we repeated these experiments using the ER-negative MDA-MB-231 cell line and obtained similar results (Supplementary Figures 2 and 3, see section on supplementary data given at the end of this article). LRH-1 knockdown cells showed 5 and 20% migration at 6 and 24 h time points respectively while scrambled shRNA control cells showed 18 and 47% migration (Supplementary Figure 2c and d). A 60% reduction in cell invasiveness was observed in LRH-1 knockdown cells compared to controls ($P<0.05$; Supplementary Figure 2e). Similarly, when LRH-1 was over-expressed, 30 and 70% migration was observed at 6

and 24 h compared to controls (15 and 43% at 6 and 24 h) ($P<0.05$ and $P<0.01$ respectively compared to controls) (Supplementary Figure 3b and c). As MDA-MB-231 is a highly invasive cell line, no further increase in cell invasion was measured in LRH-1 over-expressing cells (Supplementary Figure 3d).

LRH-1 enhances EGF-dependent migration and actin cytoskeleton changes in MCF-10A cells

To determine whether these effects of LRH-1 were confined to malignant breast cancer cells, experiments were repeated in the immortalised, non-tumourigenic mammary epithelial cell line, MCF-10A. LRH-1

Figure 2 (a) Real-time quantitation of mRNA transcripts in LRH-1 over-expressing cells and control MCF-7 transfections demonstrated a 12-fold increase in transcript levels. (b) LRH-1 and β -tubulin immunoblots of lysates collected 24 h post transfection with either pCDNA-LRH-1 or pCDNA plasmids. Densitometric analysis revealed a 200% increase in protein. (c) Wound-healing assay ($\times 4$ magnification) at 6, 24 and 48 h post wound initiation and (d) quantitation of migration. (e) Cell morphology at the wound edge ($\times 100$ magnification), stained with phalloidin (green) and ToPro (blue) demonstrating actin projections (white arrows) at the wound edge were increased in LRH-1 over-expressing cells (f). (g) Cell invasion increased with LRH-1 increase in the cells. Data are presented as mean $\pm$ S.E.M., $n=3$ separate experiments, * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ versus controls.

over-expressing cells had 62% cell migration compared to 20% in controls at 24 h post assay initiation ($P<0.05$; Fig. 3a and b). Over-expression of LRH-1 caused increased actin stress fibres in the cell architecture; membrane protrusions were observed and

these rapidly formed connections with the neighbouring cells (Fig. 3c). Cells were also larger in size compared to control transfections ($P<0.001$) and were more elongated in structure (Fig. 3c). An increase in cell number was also observed following LRH-1 over-expression ($P=0.005$; Fig. 3e). As migration and cell proliferation were rapid following LRH-1 over-expression in MCF-10A cells, experiments were conducted at the 24 h point only.

The above-described experiments demonstrated that LRH-1 is directly involved in cell migration in both normal and malignant breast epithelial cells, and this effect is independent of ER α status.

LRH-1 over-expression causes post-translational cleavage of E-cadherin in conjunction with increased MMP9 expression

It is well established that alterations in E-cadherin function are correlated to increased malignancy and metastasis of epithelial cancer cells, and particularly related to EMT. The loss of E-cadherin function occurs through several mechanisms including transcriptional or methylation repression of the gene and post-translational truncation (Graff *et al.* 1995, Hajra *et al.* 1999). Therefore, we have studied the expression of E-cadherin using qPCR and western blot analysis in MCF-7 cells over-expressing LRH-1.

E-cadherin mRNA expression was significantly altered with changes in LRH-1 expression (Fig. 4a). With LRH-1 over-expression, a twofold decrease in *E-cadherin* mRNA was observed ($P<0.005$). Conversely, with the knockdown in LRH-1, a 0.5-fold increase in *E-cadherin* mRNA was noted ($P<0.05$). Western blot analysis indicated that cells with high LRH-1 protein expression (Fig. 4b and c) exhibited little or no change in the mature 120 kDa form of E-cadherin (E-cad¹²⁰) compared with control cells (Fig. 4b and e). In the LRH-1 over-expressing cells, however, there were higher levels of the truncated, inactive 97 kDa form of E-cadherin (E-cad⁹⁷) compared with control cells (Fig. 4b and d). Densitometric quantitation was used to determine the ratio E-cad⁹⁷ to E-cad¹²⁰ in control and LRH-1 over-expressing cells (Fig. 4f). With a twofold elevation in LRH-1 expression, the E-cad¹²⁰ to E-cad⁹⁷ ratio showed a twofold decrease; indicating a twofold increase in inactive E-cad⁹⁷ levels (Fig. 4b, d and f). Since MMPs play important roles in cancer progression and are known to regulate E-cadherin cleavage, we examined MMP9 expression levels. Knockdown of LRH-1 expression in MCF-7 cells resulted in a 70% reduction in *MMP9* mRNA expression ($P<0.05$; Fig. 4g). Furthermore, in LRH-1

Figure 3 (a) Wound-healing phenotype ($\times 4$ magnification) and (b) quantitation of migration in MCF-10A cells following LRH-1 over-expression (+LRH-1) compared with cells transfected with plasmid vector only. (c–e) Photographs demonstrating differences in cell morphology and number: (c) cells photographed at $\times 40$ magnification are stained with phalloidin (green) and DAPI (blue) at 24 h post wound initiation (top panel) and DAPI-stained cells photographed at $\times 10$ magnification (bottom panel). Increases in cell size (d) and cell number (e) were observed with increased LRH-1 expression. Data are presented as mean $\pm$ S.E.M., $n =$ at least 3 separate experiments, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control.

over-expressing MCF-7 cells, a fourfold increase was observed in MMP9 expression compared with controls ($P < 0.005$; Fig. 4h). These data suggest that the changes observed in E-cadherin may be mediated by the regulation of MMP9 expression by LRH-1.

Discussion

LRH-1 has well-established roles in embryonic development, cholesterol and bile acid metabolism and steroidogenesis. Recent studies have also highlighted its effects on cancer cell proliferation, particularly in tumours of the intestine and stomach (Botrugno *et al.* 2004, Schoonjans *et al.* 2005, Wang *et al.* 2008). In breast cancer, LRH-1 stimulates aromatase expression in adipose stromal cells, thereby

increasing the availability of local oestrogen for ER-positive tumour growth (Clyne *et al.* 2002, Zhou *et al.* 2005). However, LRH-1 is also expressed in breast cancer epithelial cells where it could potentially have direct effects, independent of aromatase (Miki *et al.* 2006). In this study, using shRNA and over-expression strategies, we have shown that LRH-1 directly stimulates cell migration and invasion in both ER-positive and ER-negative breast cancer cell lines, as well as in the normal breast epithelial MCF-10A line. These effects are associated with actin remodelling, and post-translational inactivation of E-cadherin, suggestive of EMT.

LRH-1-induced motility and invasiveness was associated with decreased *E-cadherin* mRNA expression and cleavage of the membrane-bound E-cadherin protein (E-cad¹²⁰) to the inactive form (E-cad⁹⁷). In invasive breast and prostate cancer epithelial cell populations, the loss of cadherin-based adherens junctions is due to the cleavage, in the cytosolic domain, of E-cad¹²⁰ to E-cad⁹⁷ (Day *et al.* 1999, Vallorosi *et al.* 2000, Rashid *et al.* 2001). Facilitated by MMPs (Noe *et al.* 2001, Marambaud *et al.* 2002), this removes the β -catenin-binding domain from its cytoplasmic tail leaving a membrane-bound E-cad⁹⁷ and a free cytoplasmic 35 kDa form (E-cad³⁵) still bound to β -catenin (Vallorosi *et al.* 2000).

The proteolytic processing of E-cadherin adherens junctions by MMPs plays a crucial cancer progression by allowing changes in cell adhesion, invasion, signalling and apoptosis (Vallorosi *et al.* 2000, Egeblad & Werb 2002, Marambaud *et al.* 2002, Lopez-Otin & Matrisian 2007). In LRH-1 over-expressing MCF-7 cells, we found an increase in *MMP9* mRNA expression. This LRH-1-dependent effect was validated in the knockdown experiments. Interestingly, evidence of LRH-1-dependent regulation of MMPs was demonstrated recently (Duggavathi *et al.* 2008). Using a granulosa cell-specific LRH-1 knockout mouse, a downregulation of MMPs (MMP2, MMP9 and MMP19) was observed (Duggavathi *et al.* 2008). The regulation of E-cadherin and MMP9 provides some evidence in support of LRH-1 promoting processes involved in EMT. Recently, breast cancer stem cells were shown to share EMT attributes (Mani *et al.* 2008, Blick *et al.* 2010), survival as individual cells outside the tumour and resistance of cells to the current therapies (reviewed in Polyak & Weinberg (2009)). Given the role of LRH-1 in ESCs and the implication that LRH-1 may support EMT in breast cancer epithelial cells, further analysis of LRH-1 in breast cancer stem cells is warranted.

Figure 4 (a) E-cadherin mRNA expression MCF-7 cells with pCDNA (control), pCDNA-LRH-1 (+LRH-1), shcontrol and shLRH-1 plasmids. (b) LRH-1, E-cadherin and β-tubulin immunoblots of lysates collected 24 h post transfection with either pCDNA or pCDNA-LRH-1 plasmids. The densitometric analysis of immunoblots for (c) LRH-1, (d) E-cad⁹⁷ (e) E-cad¹²⁰ and (f) E-cad¹²⁰ to E-cad⁹⁷ ratio in control and +LRH-1. The E-cad¹²⁰ to E-cad⁹⁷ ratio in +LRH-1 cells reflects an increase in the inactive, truncated E-cad⁹⁷ form. MMP9 mRNA expression was analysed in (g) shLRH-1 and (h) +LRH-1 cells. Data are presented as mean $\pm$ s.e.m., n = at least 3 separate experiments, * P < 0.05, ** P < 0.01 and *** P < 0.001 versus controls.

Our data suggest that LRH-1 has both direct stimulatory effects on breast cancer cell motility and invasion, in addition to indirect proliferative effects mediated by activation of aromatase expression (Clyne et al. 2002, Zhou et al. 2005). It is therefore an attractive therapeutic target. As an orphan receptor, however, no classical ligands for LRH-1 have been identified. Recently, the crystal structure of the LRH-ligand-binding domain revealed the presence of bacterial

phospholipids derived from the *Escherichia coli* expression system used to produce the receptor (Krylova et al. 2005, Ortlund et al. 2005, Wang et al. 2005a). At present it is unclear whether mammalian phospholipids bind to LRH-1 endogenously, although they can readily be exchanged for bacterial lipids bound to the LRH-1 homologue SF-1, *in vitro* (Sablin et al. 2009). Irrespective of the existence or otherwise of an endogenous ligand, the presence of a conserved large hydrophobic pocket with the LRH-1 LBD should allow the identification of synthetic ligands that could fit into this space and potentially modulate LRH-1 activity. Indeed, compounds that bind to LRH-1 and displace bound phospholipids have been described, however, these functioned as agonists rather than antagonists (Whitby et al. 2006); to date, no small molecule LRH-1 antagonists (or inverse agonists) have been identified.

It is thought that LRH-1 activity is regulated primarily by the recruitment of co-activator (Xu et al. 2004) or co-repressor (Sablin et al. 2008) protein partners. Recently, we have described a series of short peptides, identified by phage display, that bind to the LRH-1 LBD and inhibit recruitment of co-activators (Safi et al. 2005). These peptides acted as potent antagonists, completely inhibiting LRH-1 transcriptional activity and its ability to stimulate aromatase promoter activity. Although peptides are not ideal drug candidates, small drug-like molecules that inhibit the interactions between other nuclear receptors and their co-activators have recently been discovered (Arnold et al. 2005). Taking a similar approach may identify candidate inhibitors of LRH-1:co-activator interactions that block both its direct proliferative and indirect oestrogenic effects in breast cancer cells.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1677/ERC-10-0179>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the National Health and Medical Research Council of Australia (C D Clyne), the Victorian Cancer Agency (A L Chand), the Cancer Council Victoria (C D Clyne), the United States Department of Defense (BC084667) (E W Thompson) and by the Victorian Government's Operational Infrastructure Support Program.

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