

Reversible infertility in a liver receptor homologue-1 (LRH-1)-knockdown mouse model

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Abstract. Liver receptor homologue-1 (LRH-1) is an orphan nuclear receptor that has been implicated in steroid hormone biosynthesis and fertility. Herein we describe a transgenic inducible short hairpin (sh) RNA mouse model that was used to study the effect of transient LRH-1 knockdown *in vivo*. Induction of expression of the shRNA directed against LRH-1 for 2–6 weeks resulted in 80% knockdown of LRH-1 protein in the ovary and complete infertility. Gonadotropin hyperstimulation could not rescue the observed defects in ovulation and corpus luteum formation in LRH-1-knockdown mice. The infertility phenotype was fully reversible because LRH-1-knockdown females became pregnant and delivered normal size litters and healthy pups after cessation of LRH-1 shRNA expression. Timed ovarian microarray analysis showed that, in line with the observed decrease in plasma progesterone levels, key steroid biosynthesis genes, namely *Star*, *Cyp11a1*, *Hsd3b* and *Scarb1*, were downregulated in LRH-1-knockdown ovaries. In contrast with what has been described previously, no clear effect was observed on oestrogenic activity in LRH-1-knockdown mice. Only *Sult1e1* and, surprisingly, *Hsd17b7* expression was modulated with potentially opposite effects on oestradiol bioavailability. In conclusion, the fully reversible infertility phenotype of LRH-1-knockdown mice shows the feasibility of an LRH-1 antagonist as new contraceptive therapy with a mechanism of action that most prominently affects cholesterol availability and progesterone production.

Additional keywords: estrogen, follicle culture, folliculogenesis and corpus luteum formation, microarray, NR5A2, ovary, progesterone, steroidogenesis.

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Introduction

The orphan nuclear receptor LRH-1 belongs to the nuclear receptor subfamily 5, group A (NR5A), or fushi tarazu factor-1, subfamily of nuclear receptors that bind to DNA as a monomer (Ellinger-Ziegelbauer *et al.* 1994; Galarneau *et al.* 1998; Kudo and Sutou 1997; Lin *et al.* 2000). Compounds modulating the activity of LRH-1 could represent an attractive new therapy for multiple indications because LRH-1 has been implicated in the inflammatory response, tumour formation, bile acid homeostasis and fertility (Botrugno *et al.* 2004; Schoonjans *et al.* 2005; Mueller *et al.* 2006; Venticlef *et al.* 2006, 2011; Coste *et al.* 2007; Mataka *et al.* 2007; Duggavathi *et al.* 2008; Lee *et al.* 2008; Out *et al.* 2011). The crystal structure of LRH-1 in complex with cofactor peptides reveals the presence of phospholipids in the ligand-binding domain, indicating that the activity of LRH-1 can potentially be modulated (Ortlund *et al.* 2005;

Burendahl *et al.* 2008). This was further delineated by the description of LRH-1 agonists by Whitby *et al.* (2006, 2011).

LRH-1 is expressed during embryonic development, mostly in tissues of endodermal origin. Inactivation of LRH-1 leads to early embryonic lethality at the epiblast stage due to the loss of pluripotency of embryonic stem cells (Pare *et al.* 2004; Gu *et al.* 2005). In adult tissues, LRH-1 mRNA can be detected in the liver, small intestine, adrenal gland, granulosa cells in the ovary, pre-adipocytes and testis (Fayard *et al.* 2004).

In the liver, LRH-1 has been reported to affect genes involved in lipid metabolism and transport, including *Scrab1*, *CETP* and *APOA1* (Luo *et al.* 2001; Schoonjans *et al.* 2002; Delerive *et al.* 2004). Analysis of several genetically engineered mouse models revealed an important role of LRH-1 in bile acid homeostasis and subsequent lipid absorption (Mataka *et al.* 2007; Lee *et al.* 2008; Out *et al.* 2011). In these models,

LRH-1 was shown to affect *Shp* and *Cyp8b1* expression in the liver, thereby reducing the amount cholic acid in the bile acid pool. In a more recent study, LRH-1 was shown to be critical for adequate regulation of *Cyp7a1* under conditions of bile acid sequestration (Out *et al.* 2011).

A role for LRH-1 in fertility originated from *in vitro* trans-activation studies in which LRH-1 was suggested to control *Cyp19a1*, *Star*, *Cyp11a1*, *Cyp17* and *Hsd3b2* expression (Britt *et al.* 2001; Clyne *et al.* 2002; Sirianni *et al.* 2002; Hinshelwood *et al.* 2003). Analysis of heterozygous LRH-1-knockout mice provided the first evidence that LRH-1 is important for steroid hormone biosynthesis and normal fertility *in vivo*. Progesterone, but not oestradiol, production was reduced in *LRH-1*^{+/-} mice, and this coincided with reduced fertility due to pregnancy loss (Labelle-Dumais *et al.* 2007). Analysis of granulosa cell-specific LRH-1-knockout mice confirmed the role of LRH-1 in progesterone production and in the regulation of *Scarb1*, *Star* and *Cyp11a1* expression (Duggavathi *et al.* 2008). The *Lrh1*^{sc-/-} mice were completely infertile, lacked corpora lutea (CL) in their ovaries and exhibited disturbed cumulus expansion due to dysfunction of the prostaglandin and hyaluronic acid cascades (Duggavathi *et al.* 2008). In addition, intrafollicular oestradiol levels were increased in these mice, presumably through increased *Cyp19a1* expression and reduced *Nos3* and *Sult1e1* expression (Duggavathi *et al.* 2008). Together, these findings indicate that LRH-1 is involved in follicular maturation, cumulus–oocyte expansion, CL transition and ovulation *in vivo*.

Herein we describe an inducible LRH-1-knockdown (KD) mouse model that was used to assess the value of the *in vivo* KD technology for fertility drug target validation and to further investigate the mechanism of action of LRH-1 in the ovary.

Materials and methods

Animals and husbandry

Mice were housed in macrolon cages (five animals per cage) at 19–21°C at a relative humidity was 50%–60% under an artificial 12-h light–dark cycle. Standard pelleted food (SDS, Essex, UK) and drinking water with either 2 mg mL⁻¹ doxycyclin (Dox; Sigma, St Louis, MO, USA) and 10% sucrose or 10% sucrose alone was provided *ad libitum* during the in-life period or for the time period described for the individual experiments. During mating experiments, female mice were checked daily for the presence of vaginal plugs. Mice were observed daily for morbidity and mortality during the in-life period. All animal experiments were approved by the local Animal Ethics Committee of NV Organon, Oss, The Netherlands.

Generation of LRH-1-KD mice

The LRH-1-KD mice used in the present study were obtained from Artemis (Cologne, Germany). Recombinase-mediated cassette exchange (RMCE) in embryonic stem (ES) cells was used to introduce an inducible LRH-1 short hairpin (sh) RNA in the ROSA26 locus (Seibler *et al.* 2007). The following shRNA sequence was used for LRH-1-specific vector construction: 5'-acacagaagtcgctcaattcaagattgaacgcgactctgtgt-3'. This shRNA sequence was inserted behind the human polymerase III human H1 promoter and an inducible Tet-system. The vectors

obtained were used to transfect RMCE ES cells. Three homologous recombinant clones for each shRNA vector were isolated and treated with 1 µg mL⁻¹ Dox (D-9891; Sigma) for 48 h. The LRH-1 KD efficiencies were determined by quantitative polymerase chain reaction (q-PCR). The ES cell clone with the highest level of KD (90% reduction of LRH-1 expression) was used to generate F₁ mice by injection into tetraploid blastocysts. Genotyping PCR was performed on isolated genomic DNA from tail biopsies using the following generic primers for the shRNA construct: forward, 5'-ccatggaattcgaacgctgacgctc-3'; reverse, 5'-tatggctatgaactaatgacctc-3'. As a control for primer functionality, genomic DNA of luciferase-KD mice was used (Artemis). The PCR products were analysed on a 1% Tris/Borate/EDTA (TBE) agarose gel. Adult heterozygous LRH-1-transgenic mice and wild-type littermates were used for all experiments and ranged in age from 12 to 17 weeks, unless stated otherwise.

Ovarian hyperstimulation

Dams with 2-week-old litters were treated with 2 mg mL⁻¹ Dox in 10% sucrose in their drinking water. After 1 week the mice were weaned, a tail biopsy was taken to determine the genotype and treatment with 2 mg mL⁻¹ Dox in 10% sucrose continued. When the mice were 31 days old they were injected with 10 IU, s.c., urinary FSH (Humegon; NV Organon, Oss, The Netherlands) in saline at 1430 hours. Forty-six hours after Humegon injection, the mice were injected with 5 IU, s.c., human chorionic gonadotrophin (hCG; Pregnyl; NV Organon, Oss, The Netherlands) or saline control. Mice were killed by cervical dislocation 24 or 3 h after hCG administration. After 24 h, the mice were killed, the oviduct was isolated and the number of oocytes present in the oviduct were counted.

For ovulation induction in adult mice, non-synchronised adult female transgenic mice were treated with 2 mg mL⁻¹ Dox in 10% sucrose in their drinking water for 2 weeks. After 2 weeks the mice were injected with 5 U, s.c., hCG (Pregnyl) and, 24 h later, they were killed and the number of oocytes in the oviduct was counted.

Histology

Histopathological analysis was performed on the liver, ovary, uterus and vagina of Dox-treated female LRH-1-KD and wild-type mice. At autopsy, all internal organs were examined for visual signs of abnormalities and their weights were recorded. Samples of organs and tissues from the mice were preserved in 4% buffered formaldehyde, dehydrated and embedded in paraffin wax. Sections (3–4 µm) from the different tissues were stained with haematoxylin and eosin (HE) and for subsequent microscopic evaluation.

Blood analyses

Blood was collected via the vena cava from mice under isoflurane anaesthesia. Blood was allowed to clot and the serum was separated by centrifugation at 20 800g for 15 min at room temperature. To obtain plasma samples, blood was collected in centrifuge tubes containing lithium–heparin (9 µL mL⁻¹ blood)

and separated by centrifugation at 20 800g for 15 min at room temperature.

Commercial assays were used to measure plasma 17 β -oestradiol (EIA-2693; DRG Instruments, Marburg, Germany) and plasma progesterone (catalogue no. 582601; Cayman Chemical, Ann Arbor, MI, USA). Serum concentrations of LH and FSH were determined using an immunofluorescence assay (IFMA), as described previously (van Casteren *et al.* 2000). Briefly, a maxisorb microtitre plate was coated with a mouse anti-recombinant hCG β monoclonal antibody (mAb) or mouse anti-recombinant hFHS β mAb and serum samples were added. Plates were washed and a biotin-labelled mouse anti-recombinant rat LH α polyclonal antibody was added. After the addition of europium-labelled streptavidin and enhancement solution, fluorescence was measured using a Wallac Victor2 1420 multilabel counter (Perkin Elmer, Waltham, MA, USA).

A standard curve was used to calculate a four parameter fit and to determine LH and FSH concentrations.

Real-time PCR analysis

Total RNA was isolated using Trizol (Gibco BRL, Grand Island, NY, USA). The quality of the samples was monitored using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Only RNA with an RNA integrity number (RIN) ≥ 8.5 was used. First-strand synthesis was performed using Oligo(dT)15 (Promega, Madison, WI, USA) and pd(N)6 (Amersham Pharmacia Biotech, Uppsala, Sweden) primers and Superscript II RNase H-Reverse Transcriptase (Invitrogen) according to the manufacturers' instructions. Briefly, 1.0 μ g Oligo(dT)15 primer and 1.0 μ g pd(N)6 were added to 2 μ g RNA and the volume was adjusted to 12.0 μ L with diethylpyr-carbonate (DEPC) water. The mixture was incubated at 70°C for 10 min and quickly chilled on ice for 2 min. Second-strand synthesis was performed in a volume of 20 μ L containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol (DTT), 0.5 mM dNTPs and 200 U M-MLV RNase H-reverse transcriptase. The mixture was incubated for 10 min at room temperature and then for 50 min at 40°C. The cDNA from the synthesis reaction was subjected to quantitative real-time PCR with SYBR Green mastermix (Applied Biosystems, Benelux, Bleiswijk, The Netherlands) in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The program used was 10 min at 95°C 100% ramp, followed by 40 cycles of 15 s at 95°C 100% ramp and 1 min at 60°C 100% ramp, followed by a dissociation curve step of 15 s at 95°C 100% ramp, 15 s at 60°C 100% ramp and 15 s at 95°C 2% ramp. Dissociation curves were checked for non-specific amplification. For quantification, standard curves were prepared for both the target and endogenous reference (*HPI*) genes. Levels of the target gene in each sample were normalised against those of the endogenous reference gene. The primer sequences used in the present study were as follows: for LRH-1 exon 6–7, 5'-cagaccctgttctccattgtg-3' (forward) and 5'-tcactccagcagtttgaagca-3' (reverse); for *SHP*, 5'-gagctgggtcccaagga-3' (forward) and 5'-ggcacatctgggtgaaga-3' (reverse); for *Scarb1*, 5'-gaacctaacccaaggagcat-3' (forward) and 5'-tgcatttcacagaa cagttcatg-3' (reverse); for *Cyp7a1*, 5'-ccactcatcacaactccctg-3'

(forward) and 5'-tgcttctgtgtccaatgcc-3' (reverse); for *Cyp19*, 5'-agaacaattgccttctttatg-3' (forward) and 5'-atggactccaca caaactcca-3' (reverse); for *Star*, 5'-tgccgaagacaatcatcaac-3' (forward) and 5'-tgctatgggattagtaggaag-3' (reverse); for *HPI*, 5'-gcccaagatggagcgaatc-3' (forward) and 5'-ccgagggccagcttcc-3' (reverse); for *LHCGR*, 5'-ttgaaccgggtgcttttaca-3' (forward) and 5'-ccggatgcctgtgttacaga-3' (reverse); for *FSHR*, 5'-gaattgctgatgatgtttcca-3' (forward) and 5'-gggaatagaccttg tccttgaga-3' (reverse); for *PRLR*, 5'-aagtgtcaatccctggatgg-3' (forward) and 5'-cgatgctcacctccacagaga-3' (reverse); and for *Cyp11a1*, 5'-atcagtgatgacctattccgctt-3' (forward) and 5'-ccatgcgctcccaaatata-3' (reverse).

Western blot analysis

For western blot analysis, tissues were homogenised at approximately -80°C using a Micro Dismembrator (B. Braun Biotech International, Melsungen, Germany). Frozen powder (10–100 mg) was then mixed with 200 μ L ice-cold lysis buffer (150 mM NaCl, 2 mM MgCl₂, 0.5 mM DTT, 0.25 mM EDTA, 1 mg mL⁻¹ Pefabloc (1585916; Boehringer, Ingelheim am Rhein, Germany), 10% glycerol, 1% Triton-X-100 and 50 mM Tris-HCl (pH 8.0), including protease inhibitor cocktail (1873580; Boehringer)). After 30 min incubation at 4°C, the samples were centrifuged at 14 000g for 15 min and the supernatant stored at -20°C . Protein concentrations were determined using the BCA Protein Assay kit (23227; Pierce, Rockford, IL, USA). From each sample, 30 μ g total protein was separated on a 4%–12% Precast NUPAGE Bis-Tris gel (Invitrogen) using NUPAGE 3-[*N*-morpholino]propanesulphonic acid (MOPS)–sodium dodecyl sulfate (SDS) running buffer (Invitrogen). Separated protein was transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech, Uppsala, Sweden) and the blots were blocked with 89.5% non-fat milk, 10% of 10 \times phosphate-buffered saline (PBS) and 0.5% Tween-20. Antibody incubations were performed in PBS containing 0.5% bovine serum albumin (BSA), 0.05% gelatin, 0.5% Tween-20 and 300 mM NaCl. The primary antibodies used were: anti-LRH-1 rabbit polyclonal antibody (ab18293; Abcam, Cambridge, UK), diluted 1 : 500, for the detection of LRH-1 in the ovary; anti-LRH-1 rabbit polyclonal generated against the C-terminal 20 amino acids of LRH-1, diluted 1 : 1000 for the detection of LRH-1 in the liver; and anti- β -actin rabbit polyclonal antibody (NB600-505; Novus Biologicals, Littleton, CO, USA), diluted 1 : 1500. The secondary antibody used was horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ig (ALI4404; Invitrogen). Antibody binding was visualised using an ECL Western Blotting Detection kit (Amersham) and Hyperfilm ECL (Amersham).

Gene arrays

Immature transgenic LRH-1 shRNA and wild-type mice ($n = 10$) were treated with Dox as described above. Forty-six hours after Humegon injection, the ovaries of five LRH-1-KD and wild-type mice were isolated and, 2 h after Pregnyl injection, the ovaries of the remaining LRH-1-KD and wild-type mice were isolated for RNA extraction. The RNA was isolated using an RNeasy mini extraction kit (74106; Qiagen, Valencia,

CA, USA) and RNA integrity was verified by capillary electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies). Double-stranded cDNA was synthesised from 1.5 µg total RNA using the One-Cycle Target Labelling Kit (Affymetrix, Santa Clara, CA, USA) and used for the preparation of biotin-labelled cRNA using a GeneChip IVT Labelling Kit (Affymetrix). Hybridisation of cRNA and washing of Affymetrix GeneChip Mouse Genome 430 2.0 arrays was performed according to standard Affymetrix protocols. The arrays were laser scanned with a GeneChip Scanner 300 (Affymetrix) according to the manufacturer's instructions. Data were quantified using Microarray Suite 5.0/GCOS 1.1 (Affymetrix) and were normalised using the GeneChip Robust Multi-array average (GCRMA) method; Limma (R package) was used to calculate ratios and *P*-values (Linear Models for Microarray Data, open source software). Genes were selected that were present in more than two arrays, had an expression intensity of at least 20 in one of the arrays and showed a differential expression of KD compared with wild-type mice with an adjusted *P*-value <0.05. The gene expression dataset was subsequently analysed for gene ontology and pathways to relate changes in gene expression to functional alterations using the Ingenuity Pathway Analysis tool (Ingenuity Systems, Redwood City, CA, USA) and the standard settings at the David webserver (da Huang *et al.* 2009; <http://david.abcc.ncifcrf.gov>; January 2013).

Follicle culture and induction of ovulation in vitro

Ovarian follicles were isolated and according to Rose *et al.* (1999). Briefly, female immature mice (F₁: B6BCA; 21–23 days of age) treated with Dox for 7 days were anaesthetised with isoflurane. Ovaries were removed and preantral follicles were isolated and collected. Follicles 160–210 µm in diameter with normal morphological appearance were cultured individually in Millicell-CM culture plate inserts (PICM 01250; Millipore, Bedford, MA, USA) in 250 µL α-minimum essential medium (αMEM; 22571–020; Gibco BRL) supplemented with 2 mM glutamine, 10 µg mL⁻¹ transferrin, 5 µg mL⁻¹ insulin, 50 µg mL⁻¹ ascorbic acid, 2 ng mL⁻¹ selenium (αMEM culture medium), 5% immature mouse serum, 1 µg mL⁻¹ Dox and 100 U L⁻¹ recombinant FSH (Puregon; NV Organon). Follicles were cultured in a humidified incubator gassed with 5% CO₂ in air at 37°C. The culture medium was exchanged every other day. The diameter of the follicles was measured each day at ×100 magnification using a calibrated micrometer. In addition, the survival rate of the follicles was checked by evaluating degeneration (blackening of the follicle) and bursting (loss of the oocyte). After 4–5 days, follicles were induced to ovulate with 5 U mL⁻¹ hCG (Pregnyl; NV Organon) in αMEM supplemented with 5% immature mouse serum and 1 µg mL⁻¹ Dox. After 24 h incubation, follicles were checked for ovulation (follicular rupture, oocyte extrusion, and oocyte maturation).

Statistical analysis

All data are expressed as the mean ± s.e.m. Statistical analyses were performed using non-paired Student's *t*-test or analysis of variation (ANOVA). *P* < 0.05 was considered significant.

Results

LRH-1-KD mouse model

To study the mechanism of action and the reversibility of the LRH-1 infertility phenotype, an *in vivo* inducible KD mouse model was developed. In this model the ROSA26 locus was targeted with a construct containing the human polymerase III human H1 promoter upstream of an inducible Tet-system and an shRNA directed against a highly specific sequence in exon 8 of LRH-1 (Fig. 1a). In the absence of Dox, the constitutively active Tet repressor (TetR) binds to the Tet operator (TetO) sequence located between the H1 promoter and the shRNA sequence and prevents transcription of the shRNA. Upon administration of Dox, TetR can no longer bind to TetO, and this results in the expression of the shRNA and subsequent KD of LRH-1.

The validity of the model was established by mating female transgenic LRH-1 shRNA mice, treated with 2 mg mL⁻¹ Dox and 10% sucrose in their drinking water, with wild-type male mice. The KD of LRH-1 during embryogenesis should reproduce the embryonic lethality as reported for the LRH-1-knockout mouse. Six plug-positive females delivered a total of 24 wild-type pups but no transgenic pups. It was therefore concluded that Dox-induced expression of the LRH-1 shRNA was sufficient to induce LRH-1 KD *in vivo*.

Subsequently, adult transgenic LRH-1 shRNA mice were treated with Dox for 2, 4 or 6 weeks. After 6 weeks treatment, the LRH-1-KD mice exhibited normal bodyweight compared with Dox-treated wild-type mice (31.5 ± 4.7 vs 32.3 ± 0.9 g, respectively). The transgenic LRH-1 shRNA mice exposed to 10% sucrose without Dox exhibited significantly reduced bodyweight (27.5 ± 2.9 g). Pathological examination revealed that this was caused mainly by the absence of solid food in their digestive tract. Most likely these mice preferred the intake of 10% sucrose water above solid food. Further examination of the different organs did not reveal any abnormalities in the LRH-1 KD-mice and microscopy of the liver did not show any morphological changes.

To test the efficiency of Dox-induced LRH-1 KD, q-PCR was performed on tissues samples from LRH-1-KD and wild-type mice treated either with 2 mg mL⁻¹ Dox and 10% sucrose or 10% sucrose alone. LRH-1 KD for 4 weeks resulted in an 83.5% reduction in LRH-1 mRNA expression in the liver (Fig. 1b). LRH-1 KD for 2 and 6 weeks also resulted in an 80% reduction in LRH-1 mRNA (data not shown). The transgenic LRH-1 shRNA control mice, receiving 10% sucrose without Dox, also exhibited decreased LRH-1 expression of 17% in the liver and 16% in the ovary (data not shown). This could be caused by leakage of the TetR/O regulatory system or it could be the result of physiological changes in these mice caused by the 10% sucrose treatment. In the ovary, 68% KD of LRH-1 mRNA levels was observed after 4 weeks Dox treatment compared with levels in the Dox-treated wild-type controls (Fig. 1b). We also tested several other tissues in which LRH-1 expression and a functional role for LRH-1 has been reported. In the colon of LRH-1-KD mice, the KD efficiency was approximately 60%. In adipose tissue, LRH-1 expression levels differed markedly between individual mice and no Dox-dependent KD was observed. The expression levels of LRH-1 in the testis,

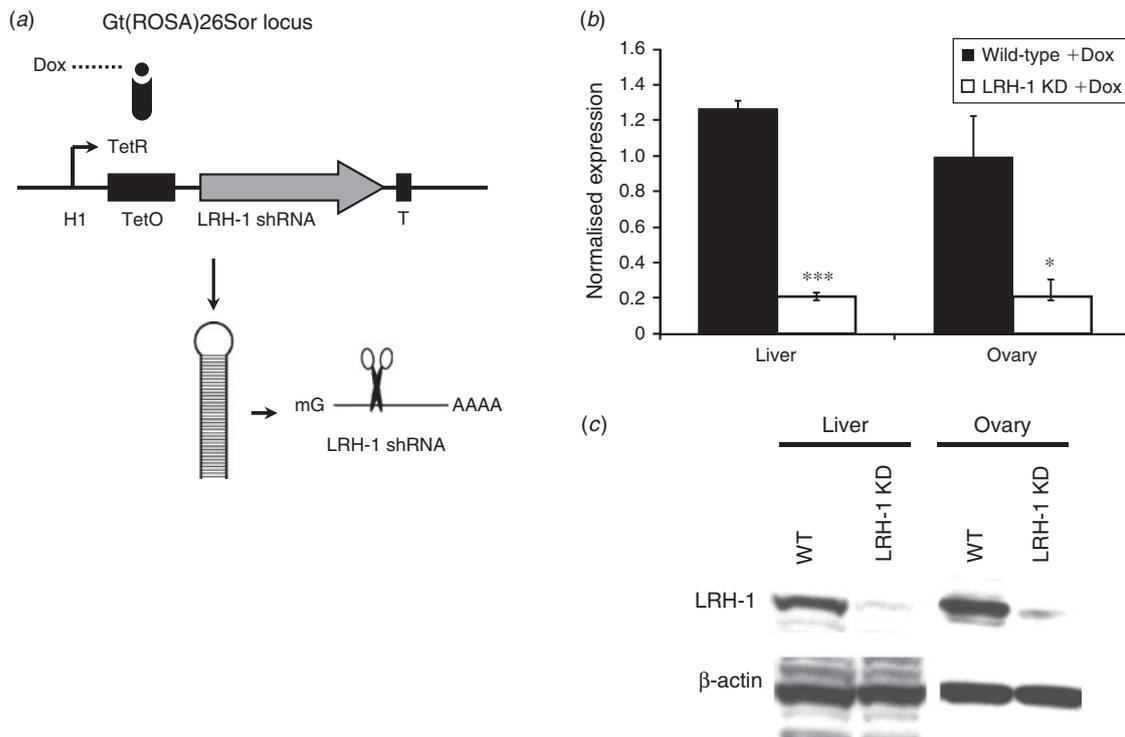


Fig. 1. Transgenic liver receptor homologue-1 (LRH-1) short hairpin (sh) RNA mouse model. (a) Schematic representation of the Tet inducible LRH-1 shRNA system. In the absence of doxycycline (Dox) the Tet repressor (TetR) is bound to the Tet operator (TetO) and no transcription takes place. In the presence of Dox, the TetR can no longer bind the TetO and the LRH-1 shRNA is transcribed. (b) Quantitative polymerase chain reaction analysis of LRH-1 mRNA expression in the liver of wild-type and LRH-1-knockdown (KD) mice treated with Dox for 4 weeks. Data are the mean \pm s.e.m. * $P < 0.05$, *** $P < 0.001$ compared with wild-type mice. (c) Western blot analysis of LRH-1 protein in the liver and ovary of wild-type (WT) and LRH-1-KD mice treated with Dox for 4 weeks.

mammary gland and adrenal gland were very low and no Dox-dependent KD of LRH-1 could be observed. Subsequently, we performed western blot analysis of liver and ovary protein extracts using LRH-1-specific antibodies. After 4 weeks Dox treatment, the liver of LRH-1-KD mice contained approximately 10%–20% of the LRH-1 protein in the liver of wild-type control mice treated with Dox (Fig. 1c). The reduction in LRH-1 protein in the ovary was also approximately 80%–90% compared with that in wild-type mice.

LRH-1-KD mice are infertile

To evaluate the fertility phenotype of LRH-1 KD, female transgenic LRH-1 shRNA mice were treated with Dox for 6 weeks and were then mated for 10 days with wild-type male mice; Dox treatment continued for another 2 weeks while the mice were breeding. Of the 17 LRH-1-KD females treated with Dox, none became pregnant (Fig. 2d). Female mice in the LRH-1 shRNA control and wild-type control groups did get pregnant and delivered normal sized litters (Fig. 2d). Even after prolonged mating with male mice for several weeks, the LRH-1-KD mice did not get pregnant.

Histological examination of the ovaries from LRH-1-KD mice that were treated with Dox for 4 weeks revealed the complete absence of CL (Fig. 2b), whereas the ovaries from

Dox-treated wild-type mice contained 5.25 ± 0.85 CL (Fig. 2a). Follicular development in the LRH-1 KD ovary proceeded up to the antral stage and the ovaries contained normal numbers of secondary, preantral and antral follicles that were not significantly different from those in wild-type ovaries. Serial sections of the complete ovary revealed that, after 4 weeks Dox treatment, the LRH-1 KD ovary contained 31.0 ± 2.9 secondary follicles (vs 41.0 ± 3.1 in Dox-treated wild-type control mice), 15.5 ± 0.9 preantral follicles (vs 14.3 ± 2.0 in Dox-treated wild-type controls) and 17.3 ± 0.6 antral follicles (vs 19.7 ± 1.5 in Dox-treated wild-type controls). In addition, no changes in the number of follicles were found after 6 weeks Dox treatment. Oocytes of normal appearance were present in the follicles of the LRH-1-KD female mice. The area of the cumulus–oocyte complex was comparable between wild-type and LRH-1-KD antral stage follicles (Fig. 2g, h). Longer treatments with Dox for up to 12 weeks resulted in an identical ovarian phenotype.

Serum hormone analysis was performed in adult non-synchronised LRH-1-KD mice. To determine the stage of the cycle, the vaginal epithelium was examined microscopically after autopsy. This indicated that 14 of 17 Dox-treated LRH-1-KD mice were in oestrus and three of 17 were in metoestrus. Of the 21 wild-type mice examined, two were in pro-oestrus, 10 were in oestrus, two were in metoestrus and seven were in dioestrus. Comparison of serum hormone levels in mice that

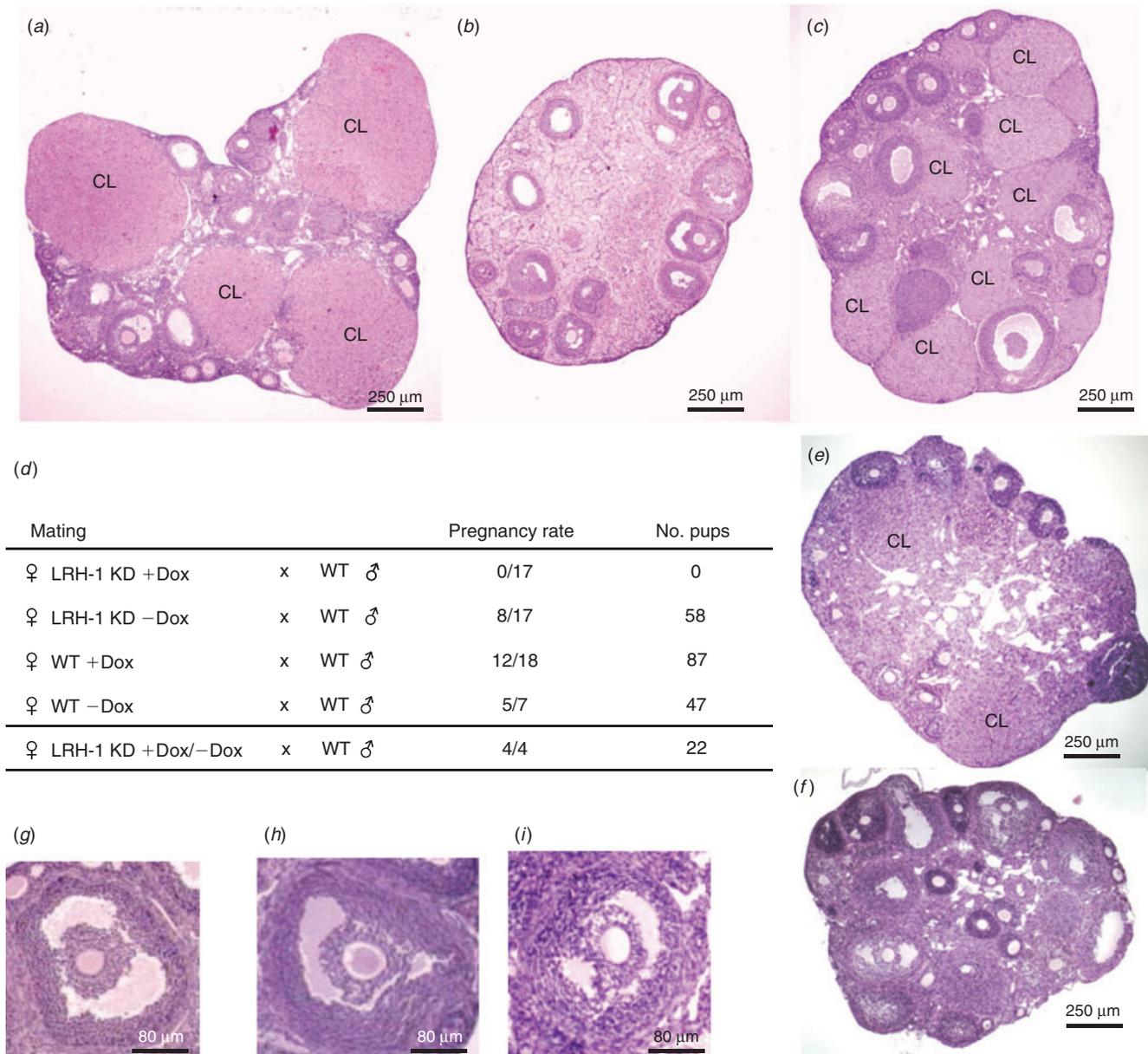


Fig. 2. Reversible infertility in liver receptor homologue-1 (LRH-1)-knockdown (KD) mice. (a, b) Representative haematoxylin–eosin (HE)-stained sections of ovaries from wild-type (a) and LRH-1-KD (b) mice treated with doxycyclin (Dox) for 6 weeks. CL, corpus luteum. In the LRH-1-KD ovary, CL are absent, whereas follicles in different stages of development are normally present. (c) Representative HE-stained section of an ovary of an LRH-1-KD mouse that, after 6 weeks Dox treatment, was reverted to vehicle treatment. Ten weeks after being switched to vehicle treatment, CL and all stages of follicular development are present. (d) Pregnancy rate and the number of pups generated by mating of female transgenic LRH-1 short hairpin (sh) RNA and wild-type (WT) mice treated with and without Dox for 6 weeks. In addition, some female LRH-1-KD mice were reverted to vehicle treatment after 6 weeks Dox treatment (+Dox/-Dox). These female mice were mated with WT male mice, became pregnant and gave birth to healthy pups. (e, f) Representative HE-stained section of the ovary of gonadotropin-stimulated, Dox-treated immature WT (e) and LRH-1-KD (f) mice 24 h after human chorionic gonadotrophin (hCG) stimulation. Although no late stage follicles are present in the WT ovaries, antral follicles are present in the LRH-1-KD ovaries. (g–h) Cumulus–oocyte complexes in the immature LRH-1-KD ovaries 24 h after hCG stimulation (i) are comparable to the area of the cumulus–oocyte complexes observed in adult WT (g) and LRH-1-KD (h) ovaries.

were in oestrus revealed significant reductions in serum progesterone levels in LRH-1-KD mice compared with Dox-treated wild-type mice (5.9 ± 0.9 vs 20.2 ± 7.7 nmol L⁻¹, respectively; $P < 0.05$). Serum FSH levels were also reduced in LRH-1-KD

mice compared with Dox-treated wild-type mice (0.74 ± 0.10 vs 1.45 ± 0.30 ng mL⁻¹, respectively; $P < 0.05$). There were no significant differences between LRH-1-KD and Dox-treated wild-type mice in terms of LH (0.169 ± 0.035 vs

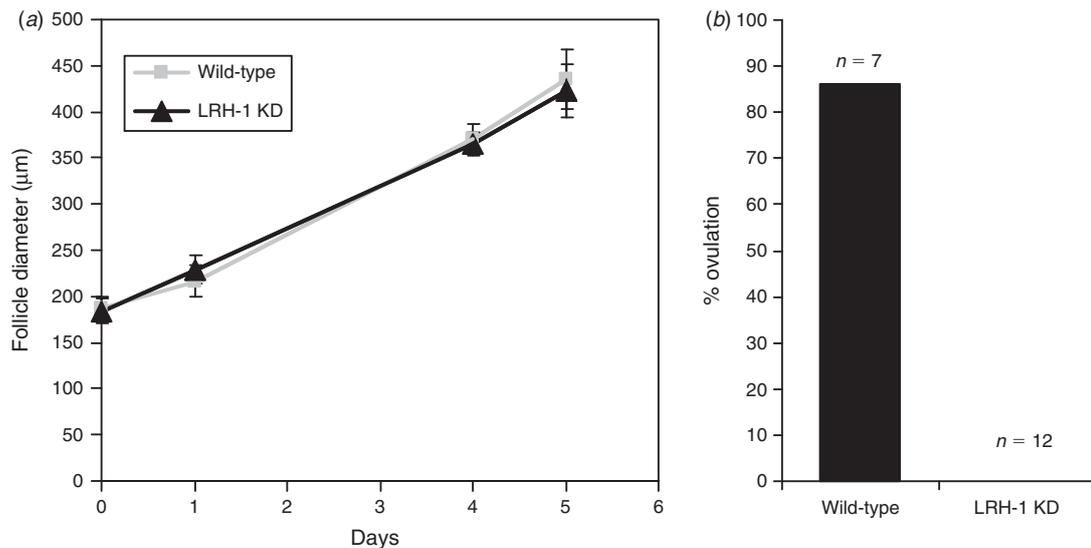


Fig. 3. Culture of liver receptor homologue-1 (LRH-1)-knockdown (KD) follicles. (a) *Ex vivo* follicle growth of doxycyclin (Dox)-treated LRH-1-KD and wild-type follicles. Data are the mean \pm s.e.m. (b) *Ex vivo* induction of ovulation in Dox-treated LRH-1-KD and wild-type mice. Wild-type follicles showed normal rates of ovulation following stimulation with human chorionic gonadotrophin. No ovulation was induced by hCG stimulation in LRH-1-KD follicles.

$0.130 \pm 0.048 \text{ ng mL}^{-1}$, respectively) and oestradiol (34.9 ± 1.8 vs $43.4 \pm 5.1 \text{ pmol L}^{-1}$, respectively) concentrations.

Subsequently, we investigated whether the infertility phenotype of the LRH-1-KD mice could be rescued by ovarian hyperstimulation treatment of both adult and synchronised immature female LRH-1-KD mice. Adult mice were treated for 4 weeks with Dox or vehicle, after which ovulation was induced via s.c. injection of hCG. Although both wild-type mice treated with Dox and untreated transgenic LRH-1 shRNA mice contained oocytes in the oviduct 24 h after hCG injection, there was no ovulation observed in Dox-treated LRH-1-KD mice, with oocyte numbers in Dox-treated wild-type, untreated LRH-1-KD and Dox-treated LRH-1-KD mice of 6.2 ± 1.0 , 6.3 ± 0.8 and 0 ± 0 , respectively.

Two-week-old immature female mice were treated with Dox for 17 days. Folliculogenesis was subsequently induced by s.c. injection of human gonadotrophins, followed by the induction of ovulation by hCG injection 2 days later. In Dox-treated LRH-1-KD female mice, there was a marked reduction in the number of oocytes in the oviduct 24 h after hCG injection compared with that in Dox-treated wild-type control (2.3 ± 2.3 vs 23.6 ± 4.0 , respectively). Histology of ovaries from immature mice showed that, 24 h after hCG, no preovulatory follicles were present in the wild-type ovaries but that in ovaries from LRH-1-KD mice the follicles had not progressed beyond the antral stage (Fig. 2e, f). In addition, the area of the cumulus–oocyte complexes in the gonadotropin-stimulated immature LRH-1 follicles (Fig. 2i) was comparable to that seen in the adult wild-type and LRH-1-KD follicles (Fig. 2g, h).

Reversible infertility in LRH-1 knockdown mice

To determine whether the infertility of LRH-1-KD mice was reversible, adult female transgenic LRH-1 shRNA and wild-type mice were treated with Dox for 2 weeks and then mated with

wild-type male mice. Dox treatment was stopped after 6 weeks when all the wild-type control females had become pregnant. None of the LRH-1-KD female mice had become pregnant at that time. Subsequently, Dox treatment was stopped and, 3 weeks later, the same LRH-1-KD and wild-type female mice were mated again with wild-type male mice. All transgenic LRH-1 shRNA female mice became pregnant and delivered a total of 22 viable pups (Fig. 2d). Analysis of genomic DNA from tail biopsies revealed the presence of both wild-type and LRH-1 shRNA transgenic pups in the litters of the LRH-1 shRNA transgenic mice. Microscopic examination showed that, 10 weeks after Dox treatment stopped, the ovaries of the LRH-1-KD mice were indistinguishable from wild-type ovaries and CL were normally present (Fig. 2c). In addition, serum progesterone levels had returned to those seen in the wild-type mice (data not shown).

Culture of LRH-1-KD follicles

To study the infertility phenotype *in vitro*, follicles were collected from the ovaries of 28-day-old immature transgenic LRH-1 shRNA mice and their wild-type littermates that had been treated with Dox for 7 days. Follicles ranging in size from 160 to 210 µm in diameter were cultured in the presence of recombinant FSH and $0.1 \mu\text{g mL}^{-1}$ Dox for 5 days, and their growth was recorded. The efficiency of LRH-1 KD was confirmed by q-PCR analysis. As shown in Fig. 3a, LRH-1 KD follicles developed at the same rate as wild-type follicles. After 5 days in culture, ovulation was induced by the addition of hCG. Ovulation was completely blocked in the LRH-1-KD follicles, whereas 86% of the wild-type follicles ovulated (Fig. 3b).

Expression of LRH-1 target genes in LRH-1-KD mice

To investigate which pathways and LRH-1 target genes are affected by LRH-1 KD, we determined the expression of several LRH-1 target genes in the liver and ovary and performed

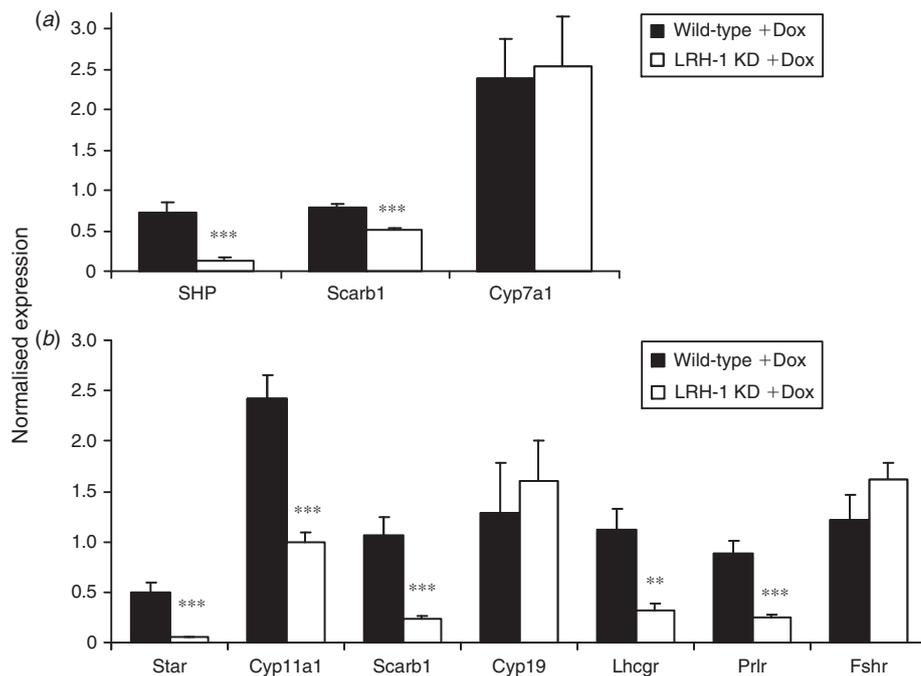


Fig. 4. Gene expression analysis in the liver and ovary from liver receptor homologue-1 (LRH-1)-knockdown (KD) and wild-type mice. Quantitative polymerase chain reaction analysis of LRH-1 target genes in the (a) liver of adult LRH-1-KD and wild-type mice treated with doxycyclin (Dox) for 4 weeks and (b) ovary of non-synchronised adult LRH-1-KD and wild-type mice treated with Dox for 4 weeks. Data are the mean \pm s.e.m. ** $P < 0.01$, *** $P < 0.001$ compared with wild-type.

microarray analysis on the ovaries of LRH-1-KD and wild-type mice. Quantitative PCR analysis revealed that *Shp* expression was downregulated by 85% in the liver of LRH-1-KD mice (Fig. 4a), *Scarb1* expression was downregulated by 43% and there was no obvious regulation of *Cyp7a1* expression in liver samples from LRH-1-KD mice (Fig. 4a).

Furthermore, q-PCR analysis of unsynchronised adult LRH-1-KD ovaries revealed that *Star*, *Cyp11a1*, *Scarb1*, *Lhcgr* and *Prlr* expression was significantly reduced compared with that in Dox-treated wild-type mice (Fig. 4b). There were no changes in *Cyp19* and *Fshr* expression. To further study the changes in expression in the LRH-1-KD ovary, we performed a microarray experiment on immature wild-type and LRH-1-KD ovaries. Two-week-old immature mice were treated with Dox for 17 days. Subsequently, the mice were treated with FSH to induce follicle maturation and, 46 h later, with hCG to induce ovulation. RNA was isolated 40 h after FSH treatment and 2 h after hCG treatment and both time points were analysed on microarray. The results showed that 40 h after FSH treatment 637 genes were differentially expressed between the LRH-1-KD and Dox-treated wild-type ovaries ($P < 0.05$). Two hours after hCG, 794 genes were differentially expressed ($P < 0.05$; see Table S1 available as Supplementary Material to this paper) and 285 genes were significantly regulated at both time points. Pathway analysis showed that regulated canonical pathways in the LRH-1-KD ovaries 40 h after FSH include C21-Steroid Hormone Metabolism and Androgen and Oestrogen Metabolism. These pathways were not regulated 2 h after hCG.

At both time points, the Interferon Signalling pathway and other immunological pathways were among the most significantly regulated pathways (data not shown).

In line with the q-PCR analysis of the adult ovary, LRH-1 expression in the immature LRH-1-KD ovary was significantly reduced by 2.4- and 2.7-fold at 40 h after FSH and 2 h after hCG, respectively, compared with Dox-treated wild-type (see Fig. S1). Microarray analysis further indicated that 40 h after FSH the genes that showed the highest regulation in the LRH-1-KD ovaries were steroidogenic genes *Star* and *Hsd17b7* (Table 1). In addition, *Scarb1*, *Cyp11a1* (Table 1) and *Hsd3b* (see Fig. S1) were significantly downregulated during folliculogenesis. Expression of *Fshr*, *Cyp19a1* and *Nos3* was not found to be significantly modulated; however, *Ccnd2* expression was significantly reduced at both time points (see Fig. S1).

Two hours after hCG, *Sult1e1* and *Hsd17b7* were among the highest regulated genes, but *Star*, *Scarb1* and *Cyp11a1* were not significantly regulated at this time point (Table 2). Seven of the highly regulated genes were significantly modulated at both time points (*Hsd17b7*, *Fetub*, *Ddc*, *Cst8*, *Dpysl4*, *Slc38a3* and *Comp*).

Other genes that showed high downregulation were previously described to be involved in: (1) the ovulation process and in CL formation and function, namely *Nts* (Hernandez-Gonzalez *et al.* 2006), *Btc* (Ashkenazi *et al.* 2005; Hernandez-Gonzalez *et al.* 2006), *Cst8* (Hsia *et al.* 2005), *Cish* (Anderson *et al.* 2009), *Runx1* (Hernandez-Gonzalez *et al.* 2006; Jo and Curry 2006), *Pappa* (Nyegaard *et al.* 2010), *Cited1* (Sriraman

Table 1. Results of microarray analysis in immature liver receptor homologue-1 (LRH-1)-knockdown (KD) ovaries showing the top 20 down-regulated genes 40 h after FSH treatment**P* < 0.05, ***P* < 0.01, ****P* < 0.001 for wild-type compared with liver receptor homologue-1 knockdown. hCG, human chorionic gonadotrophin

Gene symbol	Gene name	Fold regulation 40 h after FSH	Fold regulation 2 h after hCG 2 h
<i>Star</i>	Steroidogenic acute regulatory protein	-8.4*	-1.6
<i>Hsd17b7</i>	Hydroxysteroid (17-β) dehydrogenase 7	-8.4**	-10.4**
<i>Rasl10a</i>	RAS-like, family 10, member A	-6.2***	-2.6*
<i>Dpysl4</i>	Dihydropyrimidinase-like 4	-5.5*	-4.7*
<i>Cst8</i>	Cystatin 8 (cystatin-related epididymal spermatogenic)	-4.8*	-5.8*
-	Plasma membrane associated protein, S3-12	-4.7**	-2.1
<i>Sfrp4</i>	Secreted frizzled-related protein 4	-4.2*	-2.6
<i>Fetub</i>	Fetuin β	-4.1***	-7.3***
<i>Slc38a3</i>	Solute carrier family 38, member 3	-3.9**	-4.6***
<i>Dusp1</i>	Dual specificity phosphatase 1	-3.5*	-1.5
<i>Trappc2L</i>	Trafficking protein particle complex 2-like	-3.3***	-3.6***
<i>Comp</i>	Cartilage oligomeric matrix protein	-3.2*	-4.3**
<i>Cyp11a1</i>	Cytochrome P450, family 11, subfamily a, polypeptide 1	-2.8**	-1.7
<i>Hao3</i>	Hydroxyacid oxidase (glycolate oxidase) 3	-2.7***	-1.9*
<i>Ddc</i>	Dopa decarboxylase	-2.6*	-6.5***
<i>Scarb1</i>	Scavenger receptor class B, member 1	-2.6**	-1.5
<i>Lhcgr</i>	Luteinising hormone/choriogonadotropin receptor	-2.5*	-3.1*
<i>Scx</i>	Scleraxis	-2.5	-4.2*
<i>Rcc2</i>	Regulator of chromosome condensation 2	-2.5***	-2.4***
<i>Sh2d4a</i>	SH2 domain containing 4A	-2.5*	-2.2*

Table 2. Results of microarray analysis in immature liver receptor homologue-1 (LRH-1)-knockdown (KD) ovaries showing the top 20 down-regulated genes 2 h after human chorionic gonadotrophin treatment**P* < 0.05, ***P* < 0.01, ****P* < 0.001 for wild-type compared with liver receptor homologue-1 knockdown. hCG, human chorionic gonadotrophin

Gene symbol	Gene name	Fold regulation 40 h after FSH	Fold regulation 2 h after hCG 2 h
<i>Sult1e1</i>	Sulfotransferase family 1E, member 1	-1.0	-11.4***
<i>Nmu</i>	Neuromedin U	-1.1	-10.8***
<i>Hsd17b7</i>	Hydroxysteroid (17-β) dehydrogenase 7	-8.4**	-10.4**
<i>Nts</i>	Neurotensin	-1.1	-9.4***
<i>Fetub</i>	Fetuin β	-4.1***	-7.3***
<i>Btc</i>	Betacellulin, epidermal growth factor family member	-1.0	-7.0***
<i>Slc27a3</i>	Solute carrier family 27 (fatty acid transporter), member 3	-1.5	-6.8***
<i>Ddc</i>	Dopa decarboxylase	-2.6*	-6.5***
<i>Cst8</i>	Cystatin 8 (cystatin-related epididymal spermatogenic)	-4.8*	-5.8*
<i>Cish</i>	Cytokine inducible SH2-containing protein	-2.0	-5.1**
<i>Slc26a7</i>	Solute carrier family 26, member 7	-1.1	-4.8**
<i>Mfsd4</i>	Major facilitator superfamily domain containing 4	-2.0	-4.8*
<i>Dpysl4</i>	Dihydropyrimidinase-like 4	-5.5*	-4.7*
<i>Slc38a3</i>	Solute carrier family 38, member 3	-3.9**	-4.6***
<i>2200002K05Rik</i>	RIKEN cDNA 2200002K05 gene	-1.0	-4.4*
<i>Cited1</i>	Cbp/p300-interacting transactivator with Glu/Asp-rich C-terminal domain 1	-2.1	-4.4**
<i>Runx1</i>	Runt related transcription factor 1	-1.0	-4.4***
<i>Comp</i>	Cartilage oligomeric matrix protein	-3.2*	-4.3**
<i>Pappa</i>	Pregnancy-associated plasma protein A	-1.9	-4.2*
<i>Scx</i>	Scleraxis	-2.5	-4.2*

et al. 2010) and *Sfrp4* (Hernandez-Gonzalez *et al.* 2006; Maman *et al.* 2011), in addition to *Sult1e1*, *Star*, *Hsd17b7*, *Cyp11a*, *Lhcgr*, *Scarb1* (Table 1) and *Oxtr* (Shirasuna *et al.* 2007) and *Prlr* (Le *et al.* 2012; see Fig. S1 and Table S1); (2) proliferation,

cell cycle and apoptosis, namely *Btc* (Oh *et al.* 2011), *Rasl10a* (Elam *et al.* 2005), *Dpysl4* (Kimura *et al.* 2011), *Sfrp4* (Jacob *et al.* 2012), *Dusp1* (Chen *et al.* 2011) and *Rcc2* (Grigera *et al.* 2012); and (3) neuronal processes, namely *Nmu* (Chu *et al.*

Table 3. Results of microarray analysis in immature liver receptor homologue-1 (LRH-1)-knockdown (KD) ovaries showing the top 20 upregulated genes 40 h after FSH treatment* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for wild-type compared with liver receptor homologue-1 knockdown. hCG, human chorionic gonadotrophin

Gene symbol	Gene name	Fold regulation 40 h after FSH	Fold regulation 2 h after hCG 2 h
<i>Wfdc2</i>	WAP four-disulfide core domain 2	11.4*	2.3
<i>Ifit1</i>	Interferon-induced protein with tetratricopeptide repeats 1	9.4***	15.8***
<i>Uox</i>	Urate oxidase	9.1*	1.0
<i>Rnf128</i>	Ring finger protein 128	7.1*	1.3
<i>Usp18</i>	Ubiquitin specific peptidase 18	6.9***	8.0***
<i>Klf5</i>	Kruppel-like factor 5	6.3*	2.0
<i>Ifi44</i>	Interferon-induced protein 44	5.4***	7.1***
<i>Cd24a</i>	CD24a antigen	4.9*	1.9
<i>Trim30</i>	Tripartite motif-containing 30	4.9***	6.0***
<i>Rsad2</i>	Radical S-adenosyl methionine domain containing 2	4.9***	5.4***
<i>Ccl8</i>	Chemokine (C-C motif) ligand 8	4.8**	4.2**
<i>Galnt3</i>	UDP-N-acetyl- α -D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3	4.8*	2.0
<i>Slc2a3</i>	Solute carrier family 2 (facilitated glucose transporter), member 3	4.8*	1.1
<i>Oas2</i>	2',5'-Oligoadenylate synthetase 2	4.7***	6.8***
<i>St14</i>	Suppression of tumorigenicity 14 (colon carcinoma)	4.7**	1.3
<i>Ier3</i>	Immediate early response 3	4.6**	-1.3
<i>Isg20</i>	Interferon-stimulated protein	4.5*	1.6
<i>Ifi47</i>	Interferon gamma inducible protein 47	4.5***	4.9***
<i>ligp1</i>	Interferon inducible GTPase 1	4.4***	5.7***
<i>Lrrtm3</i>	Leucine rich repeat transmembrane neuronal 3	4.4***	1.7**

2012), *Nts* (Hernandez-Gonzalez *et al.* 2006; Hanson *et al.* 2012) and *Ddc* (Børglum *et al.* 2001). Significant regulation was also observed for *Ptgs2*, *Cd44* and *Ptx3* genes involved in the cumulus expansion process, but not for *C1qbp*, *Tnfaip6*, *Mmp2*, *Mmp9*, *Mmp19*, *Adamts4*, *Adamts1* and *Has2* (see Fig. S1; Duggavathi *et al.* 2008).

Most of the most upregulated genes at both 40 h after FSH and 2 h after hCG are linked to interferon and immunology-related processes (*Wfdc2*, *Ifit1*, *Uox*, *Rnf128*, *Usp18*, *Ifi44*, *Cd24a*, *Trim30*, *Rsad2*, *Ccl8*, *Oas2*, *Ier3*, *Isg20*, *Ifi47*, *ligp1*, *Ifit2*, *Cxcl10*, *Irf7*, *Igtp*, *Ifi27*, *Psm8* and *Sp100*; Tables 3, 4; de Veer *et al.* 2005) and nine these highly modulated genes were significantly regulated at both time points.

Discussion

In the present study, temporal *in vivo* KD of LRH-1 generally recapitulated phenotypic aspects of previous studies. However, some interesting differences and additional findings were observed. With respect to the liver, *Shp* and *Scarb1* expression was reduced, but *Cyp7a1* expression was unaffected (Mataki *et al.* 2007; Lee *et al.* 2008; Out *et al.* 2011). Liver histology was normal and plasma liver parameters were not significantly changed (data not shown); these findings are in line with other studies of LRH-1-deficient mouse models.

In the ovaries of transgenic LRH-1 shRNA mice, Dox treatment resulted in 80–90% KD of LRH-1 protein in the ovary. Remarkably, the LRH-1 mRNA level was only reduced by 68% in the LRH-1-KD ovary. A possible explanation could be the presence of a partial LRH-1 transcript after Dox treatment.

The reduced LRH-1 expression resulted in complete infertility, the absence of CL and an abnormal oestrous cycle. The altered expression profile at 40 h after FSH stimulation, exemplified by the reduced expression of *Ccnd2*, an FSH/oestradiol-responsive gene involved in granulosa cell proliferation (Sicinski *et al.* 1996; Robker and Richards 1998), suggests that LRH-1 deficiency also affects folliculogenesis. However, morphologically, follicular maturation (including granulosa cells proliferation) appeared to progress normally up to the antral stage in LRH-1-KD mice both *in vitro* and *ex vivo*. Ovulation was blocked in the LRH-1-KD mice and examination of serial sections of the ovaries of LRH-1-KD mice revealed that oocytes were present in all antral stage follicles. Stimulation with gonadotrophins in adult and immature mice and in follicle culture could not force oocyte rupture. This indicates that the observed reduction in serum FSH itself is most likely not causing the infertility phenotype, and this is supported by the normal fertility observed in haploinsufficient FSH β -knockout mice (Kumar *et al.* 1997; Sun *et al.* 2006). Instead of progressing to the luteal stage, late stage follicles in LRH-1-KD mice most likely become atretic, because there was no indication of accumulation of antral follicles or luteinised unruptured follicles in the ovaries.

Plasma progesterone levels were reduced and the expression of progesterone steroid biosynthesis genes *Scarb1*, *Cyp11a*, *Star* and *Hsd3b* was downregulated in the ovaries of LRH-1-KD mice, most prominently 40 h after FSH treatment. Duggavathi *et al.* (2008) reported an increase in intrafollicular oestradiol production in granulosa cell-specific LRH-1-knockout mice. This was accompanied by increased *Cyp19a1* expression and

Table 4. Results of microarray analysis in immature liver receptor homologue-1 (LRH-1)-knockdown (KD) ovaries showing the top 20 upregulated genes 2 h after human chorionic gonadotrophin treatment**P* < 0.05, ***P* < 0.01, ****P* < 0.001 for wild-type compared with liver receptor homologue-1 knockdown. hCG, human chorionic gonadotrophin

Gene symbol	Gene name	Fold regulation 40 h after FSH	Fold regulation 2 h after hCG 2 h
<i>Ifit1</i>	Interferon-induced protein with tetratricopeptide repeats 1	9.4***	15.8***
<i>Coch</i>	Coagulation factor C homologue (<i>Limulus polyphemus</i>)	-1.1	8.2**
<i>Usp18</i>	Ubiquitin specific peptidase 18	6.9***	8.0***
<i>Ifi44</i>	Interferon-induced protein 44	5.4***	7.1***
<i>Oas2</i>	2',5'-Oligoadenylate synthetase 2	4.7***	6.8***
<i>Ifit2</i>	Interferon-induced protein with tetratricopeptide repeats 2	3.1***	6.3***
<i>Trim30</i>	Tripartite motif-containing 30	4.9***	6.0***
<i>AW112010</i>	Expressed sequence AW112010	3.7**	5.8***
<i>Iigp1</i>	Interferon inducible GTPase 1	4.4***	5.7***
<i>Cxcl10</i>	Chemokine (C-X-C motif) ligand 10	4.2***	5.5***
<i>Rsad2</i>	Radical S-adenosyl methionine domain containing 2	4.9***	5.4***
<i>Irf7</i>	Interferon regulatory factor 7	3.6***	5.4***
<i>Ifi47</i>	Interferon gamma inducible protein 47	4.5***	4.9***
<i>D14Ert668e</i>	DNA segment, Chr 14, ERATO Doi 668, expressed	4.0***	4.8***
<i>Igtp</i>	Interferon gamma induced GTPase	3.6***	4.8***
<i>Ifi27</i>	Interferon, α -inducible protein 27	3.6***	4.8***
<i>Psmb8</i>	Proteasome (prosome, macropain) subunit, β type 8 (large multifunctional peptidase 7)	3.4***	4.3***
<i>Sp100</i>	Nuclear antigen Sp100	2.8***	4.3***
<i>Ccl8</i>	Chemokine (C-C motif) ligand 8	4.8**	4.2**
<i>Ih1</i>	Interferon induced with helicase C domain 1	2.7***	4.2***

reduced *Nos3* and *Sult1e1* expression. We did not observe this in the present study. Plasma oestradiol concentrations were not significantly increased in LRH-1-KD mice, *Cyp19a1* and *Nos3* expression was not significantly modulated (see Fig. S1) and no effect on the thickness of the epithelial cells of the endometrium was observed (data not shown). We did observe a markedly reduced expression of *Sult1e1* 2 h after hCG and, surprisingly, *Hsd17b7* expression was highly regulated both before and after hCG stimulation. *Hsd17b7* has been described to convert oestrone to oestradiol in luteal cells and also has a role in the final steps of cholesterol biosynthesis (Shehu *et al.* 2008). This finding would support a decrease, rather than an increase, in oestradiol synthesis during folliculogenesis. The regulation of both *Sult1e1* and *HSD17b7* 2 h after hCG would lead to opposite effects on oestrogen bioavailability. Together, these observations suggest that, in the LRH-1 KD model, oestradiol is not the main component that causes the infertility phenotype, and this is further supported by the fact that mouse models of increased oestrogenic activity do not show complete female infertility (Jablonka-Shariff *et al.* 1998; Gershon *et al.* 2007). The different effect on oestradiol in LRH-1-KD mice compared with granulosa cell-specific LRH-1-knockout mice (Duggavathi *et al.* 2008) could be explained by the incomplete and temporal nature of KD of LRH-1 in the LRH-1-KD mice. Downstream of oestradiol we found some changes in the expression of genes involved in cumulus expansion (see Fig. S1). *Ptgs2* and *Ptx3* expression was significantly downregulated in LRH-1-KD mice 2 h after hCG, and *Cd44* expression was significantly increased 40 h after FSH. The expression of *Tnfaip6*, *C1qbp*, *Adamts4*, *Adamts1* and other proteases was not significantly changed or

was undetectable in our experiments. Nevertheless, cumulus expansion appeared to be affected because, in the gonadotropin-stimulated immature LRH-1-KD mice, the cumulus-oocyte complex was still not fully expanded 24 h after hCG (Duggavathi *et al.* 2008; Richards and Pangas 2010).

The reduced progesterone level in the LRH-1-KD mice is likely to represent a factor with greater impact on fertility. In rodents, ovulation is preceded by a small increase in progesterone as well as an increase in oestradiol, prolactin and LH. The preovulatory rise in progesterone has been shown to be mandatory for ovulation (Hibbert *et al.* 1996; Robker *et al.* 2000) and its absence in LRH-1-KD mice could largely explain the observed infertility phenotype. The progesterone receptor-knockout (PRKO) mice, in which both progesterone receptors have been inactivated, are completely infertile (Robker *et al.* 2000; Conneely *et al.* 2003), like LRH-1-KD mice. In PRKO mice, folliculogenesis progresses normally, but ovulation is blocked and cannot be induced by exposure to superovulatory levels of gonadotrophins. The main difference between the LRH-1 KD and PRKO phenotypes resides in the observation that PRKO granulosa cells can still differentiate into luteal cells (Robker *et al.* 2000), indicating that additional defects are present in LRH-1-KD mice.

We were unable to show that progesterone supplementation rescued the LRH-1-KD phenotype. Subcutaneous injection of 100 μ L of 10 mg mL⁻¹ progesterone 3 h prior and 2 h after hCG injection in adult LRH-1-KD mice failed to rescue ovulation (data not shown).

The absence of luteinisation may be caused by reduced *Lhcgr* and *Prlr* expression in LRH-1-KD mice. This could indicate that

LRH-1 KD antral follicles are not fully prepared for ovulation and progression of granulosa cells to the luteal phase and have reduced sensitivity to the LH surge.

Microarray analysis revealed significant upregulation of interferon and immunology pathways in the LRH-1-KD model. This could be caused by LRH-1 shRNA expression provoking cellular antiviral RNA interference machinery (de Veer *et al.* 2005). However, if the regulation of these genes indeed represents cellular antiviral defence or off-target effects, they do not seem to cause a deviant liver or ovarian phenotype in the LRH-1-KD mice. Alternatively, regulation of these pathways could be a consequence of the role of LRH-1 in immunological responses (Lin *et al.* 2000; Mueller *et al.* 2006; Venticlef *et al.* 2006, 2011; Coste *et al.* 2007), or an immunological response to the increase cellular debris from atretic antral follicles (Hakuno *et al.* 1996).

Previously described LRH-1-deficient mouse models did not establish whether the defects caused by LRH-1 deficiency are reversible. We successfully applied *in vivo* inducible RNA interference (RNAi) technology in the LRH-1-KD model to address whether fertility is restored after transient LRH-1 KD. Three weeks after withdrawal of Dox treatment, the LRH-1 shRNA transgenic mice were again fully fertile and delivered healthy pups with no signs of irreversible changes due to the transient reduction in LRH-1 expression. Therefore, analysis of the LRH-1-KD mouse model revealed a reversible infertility phenotype that was predominantly caused by reduced progesterone synthesis and, in contrast with earlier studies, with no obvious effects on oestradiol activity. In addition, the data obtained indicated a unique mechanism of action in which the processes of ovulation and luteinisation are disturbed due to defects caused by LRH-1 deficiency during folliculogenesis before the LH surge. Because this model mimics the effects of an LRH-1 antagonist, the results further support the feasibility of the use of LRH-1 antagonising compounds as contraception therapy. It should be mentioned though that the unopposed oestrogenic effects, the possible need for continuous treatment and the potentially teratogenic effects of LRH-1 antagonists during embryogenesis make the development of LRH-1 antagonist for contraception challenging.

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