The *INSL3* gene is a direct target for the orphan nuclear receptor, COUP-TFII, in Leydig cells

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Abstract

Insulin-like 3 (INSL3), a hormone produced by Leydig cells, regulates testicular descent during foetal life and bone metabolism in adults. Despite its importance, little is known about the molecular mechanisms controlling *INSL3* expression. Reduced *Ins3* mRNA levels were reported in the testis of mice deficient for chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII), an orphan nuclear receptor known to play critical roles in cell differentiation and lineage determination in several tissues. Although COUP-TFII-deficient mice had Leydig cell dysfunction and impaired fertility, it remained unknown whether *Ins3* expression was directly regulated by COUP-TFII. In this study, we observed a significant decrease in *Ins3* mRNA levels in MA-10 Leydig cells depleted of COUP-TFII. Furthermore, a –1087 bp mouse *Ins3* promoter was activated fourfold by COUP-TFII in MA-10 Leydig cells. Using 5' progressive deletions, the COUP-TFII-responsive element was located between –186 and –79 bp, a region containing previously uncharacterised direct repeat 0-like (DR0-like) and DR3 elements. The recruitment and direct binding of COUP-TFII to the DR0-like element were confirmed by chromatin immunoprecipitation and DNA precipitation assay respectively. Mutation of the DR0-like element, which prevented COUP-TFII binding, significantly decreased COUP-TFII-mediated activation of the –1087 bp *Ins3* reporter in CV-1 fibroblast cells but not in MA-10 Leydig cells. Finally, we found that COUP-TFII cooperates with the nuclear receptor steroidogenic factor 1 (SF1) to further enhance *Ins3* promoter activity. Our results identify *Ins3* as a target for COUP-TFII in Leydig cells and revealed that COUP-TFII acts through protein–protein interactions with other DNA-bound transcription factors, including SF1, to activate *Ins3* transcription in these cells.

Key Words

- insulin-like 3
- NR2F2
- nuclear receptor
- Leydig cells
- cooperation

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Introduction

Insulin-like 3 (INSL3), also known as relaxin-like factor, belongs to the insulin–insulin-like growth factor–relaxin family of growth factors and hormones that was originally discovered by screening a boar testicular cDNA library

(Adham *et al.* 1993). The corresponding sequences were later obtained by screening genomic or cDNA libraries and by RT-PCR from humans (Burkhardt *et al.* 1994) and a variety of other species (Bathgate *et al.* 1996, Spiess *et al.* 1999,

Zarreh-Hoshyari-Khah *et al.* 1999, Klonisch *et al.* 2001). In males, INSL3 is specifically produced by testicular Leydig cells from the onset of gonadal development throughout adulthood, whereas it is only secreted by the adult ovary (theca and luteal cells) in females (Zimmermann *et al.* 1997, Balvers *et al.* 1998). In foetal Leydig cells, INSL3 is strongly expressed from embryonic day 13.5 (E13.5) in mice (Zimmermann *et al.* 1997) and its expression decreases after birth with the disappearance of the foetal Leydig cell population (Zimmermann *et al.* 1997, Balvers *et al.* 1998). INSL3 levels in the adult Leydig cell population rise just before puberty, peak in adult animals and then decline in ageing animals (Pusch *et al.* 1996, Zimmermann *et al.* 1997, Balvers *et al.* 1998, O'Shaughnessy *et al.* 2002, Paust *et al.* 2002).

INSL3 plays important roles in male and female reproductive function. In males, INSL3 is involved in two very different, but critical, processes. During embryonic development, INSL3 was found to be a master regulator of gonadal descent, an essential step of the male sex differentiation process. *Insl3*^{-/-} null mice present bilateral cryptorchid testes, located high in the abdominal cavity close to the kidneys (Nef & Parada 1999, Zimmermann *et al.* 1999). Furthermore, INSL3 expression in pancreatic β -cells of *Insl3* null mice restored normal testis descent (Adham *et al.* 2002), while its expression in females led to descended ovaries (Adham *et al.* 2002, Koskimies *et al.* 2003). In adults, the role of INSL3 in males is not yet fully understood. INSL3 was found to be involved in bone metabolism (Ferlin *et al.* 2008) and proposed to act as a germ cell survival factor (Kawamura *et al.* 2004). In adult females, INSL3 plays a role in oocyte maturation and in follicle selection and survival (Spaniel-Borowski *et al.* 2001, Irving-Rodgers *et al.* 2002, Kawamura *et al.* 2004).

Despite its crucial roles in reproductive development and function, surprisingly, very little is known about the mechanisms regulating *Insl3* expression in Leydig cells. To date, only two transcription factors have been shown to regulate *Insl3* promoter activity in Leydig cells: the nuclear receptors steroidogenic factor 1 (SF1, Ad4BP, NR5A1) and NUR77 (NGFI-B, NR4A1) (Zimmermann *et al.* 1998, Koskimies *et al.* 2002, Sadeghian *et al.* 2005, Tremblay & Robert 2005, Robert *et al.* 2006). As these nuclear receptors are co-expressed in other cell types that do not produce INSL3, other transcription factors must participate in *Insl3* transcription in Leydig cells.

The orphan nuclear receptor, chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII, NR2F2), is strongly expressed in tissues originating from

the mesenchyme and plays critical roles during mouse development (Tsai & Tsai 1997, Lin *et al.* 2011). *Coup-tfii* null mice die before E10.5 due to angiogenesis and cardiovascular defects (Pereira *et al.* 1999). Temporal and tissues-specific *Coup-tfii* ablation in the stomach, limbs, diaphragm, uterus, and endothelial cells have revealed important roles in cell growth and differentiation and in organogenesis (Lee *et al.* 2004, Takamoto *et al.* 2005, You *et al.* 2005, Kurihara *et al.* 2007, Petit *et al.* 2007). It was previously reported that COUP-TFII might be involved in *Insl3* expression (Qin *et al.* 2008). Indeed, inactivation of *Coup-tfii* during pre-pubertal stages of male development in mice led to a blockade in Leydig cell differentiation at the progenitor stage. This resulted in infertility, hypogonadism and spermatogenetic arrest due to defective testosterone synthesis in *Coup-tfii*^{-/-} male mice. A decrease in the expression of several genes (*Hsd3b1*, *Cyp11a1* and *Cyp17a1*) required for testosterone biosynthesis was observed in these mice (Qin *et al.* 2008). A decrease in *Insl3* mRNA levels was also reported (Qin *et al.* 2008). However, it remains unknown whether the decrease in gene expression, particularly *Insl3*, was due to a defect in the Leydig cell differentiation process and/or to a direct role of COUP-TFII in regulating their expression.

COUP-TFII regulates gene expression by binding to direct repeat (DR) motifs (AGGTCA) separated by variable spacing (from zero to six nucleotides) between the two half-sites (Pereira *et al.* 2000). Herein, we report that COUP-TFII directly and positively regulates *Insl3* gene expression by binding to a previously uncharacterised DR0-like response element in the *Insl3* promoter. In addition, we found that COUP-TFII cooperates with the nuclear receptor SF1 to further enhance *Insl3* transcription.

Materials and methods

Double immunohistochemistry

Immunohistochemistry was done using an anti-COUP-TFII antiserum (1:100, R&D Systems, Minneapolis, MN, USA) and was carried out as described in Martin *et al.* (2008). For double immunodetection, the counterstaining with haematoxylin following COUP-TFII detection was omitted and replaced by the incubation with an anti-INSL3 antiserum (1:100 dilution, M-122, Santa Cruz Biotechnology) overnight at 25 °C. The samples were washed twice with 1× PBS for 10 min each time and an alkaline phosphatase (ALKP)-conjugated secondary antibody (1:1000, Sigma-Aldrich Canada) was added and incubated for 1 h at 25 °C. Final detection was done using

an ALKP detection solution (Tris-HCl 0.1 M, pH 9.2, 1 mM levamisole, naphthol 0.04% and Fast Blue BB diazonium salt 0.2%). The negative control corresponds to the same procedures with the omission of anti-COUP-TFII antiserum (data not shown). All experiments were conducted according to the Canadian Council for Animal Care and have been approved by the Animal Care and Ethics Committee of Laval University (protocol #2009011). Images were obtained using an Axioskop2 Plus microscope (Carl Zeiss Canada, Toronto, ON, Canada) and the Image-Pro Plus Software (MediaCybernetics Rockville, MD, USA).

siRNA transfection

MA-10 Leydig cells were transfected with 150 nM siRNA (Life Technologies) directed against *Coup-tfii* transcripts (sequence: ACU GGC CAU AUA UGG CAA UUC AAU A) or with a control non-targeting siRNA for 48 h using the JetPRIME Transfection Reagent according to manufacturer's protocol for siRNA transfection (PolyPlus, Illkirch, France). Three different siRNAs directed against different regions of COUP-TFII were used and they all decreased COUP-TFII protein levels, albeit with different efficacy. The one that decreased COUP-TFII protein by about 80% was chosen. Less than 10% difference was observed between scrambled siRNAs and no siRNA (data not shown). siRNA transfection had no effect on cell viability (Trypan Blue exclusion) and cell number.

RNA isolation and real-time PCR

Following siRNA treatment, RNA isolation and cDNA synthesis were carried out as previously described (Martin *et al.* 2008). Quantitative RT-PCR was carried out using a LightCycler 1.5 instrument and the LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics) according to the manufacturer's protocol. PCRs were carried out on *Ins13* using the forward primer 5'-TGG CTA GAG CAG AGA CAT C-3' and the reverse primer 5'-CCT GTG GTC CTT GCT TAC-3' and under the following conditions: 10 min at 95 °C followed by 35 cycles of denaturation (5 s at 95 °C), annealing (5 s at 62 °C) and extension (20 s at 72 °C) with single acquisition of fluorescence at the end of each extension steps. As internal control, PCRs were carried out using previously described *Rpl19*-specific primers and conditions (Martin *et al.* 2008). The specificity of the PCR products was confirmed by analysis of the melting curve and agarose gel electrophoresis. Quantification of gene expression was carried out using the Relative

Quantification Software (Roche Diagnostics) and is expressed as a ratio of *Ins13*:*Rpl19* levels.

Protein purification and western blotting

After siRNA transfections, MA-10 Leydig cells were rinsed twice with ice-cold PBS and nuclear proteins were prepared as described previously (Martin *et al.* 2008). Protein concentrations were determined using standard Bradford assays. Nuclear proteins (15 µg) were boiled 10 min in a denaturing loading buffer, fractionated by SDS-PAGE, and transferred onto nitrocellulose membrane (Bio-Rad). Immunodetection was carried out using a horseradish peroxidase coupled antibody approach according to the manufacturer's instructions (Amersham ECL and ECL Prime Western Blotting Detection Reagents, GE Healthcare Life Sciences, Baie-D'Urfé, QC, Canada). COUP-TFII and LAMIN B proteins were detected using a monoclonal anti-COUP-TFII antibody (1:1000, R&D Systems) and an anti-LAMIN B antiserum (1:1000, C-20, Santa Cruz Biotechnology) respectively.

Plasmids

The -1087 to +5 bp murine *Ins13* luciferase promoter construct was generated by PCR amplification from mice genomic DNA using the following set of oligonucleotide primers: forward containing a BamHI cloning site (italicised), 5'-GCG GAT CCT GGT TCC TAT GAT CTG GCT G-3' and reverse containing a KpnI cloning site (italicised), 5'-GGG GTA CCG TGG CAG GAG GCA GTG GGC AG-3'. Deletions to -800, -600, -400, -333, -284, -234, -186 and -79 bp were obtained by PCR using the -1087 bp *Ins13* promoter as template, along with a common reverse primer mentioned earlier and the following forward primers containing a BamHI (italicised) cloning site: -800 bp, 5'-GCG GAT CCC CCT TGC TCC CCT GAC TGT G-3'; -600 bp, 5'-GCG GAT CCC TGG GAG AGT AGA GGT CTT G-3'; -400 bp, 5'-GCG GAT CCA ACA GGA AAG GAA CCA TTT A-3'; -333 bp, 5'-GCG GAT CCA AAC CAA GTC ATA AAT ACC TCC-3'; -284 bp, 5'-GCG GAT CCT CCA ACT CAC AGC AAT TCT CC-3'; -234 bp, 5'-GCG GAT CCA TGT GGG CTT CAC CCT TAC AAA GG-3'; -186 bp, 5'-CGG GAT CCA ATG TTG GGG AGC GGC TCC TG-3' and -79 bp, 5'-GGG GAT CCT GCT GCT TGC CTG TGT TC-3'. All promoter fragments were cloned into a modified pXP1 luciferase reporter plasmid (Tremblay & Viger 1999). The -1087 bp *Ins13* reporter constructs harbouring inactivating mutations in both half-site of the DR0-like or of the DR3 elements were

generated in a two-step process using the QuikChange XL Mutagenesis Kit (Stratagene, La Jolla, CA, USA) with the following oligonucleotides (mutated nucleotides are in bold, underlined and lowercase): first half-site mutation in DR3 (sense, 5'-CCT GGG AGA GGA CTT CAA aac aCC AAG CTG GAC ACA CAG C-3'; antisense, 5'-GCT GTG TGT CCA GCT TGG tgt tTT GAA GTC CTC TCC CAG G-3'), second half-site mutation in DR3 (both half-site mutated; sense, 5'-CCG CAC CTG GGA Gct tAC TTC AAA ACA CC-3'; antisense, 5'-GGT GTT TTG AAG Taa gCT CCC AGG TGC GG-3'), first half-site mutation in DR0-like (sense, 5'-CCG TGA CTC GAG CCT Cac aaT TTT GGG TGC TGC TTG C-3'; antisense, 5'-GCA AGC AGC ACC CAA AAt tgt GAG GCT CGA GTC ACG G-3') and second half-site mutation in DR0-like (both half-sites mutated; sense, 5'-CCG TGA CTC GAG aag CAC AAT TTT GGG-3'; antisense, 5'-CCC AAA ATT GTG ctt CTC GAG TCA CGG-3'). The -1087 bp *Insl3* reporter construct harbouring mutations in both DR0-like and DR3 elements was generated via the same two-step process using the -1087 bp *Insl3* DR3 mutated construct as template and the sense and antisense oligonucleotides containing the mutations for the both half-site of the DR0-like element described above. The human -1137 bp *INSL3* and rat -508 bp *Insl3* promoter constructs have been described previously (Robert *et al.* 2006, Laguë & Tremblay 2008). The mouse COUP-TFII expression vector (Pereira *et al.* 1999) was obtained from Dr Ming Tsai (Baylor College of Medicine, Houston, TX, USA). The mouse SF1 expression plasmid has been described previously (Tremblay & Viger 2001). All plasmids were verified by sequencing (Centre de Génomique de Québec, CHUL Research Centre, Quebec City, QC, Canada).

Cell culture, transfections and reporter assays

Mouse MA-10 Leydig cells (Ascoli 1981), provided by Dr Mario Ascoli (University of Iowa, Iowa City, IA, USA) were grown in DMEM/F12 medium supplemented with 2.438 g/l sodium bicarbonate, 3.57 g/l HEPES, 15% horse serum and 50 mg/l of gentamicin and streptomycin sulfate. African Green monkey kidney fibroblast CV-1 cells were grown in DMEM medium supplemented with 3.7 g/l HEPES, 10% newborn calf serum and 50 mg/l of gentamicin and streptomycin sulfate. The MA-10 Leydig cells were originally derived from the mouse M5480P tumour (Ascoli 1981) and have since been well characterised. They produce steroids and respond to luteinizing hormone/human chorionic gonadotrophin (as well as Forskolin and cAMP) stimulation. The MA-10 Leydig cell

line corresponds to the adult Leydig cells (reviewed in Rahman & Huhtaniemi (2004)). The expression of *Insl3* in MA-10 Leydig cells is similar to what has been reported in primary Leydig cell cultures and in rodents. For instance, *Insl3* expression is known to be repressed by estradiol *in vivo* in mice and rats (Nef *et al.* 2000, Cederroth *et al.* 2007, Strauss *et al.* 2009, Zhou *et al.* 2011) and this repression was also observed in MA-10 Leydig cells (Laguë & Tremblay 2009). *Insl3* expression is upregulated by androgens *in vivo* (Zhou *et al.* 2010) as well as in primary Leydig cells and in MA-10 Leydig cells (Laguë & Tremblay 2008, Tremblay *et al.* 2009). Therefore, the MA-10 Leydig cell line is an appropriate model to study *Insl3* gene regulation in the adult Leydig cell population. MA-10 and CV-1 cells were maintained at 37 °C in 5% CO₂. MA-10 and CV-1 cells were transfected and lysates were analysed as described previously (Martin *et al.* 2008, 2009). Data reported represent the average of at least three experiments, each performed in triplicate. The cell passage number was lower than 35 for all experiments.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were carried out essentially as described previously (Robert *et al.* 2006) with some modifications. Briefly, MA-10 Leydig cells were fixed with 1% formaldehyde at 20 °C for 10 min. After harvesting in PBS, pelleted cells were washed for 10 min at 4 °C each time with buffer I (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES and pH 6.5), buffer II (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES and pH 6.5) and protease inhibitors. The samples were then centrifuged at 5900 *g* for 5 min at 4 °C. The pellets were resuspended (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.0, protease inhibitors) and incubated on ice for 10 min. The samples were next sonicated with the Misonix S-400 sonicator at 80% power and the lysates were centrifuged at 15 700 *g*. The supernatants were diluted and incubated at 4 °C for 2 h with 30 µl of magnetic beads (Dynabeads, Life Technologies) which were previously blocked (with 0.2 µg/µl of sheared salmon sperm DNA and 0.5% BSA in PBS for 1 h at 4 °C) along with 5 µg of an anti-NUR77 (M210, Santa Cruz Biotechnologies) antiserum (used as an immunoprecipitation positive control), an anti-COUP-TFII antiserum or non-specific immunoglobulin Gs (IgGs) (Santa Cruz Biotechnologies). The precipitates were washed four times in a rotating wheel with ChIP wash buffer (100 mM Tris, pH 7.4, 500 mM LiCl, 1% Igepal and 1% sodium deoxycholate) for 5 min at 4 °C and once again with TE (10 mM Tris, pH 8 and 1 mM EDTA).

They were extracted with freshly prepared Elution buffer (1% SDS and 0.1 M NaHCO₃). The eluates were kept at 65 °C overnight with 0.2 M NaCl to reverse the crosslinks. DNA was purified by phenol/chloroform extraction and ethanol precipitation and resuspended in 10 mM Tris, pH 8. COUP-TFII-immunoprecipitated DNA fragments were analysed by PCR using primers specific for the proximal (−225 to −26 bp) (forward, 5′-CCC TTA CAA AGG GGC GCT TGG C-3′ and reverse, 5′-GGA TGC TCC TTA TAA CTG CCT CG-3′) or distal (−3133 to −2923 bp) (forward, 5′-GGT ACA AGA CCA AGA CTC CAG ACG TCA CG-3′ and reverse, 5′-GGG GTA GTC CTG ACT AAG TTG AGA GGG G-3′) region of the mouse *Insl3* promoter. The PCRs were carried out on a T_{gradient} thermocycler (Biometra, Montreal Biotech, Dorval, Québec, Canada) using Vent polymerase (New England Biolabs, Whitby, ON, Canada) and under the following conditions: 5 min at 95 °C followed by 40 cycles of denaturation (60 s at 95 °C), annealing (proximal region 30 s at 60 °C and distal region 60 s at 64 °C) and extension (30 s at 72 °C). The specificity of the PCR products was confirmed by agarose gel electrophoresis and sequencing. Input DNA represents 10% of total DNA used for a ChIP experiment. ChIP results were confirmed by three separate experiments.

DNA precipitation assays

DNA precipitation assays were carried out with 30 µl of streptavidin magnetic beads (Promega), which were washed twice with 1× B&W buffer (5 mM Tris, pH 7.5, 0.5 mM EDTA and 1 M NaCl), and then 100 ng of WT and mutated double-stranded biotinylated oligonucleotides were bound to the beads in 1× B&W buffer for 1 h at room temperature (beads without oligonucleotides were used as a negative control). The beads were washed twice in 1× B&W buffer, once with 1× binding buffer (5% glycerol, 20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 0.15% Triton X-100, 100 mM NaCl and 4 mM MgCl₂) and blocked with 1% BSA and sperm salmon DNA (0.2 mg/ml) for 1 h at 4 °C. The bound biotinylated oligonucleotides were then mixed with 10 µg of poly (dI-dC) and 100 µg of nuclear extracts from MA-10 Leydig cells in a total volume of 500 µl of 1× binding buffer with protease inhibitors, and incubated for 2 h (1 h 30 min at 4 °C and 30 min at room temperature) with rotation, followed by five washes in 1× binding buffer. The bound proteins were then eluted in western blotting-loading buffer and analysed by SDS-PAGE. The following oligonucleotides were biotinylated, annealed with the corresponding non-biotinylated oligonucleotide and

used as probes containing: i) the WT COUP-TFII high-affinity response element (underlined; [Kliwer et al. 1992](#)) 5′-AGC TTC AGG TCA GAG GTC AGA GAG CT-3′; ii) the mutated (shown in lowercase); COUP-TFII high-affinity response element 5′-AGC TTC Aaa TCA Gaa aTC AGA GAG CT-3′; iii) the WT DR0-like from the *Insl3* promoter 5′-CCG TGA CTC GAG CCT CGA CCT TTT GGG TGC-3′; iv) the mutated (shown in lowercase) DR0-like from the *Insl3* promoter 5′-CCG TGA CTC GAG aaT CGA aaT TTT GGG TGC-3′; v) the WT DR3 element from the *Insl3* promoter 5′-ACC TGG GAG AGG ACT TCA AGG TCC CAA GCT GGA-3′ and vi) the mutated (shown in lowercase) DR3 element from the *Insl3* promoter 5′-ACC TGG GAG Aaa ACT TCA Aaa TCC CAA GCT GGA-3′.

Statistical analyses

For all single comparisons between two experimental groups, paired Student's *t*-tests were carried out. For all statistical analyses, *P* < 0.05 was considered significant. For multiple group comparisons, statistical analyses were done using one-way ANOVA followed by the Newman-Keuls *post hoc* test. All statistical analyses were done using the GraphPad Prism Software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

COUP-TFII present in MA-10 Leydig cells regulates *Insl3* gene expression

To determine whether COUP-TFII is present in INSL3-expressing Leydig cells within the mouse testis, double immunohistochemistry was carried out on mice testis sections at E19.5 and post-natal day 32 (P32). As shown in [Fig. 1A](#), at E19.5 COUP-TFII was detected in interstitial cells that were negative for INSL3 (red-brown nuclear staining, open arrowheads). On the other hand, P32, COUP-TFII and INSL3 (blue-purple cytoplasmic staining) were co-localised in interstitial Leydig cells (solid arrowheads). COUP-TFII was also detected in some peritubular cells at E19.5 and P32 that do not express INSL3 (arrows in [Fig. 1A and B](#)). Thus, COUP-TFII and INSL3 are co-expressed in adult Leydig cells but not in foetal Leydig cells of the mouse testis.

Next, to determine whether COUP-TFII is involved in *Insl3* gene expression, MA-10 Leydig cells were transfected with siRNA directed against COUP-TFII or non-targeting siRNA, and *Insl3* mRNA levels were determined by qPCR. COUP-TFII knockdown (to about 20%) was confirmed by

COUP-TFII/INSL3

Figure 1

COUP-TFII co-localises with INSL3 in mouse adult Leydig cells. Double immunohistochemistry was carried out on (A) embryonic (E19.5) and (B) adult (P32) mouse testis sections using an anti-COUP-TFII antiserum and an anti-INSL3 antiserum. COUP-TFII (red-brown staining) is present in the nuclei of interstitial cells (solid and open arrowheads). Leydig cells are positive for INSL3 (cytoplasmic blue-purple staining). COUP-TFII was also detected in the nuclei of some peritubular cells (arrows). Omission of the primary antibody served as negative control (data not shown). IS, interstitium; ST, seminiferous tubules. Magnification, 400 $\times$; scale bar, 50 μ m.

western blotting (Fig. 2). In COUP-TFII-depleted MA-10 Leydig cells, *Insl3* mRNA levels were decreased by 40% when compare with cells transfected with scrambled siRNAs (Fig. 2). Thus COUP-TFII contributes to the regulation of *Insl3* expression in MA-10 Leydig cells.

In order to determine whether COUP-TFII could activate an *Insl3* promoter construct and to avoid non-specific activations, a dose-response assay was carried out. MA-10 Leydig cells were transfected with a -1087 and a -79 bp mouse *Insl3* reporters in the presence of increasing doses of a COUP-TFII expression vector. As shown in Fig. 3, the -1087 bp mouse *Insl3* promoter was activated (3.5- to 4.3-fold) by COUP-TFII at all doses tested (25, 50, 125 and 250 ng). However, at 250 ng the minimal -79 bp *Insl3* reporter was significantly activated by about sevenfold (Fig. 3), which indicates non-specific

effects at a dose of 250 ng. A dose of 50 ng of COUP-TFII was therefore chosen as the optimal dose to significantly and specifically activate the -1087 bp *Insl3* promoter.

COUP-TFII activates the *Insl3* promoter via a novel DR0-like element at -97 bp

To locate the COUP-TFII-responsive element, a series of 5' progressive deletions of the mouse *Insl3* promoter were transfected in MA-10 Leydig cells. As shown in Fig. 4, deletion from -1087 to -186 bp did not significantly impair COUP-TFII responsiveness. However, further deletion to -79 bp resulted in a substantial decrease in COUP-TFII-dependent activation (Fig. 4). These results indicate that a COUP-TFII-responsive element is likely located between -186 and -79 bp. *In silico* analysis of this sequence revealed the presence of two previously uncharacterised DR elements, a DR0-like and a DR3, that could mediate the COUP-TFII-dependent activation of the

Figure 2

Knockdown of COUP-TFII in MA-10 Leydig cells decreases *Insl3* gene expression. MA-10 Leydig cells were transfected with 150 nM of siRNA directed against COUP-TFII (black bar) or with a control non-targeting siRNA (open bar) for 48 h. The cells were then harvested to obtain total RNA and nuclear extracts. *Insl3* mRNA levels were quantified by qPCR, while COUP-TFII protein levels were determined by western blotting. The qPCR results were corrected with the *Rpl19* mRNA. An asterisk (* $P < 0.05$) indicates a statistically significant difference from control.

Figure 3

COUP-TFII activates the mouse *Insl3* promoter. MA-10 Leydig cells were co-transfected with a -1086 to +5 bp (black bars) or a minimal -79 to +5 bp (open bars) mouse *Insl3* promoter construct along with an empty expression vector or different quantities (25, 50, 125 and 250 ng) of a COUP-TFII expression vector. Results are shown as fold activation over control ($\pm$ S.E.M.). An asterisk (* P <0.05) and a hashtag (# P <0.05) indicate a statistically significant difference from control (no COUP-TFII) for a given reporter.

Insl3 promoter (Fig. 5A). Alignment of this sequence from different species (mice, rat, humans and bovine) revealed that the DR0-like element was well conserved in the *Insl3* promoter of all species analysed, whereas the DR3 element was not (Fig. 5A). Consistent with this, COUP-TFII could equally activate the humans, rat and mice *Insl3* promoter in transient transfections of MA-10 Leydig cells (Fig. 5B).

To determine the contribution of the DR0-like and DR3 elements to *Insl3* promoter activity, MA-10 Leydig cells and CV-1 heterologous fibroblast cells were transfected with various -1087 bp *Insl3* reporter constructs either WT harbouring mutations in the DR0-like and/or the DR3 elements. As shown in the left panel of Fig. 6A, mutation of the DR3 element led to a 75% decrease in *Insl3* promoter activity in MA-10 Leydig cell. A mutation of the DR0-like element was more dramatic with a 93% reduction in *Insl3* promoter activity, which is similar to what was observed with the minimal -79 bp *Insl3* promoter. Mutation of both DR3 and DR0-like produced results identical to the DR0-like mutation (left panel of Fig. 6A). In CV-1 fibroblast cells, mutations of the DR3 and DR0-like elements also decreased *Insl3* promoter activity, albeit to a much lesser extent than in MA-10 Leydig cells

(right panel of Fig. 6A). These data indicate that the DR3 and DR0-like elements are both essential for maximal *Insl3* promoter activity in MA-10 Leydig cells.

Next, we sought to determine whether the DR3 and/or the DR0-like elements were involved in the COUP-TFII-dependent activation of the *Insl3* promoter. As shown in the left panel of Fig. 6B, mutation of the DR3 or the DR0-like individually or in combination (double DR3/DR1-like mutant) in the *Insl3* promoter had no impact on COUP-TFII responsiveness in MA-10 Leydig cells. This could indicate that COUP-TFII activates the *Insl3* promoter in association with other DNA-bound transcription factors in Leydig cells. In agreement with this, transfections in CV-1 fibroblast cells revealed that mutation of the DR0-like element at -103/-91 bp abrogated COUP-TFII responsiveness while mutation of the DR3 element had no impact (right panel in Fig. 6B). This indicates that the DR0-like element is necessary for the COUP-TFII-mediated activation of the -1087 bp *Insl3* promoter and that in MA-10 Leydig cells, COUP-TFII may act independently of DNA binding.

COUP-TFII binds to the DR0-like element in the proximal *Insl3* promoter

Next, ChIP assays were carried out to determine whether COUP-TFII was recruited to the proximal *Insl3* promoter. Proteins were cross-linked to the chromatin, which was then sheared and immunoprecipitated using an anti-COUP-TFII antiserum. By PCR, a band of ~200 bp (-225 to -26 bp) was detected in the input sample (Fig. 7A, upper panel lane 4) as well as in the samples immunoprecipitated with the anti-COUP-TFII antiserum (Fig. 7A, upper panel lane 5) and with the anti-NUR77 antiserum (Fig. 7A, upper panel lane 7; used as a positive control). No bands were observed in the IgG control sample (Fig. 7A, upper panel lane 3). An upstream genomic region (-3133 to -2923 bp) that does not contain any COUP-TFII binding site was also used (Fig. 7A, lower panel). No specific recruitment of COUP-TFII was observed on this distal region located at ~3000 bp upstream to the *Insl3* transcriptional start site (Fig. 7A, lower panel lane 5) or when using an IgG as negative control (Fig. 7A, lower panel lane 3). COUP-TFII is thus recruited to the proximal *Insl3* promoter in a native chromatin environment in MA-10 Leydig cells.

Although our ChIP data confirm the recruitment of COUP-TFII to the proximal *Insl3* promoter, this approach cannot discriminate between direct DNA binding and indirect recruitment through protein-protein

Figure 4

The COUP-TFII-responsive element is located within the proximal *InsI3* promoter. MA-10 Leydig cells were co-transfected with either an empty expression vector (–, open bars) or a COUP-TFII expression vector (+, black bars), along with a series of 5' deletion constructs of the mouse *InsI3*

promoter as indicated. The number of experiments, each performed in triplicate, is indicated. Results are shown as fold activation over control ($\pm$ s.e.m.). A different letter indicates a statistically significant difference ($P < 0.001$).

interactions. Furthermore, our functional data (Fig. 6) suggest that COUP-TFII can act independently of DNA binding in MA-10 Leydig cells. Therefore, to determine whether COUP-TFII directly binds to the DR0-like element *in vitro*, we used a DNA precipitation approach. As shown in Fig. 7B, COUP-TFII was found to bind to oligonucleotides containing a high-affinity DR1 element (Fig. 7B, lane 3) as well as the DR0-like element from the *InsI3* promoter (Fig. 7B, lane 5), but it did not bind the DR3 element from the *InsI3* promoter (Fig. 7B, lane 7) or oligonucleotides harbouring mutations in these elements (Fig. 7B, lanes 4, 6 and 8). Taken together, these results indicate that COUP-TFII directly and specifically binds to the DR0-like element in the proximal *InsI3* promoter.

COUP-TFII cooperates with SF1 on the *InsI3* promoter

As our activation data (Fig. 6) indicate that COUP-TFII may act in association with other DNA-bound transcription factors and as the DR0-like element (–103/–91 bp) is in proximity to binding sites for the nuclear receptor SF1 (Zimmermann *et al.* 1998, Koskimies *et al.* 2002), one of which is located within the DR3 element (–151/–135 bp), we tested the possibility that these two nuclear receptors

might cooperate to modulate *InsI3* promoter activity. Co-transfections in MA-10 Leydig cells revealed that COUP-TFII and SF1 individually activate the –1087 bp *InsI3* promoter about fivefold while both nuclear receptors in combination had additive effects (left panel in Fig. 8). This cooperation was more apparent in heterologous CV-1 cells, where co-transfection of both COUP-TFII and SF1 led to a synergistic activation (about 30-fold) of the *InsI3* promoter (right panel in Fig. 8).

Discussion

INSL3 is produced exclusively by the male foetus primarily to control the first phase of testicular descent (Ivell & Anand-Ivell 2011). In adult males, INSL3 was identified as a major circulating testicular hormone that directly correlates with Leydig cell capacity and development (Ivell & Anand-Ivell 2011, Ivell *et al.* 2013a). Recent evidence has identified a novel role for INSL3 in bone metabolism (Ferlin *et al.* 2008) in addition to its proposed role in the prevention of male germ cells apoptosis (Kawamura *et al.* 2004, Del Borgo *et al.* 2006, Amory *et al.* 2007). In adult females, despite its low expression compared with males, INSL3 was found to be a paracrine factor within the ovary

Figure 5

Species conservation of COUP-TFII responsiveness. (A) Sequence alignment of the two direct-repeat (DR) elements (grey shaded boxes) present in the *InsL3* promoter from the mice, rat, human and bovine. The DR3 element is located at -143 bp and the DR0-like element is located at -97 bp. Conserved residues are indicated by capital letter while one mismatch is represented by a lowercase letter. Non-conserved residues are indicated by asterisks. The two potential DR elements are represented by the white

(DR3) and grey (DR0-like) diamonds. (B) MA-10 Leydig cells were co-transfected with either an empty expression vector (-) or a COUP-TFII expression vector (+), along the mice (black bars), human (open bars) or rat (grey bars) *InsL3* promoter as indicated. The number of experiments, each performed in triplicate, is indicated. Results are shown as fold activation over control ($\pm$ s.e.m.). An asterisk (* P <0.05) indicates a statistically significant difference from control.

and may be involved in follicle selection and survival (Ivell & Anand-Ivell 2011). Despite these important physiological roles and the identification of INSL3 as a unique marker of Leydig cells, very little is known about the molecular mechanisms that regulate *InsL3* expression in these cells.

INSL3 is constitutively expressed in Leydig cells and, unlike testicular steroids, it is regulated independently of the hypothalamo-pituitary-gonadal axis (Anand-Ivell

et al. 2006, Atlantis *et al.* 2009, Ivell & Anand-Ivell 2009). This absence of direct hormonal regulation was also reported in studies of the rat and mice *InsL3* gene promoter (Zimmermann *et al.* 1998, Koskimies *et al.* 2002). From these studies, a relatively short promoter region of ~200 bp was sufficient to drive *InsL3* gene transcription in various Leydig cell lines. Within this proximal region, three binding sites for the transcription factor SF1 were

Figure 6

COUP-TFII activates the *InsL3* promoter via the DR0-like element at -97 bp. (A) The DR0-like and DR3 elements are critical for *InsL3* promoter activity. MA-10 Leydig (left panel) and CV-1 fibroblast (right panel) cells were co-transfected with either a WT -1087 bp *InsL3* reporter, reporters harbouring mutations (depicted by a large X) in the DR0-like and DR3 elements as indicated, or a minimal -79 bp reporter. The two DR elements are represented by the white (DR3) and grey (DR0-like) diamonds. The number of experiments, each performed in triplicate, is indicated.

Results are shown as relative activity ($\pm$ s.e.m.). A different letter indicates a statistically significant difference (P <0.001). (B) MA-10 Leydig (left panel) and CV-1 (right panel) cells were transfected with the same reporters described in A along with either an empty expression vector (-, open bars) or an expression vector for COUP-TFII (+, black bars). The number of experiments, each performed in triplicate, is indicated. Results are shown as fold activation over control ($\pm$ s.e.m.). A different letter indicates a statistically significant difference (P <0.001).

Figure 7

COUP-TFII is recruited to the proximal *InsL3* promoter and binds specifically to the DR0-like element. (A) COUP-TFII recruitment to the proximal *InsL3* promoter in MA-10 cells was determined by ChIP. An aliquot of chromatin preparation before immunoprecipitation (input) was used as positive control. A 200-bp DNA fragment containing the DR0-like element present in the mouse *InsL3* promoter was amplified by PCR in the input and in the sample immunoprecipitated with the anti-COUP-TFII antiserum but not in the IgG control sample. Recruitment of NUR77 in the same region was used as positive control. The recruitment of COUP-TFII and NUR77 was also assessed on the distal *InsL3* promoter region (~3000 bp) and the signals were similar to those of the IgG-negative control sample. Water was used as negative control for the PCR (Ctrl (-) PCR). The PCR is representative of three independent experiments. (B) DNA precipitation assays were carried out using oligonucleotides containing a high affinity DR1 element (HA DR1, AGGTCAAGGTCA, positive control), the DR0-like element and the DR3 element from the *InsL3* promoter either WT or with a mutation that destroys the binding site (Mut) along with nuclear extracts from MA-10 Leydig cells. Western blottings were used to detect COUP-TFII. Input corresponds to 15 µg of the nuclear extracts used in the DNA precipitation assay. The western blotting shown is the representative of three independent experiments.

identified (Zimmermann *et al.* 1998, Koskimies *et al.* 2002). Mutagenesis studies showed that all three SF1 elements were needed for maximal SF1-dependent activation of the *InsL3* promoter. However, SF1 displayed differential binding affinities for the three SF1 elements and was found to have the highest affinity for the most distal element at -144 to -136 bp (which represents a half-site of the DR3 we have identified in our study; Koskimies *et al.* 2002). Yet it was the element at -115 to -107 bp that showed the strongest response to SF1 in

terms of transcriptional activation (Koskimies *et al.* 2002). In addition to the mice and rat *InsL3* promoter, SF1 was also identified as a potent activator of the canine *INSL3* promoter (Truong *et al.* 2003). Binding of SF1 to each site may very well differ in intensity between species as shown by Sadeghian *et al.* (2005), but the motif at -115 to -107 bp was always found to be critical for *InsL3* gene expression in all species analysed (Zimmermann *et al.* 1998, Koskimies *et al.* 2002, Truong *et al.* 2003, Sadeghian *et al.* 2005, Robert *et al.* 2006). In addition to SF1, our laboratory has identified the orphan nuclear receptor NUR77 as a positive regulator of human and mice *INSL3* transcription in Leydig cells. NUR77 was found to bind to an element located at -100 bp within the proximal *INSL3* promoter, an element essential and sufficient to confer NUR77 responsiveness (Robert *et al.* 2006).

In our present work, we found that the orphan nuclear receptor COUP-TFII was essential for maximal *InsL3* expression in MA-10 Leydig cells. Our results complement the *in vivo* findings by Qin *et al.* (2008) which showed that *InsL3* mRNA levels were decreased in *Coup-tfii*^{-/-} mice at the pre-pubertal stage. However, as Leydig cell differentiation was impaired in these animals, it remained unknown whether the decrease in *InsL3* was due to improper Leydig cell differentiation and/or to a direct action of COUP-TFII in *InsL3* transcription. The fact that depletion of COUP-TFII in MA-10 Leydig cells using siRNA led to a significant decrease in *InsL3* mRNA levels indicates that COUP-TFII could directly regulate *InsL3* gene expression in these cells. This is further supported by the

Figure 8

COUP-TFII and SF1 cooperate on the *InsL3* promoter. MA-10 Leydig (left panel) and CV-1 (right panel) cells were co-transfected with an empty expression vector (open bars) or expression vectors for COUP-TFII (grey bars) and SF1 (hatched bars) either alone or in combination (solid bars) along with a -1087 bp and a -79 bp *InsL3* reporter. The number of experiments, each carried out in triplicate, is indicated. Results are shown as fold activation over control ($\pm$ S.E.M.). Different letters indicate statistically significant differences ($P < 0.05$).

fact that COUP-TFII and INSL3 co-localise in Leydig cells of the adult mouse testis. In foetal testis, however, COUP-TFII is not present in INSL3 positive cells, indicating that this nuclear receptor does not directly regulate *Insl3* expression in the foetal testis.

A previously uncharacterised DR0-like sequence (GAGCCTCGACCT) was identified for COUP-TFII binding and promoter activation in the proximal *Insl3* promoter, the region known to be sufficient for the *Insl3* promoter activity (Zimmermann *et al.* 1998, Koskimies *et al.* 2002, Sadeghian *et al.* 2005). This DR0-like sequence at -97 bp was also present in the *Insl3* promoter from various species including mice, rat, human and bovine, and thus has been evolutionarily conserved supporting a crucial function for the associated DNA-binding protein. Consistent with this, the human and rat *Insl3* promoter were also significantly activated by COUP-TFII.

COUP-TFII is known to bind to a variety of DR elements with variable spacing. Although it shows the highest affinity for a DR1 element, COUP-TFII can also bind to DR0 and DR2 elements with the second highest affinity (Cooney *et al.* 1992, Kadowaki *et al.* 1992, Kliewer *et al.* 1992). Mutation of the DR0-like element severely decreased *Insl3* promoter activity in MA-10 Leydig cells thus identifying this element as critical for *Insl3* transcription. Furthermore, DNA-protein interaction experiments revealed that COUP-TFII binds to the DR0-like element. Yet, mutation in the DR0-like element that prevents COUP-TFII binding had no effect on COUP-TFII-dependent activation of the *Insl3* promoter in MA-10 Leydig cells. However, the same mutation abrogated COUP-TFII-responsiveness of the *Insl3* promoter in heterologous CV-1 fibroblast cells. These results indicated that although COUP-TFII can bind to and activate the *Insl3* promoter via the DR0-like element, it may also do so in a DNA binding-independent manner most likely via interactions with other Leydig cell-enriched, DNA-bound transcription factors. The proximity of the SF1 binding site within the DR3 element, which is also important for *Insl3* promoter activity in Leydig cells (this study and Koskimies *et al.* (2002)), prompted us to test whether COUP-TFII and SF1 function together on the *Insl3* promoter. We found that COUP-TFII and SF1 had additive effects on *Insl3* promoter activity when transfected in MA-10 Leydig cells whereas in CV-1 heterologous cells, the nuclear receptors had strong synergistic effects. This is the first report of a transcriptional cooperation between COUP-TFII and SF1. Additional work is needed to fully decipher the mechanisms of COUP-TFII and SF1 cooperation in *Insl3* gene expression.

In conclusion, we reported that *Insl3* mRNA levels were decreased in COUP-TFII-depleted MA-10 Leydig cells and found that the *Insl3* promoter is directly and positively regulated by COUP-TFII. Furthermore, we identified a transcriptional cooperation between the nuclear receptors COUP-TFII and SF1 to further enhance *Insl3* promoter activity. It is noteworthy that the concentration of INSL3 circulating in the bloodstream parallels the mRNA levels found within the testes (Irving-Rodgers *et al.* 2002, Ivell *et al.* 2013b) further supporting that this hormone is constitutively secreted as soon as it is synthesised. For this reason, quantification of *Insl3* mRNA levels in the testis, or of INSL3 as the secreted peptide circulating in the bloodstream, provides an excellent assessment of Leydig cell differentiation and function (Ivell *et al.* 2013a). Our findings contribute to a better understanding of the regulation of INSL3 expression, a hormone now considered as an informative clinical parameter of gonadal function (Ivell *et al.* 2013a).

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.

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