KLF6 cooperates with NUR77 and SF1 to activate the human INSL3 promoter in mouse MA-10 Leydig cells

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Abstract

Insulin-like 3 (INSL3), a Leydig cell-specific hormone, is essential for testis descent during foetal life and bone metabolism in adults. Despite its essential roles in male reproductive and bone health, very little is known regarding its transcriptional regulation in Leydig cells. To date, few transcription factors have been shown to activate INSL3 promoter activity: the nuclear receptors AR, NUR77, COUP-TFII and SF1. To identify additional regulators, we have isolated and performed a detailed analysis of a 1.1 kb human INSL3 promoter fragment. Through 5′ progressive deletions and site-directed mutagenesis, we have mapped a 10 bp element responsible for about 80% of INSL3 promoter activity in Leydig cells. This element is identical to the CPE element of the placental-specific glycoprotein-5 (PSG5) promoter that is recognized by the developmental regulator Krüppel-like factor 6 (KLF6). Using PCR and western blotting, we found that KLF6 is expressed in several Leydig and Sertoli cell lines. Furthermore, immunohistochemistry on adult mouse testis revealed the presence of KLF6 in the nuclei of both Leydig and Sertoli cells. KLF6 binds to the 10 bp KLF element at −108 bp and activates the −1.1 kb human, but not the mouse, INSL3 promoter. KLF6-mediated activation of the human INSL3 promoter required an intact KLF element as well as Leydig/Sertoli-enriched factors because KLF6 did not stimulate the human INSL3 promoter activity in CV-1 fibroblast cells. Consistent with this, we found that KLF6 transcriptionally cooperates with NUR77 and SF1. Collectively, our results identify KLF6 as a regulator of human INSL3 transcription.

Introduction

Insulin-like 3 (INSL3), also known as Leydig-insulin-like (Ley-I-L) and relaxin-like factor (RLF), is a small peptide hormone (20 aa) belonging to the insulin–IGF–relaxin family of growth factors and hormones (Adham et al. 1993, Pusch et al. 1996). During development, INSL3 is expressed in a sexually dimorphic pattern and produced almost exclusively by Leydig cells. During foetal life, INSL3 was found to be a critical regulator of testicular descent. Insl3-deficient mice exhibit bilateral undescended testes located high in the abdominal cavity (Nef & Parada 1999,
Zimmermann et al. 1999). In Insl3−/− mice, testicular descent was restored by expressing INSL3 in pancreatic β-cells (Adham et al. 2002). Further support for a role for INSL3 as a fundamental regulator of gonadal positioning is the fact that female transgenic mice expressing INSL3 had descended ovaries (Adham et al. 2002, Koskimies et al. 2003). In adults, Insl3 is expressed in both males (Leydig cells) and females (theca and luteal cells) (Adham et al. 1993, Pusch et al. 1996, Zimmermann et al. 1997, Balvers et al. 1998), albeit expression in adult males is an order of magnitude higher than in adult females (Bullesbach et al. 1999). During adult life, INSL3 was found to regulate bone metabolism in males (Ferlin et al. 2008, 2013). In addition to its important functional roles, INSL3 constitutes a highly specific marker of Leydig cell differentiation and functional status (Forresta et al. 2004, Ivell et al. 2013).

Despite the fact that the INSL3 promoter has been isolated from various species, very little is known regarding its transcriptional regulation. We and others have shown that transcription factors belonging to the nuclear receptor family are involved in INSL3 promoter activity in various species. These include SF1 (Ad4BP, NR5A1) (Zimmermann et al. 1998, Koskimies et al. 2002, Truong et al. 2003, Sadeghian et al. 2005), NUR77 (NGFI-B, NR4A1) (Tremblay & Robert 2005, Robert et al. 2006), the testosterone-activated androgen receptor (AR, NR3C4) (Lague & Tremblay 2008, Tremblay et al. 2009) and more recently COUP-TFII (NR2F2) (Mendoza-Villarroel et al. 2014).

Krüppel-like factors (KLF) are C2H2 zinc finger transcription factors that belong to the SP1/KLF family. So far, sixteen KLF factors (KLF1-16) have been isolated in mammalian species (reviewed in Pearson et al. 2008). Members of the KLF family act as transcriptional activators, repressors, or both by binding to the consensus CA-rich sequence CACCC present in the promoter region of target genes (Pearson et al. 2008). KLFs are found in a wide variety of tissues and have been implicated in diverse biological processes including cell proliferation, tissue-specific gene expression, apoptosis, cell growth, differentiation and tumorigenesis (Pearson et al. 2008). Although KLFs have been detected in numerous tissues, very little is known regarding their expression and roles in the testis. So far, KLF4 has been immunolocalized to germ cells and Sertoli cells (Behr & Kaestner 2002, Godmann et al. 2008) whereas mRNA for Klf2 (Anderson et al. 1995), Klf5 (Sogawa et al. 1993), Klf6 (Sogawa et al. 1993, Inuzuka et al. 1999), Klf13 and Klf14 (Scobey et al. 2000) were detected in adult testis by Northern blotting or RT-PCR.

In this study, we have identified a key regulatory element specifically in the human INSL3 promoter and found that this element is recognized and activated by the KLF6 transcription factor, which we have located in the nuclei of somatic cells of the mouse testis. Furthermore, we report that KLF6 functionally cooperates with the nuclear receptors NUR77 and SF1 to further stimulate human INSL3 promoter activity.

**Materials and methods**

**Plasmids**

The −1137, −920, −656, −322 and −93 bp to +11 bp human INSL3 promoter fragment and the −1137 bp

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**Figure 1** Localization of an essential regulatory region in the proximal human INSL3 promoter. MA-10 Leydig cells (left panel) and CV-1 fibroblasts (right panel) were co-transfected with various 5′ deletion constructs of the human INSL3 promoter (the 5′-end point of each construct is indicated on the left of the graph) or a construct harbouring a mutation in the previously characterized NBRE/SF1 (Robert et al. 2006). The mutation is depicted by a large X. Thick lines below the promoter diagrams indicate the position of the NBRE/SF1, SF1, COUP-TF and AR elements. Results are shown as % activity (± S.E.M.) relative to the activity of the −1137 bp wild-type reporter in MA-10 cells. For each cell line, a different letter indicates a statistically significant difference.
containing a mutation in the NBRE at −95 bp have been described previously (Robert et al. 2006). The deletion constructs to −132 and −85 bp have been described in the study by Laguë & Tremblay (2008). Various trinucleotide and dinucleotide mutant constructs in the context of the −1137 bp reporter were generated using the QuikChange XL Mutagenesis Kit (Stratagene) along with the following oligonucleotides (only the sequence of the sense oligonucleotide is shown), where the mutations are in lowercase: M1 5′-CCC TGG CCC TGG GAG AAA tta TCT GGC ACT AAC CCC ACC C-3′, M2 5′-CCT GGG AGA AAG GCT CTG Gac aTa ACC CCA CCC TTG ACC C-3′, M3 5′-AAG GCT CTG GCA CTA ACC acC CCC TTG ACC TTT TTC CTG GGC-3′, M4 5′-GGG ACT AAG CCC ACC ACC TTt caC TTT TTC CTG GGC GGT CC-3′, M5 5′-CCC CAC CCT GAC CTT TTt aaT GGG CGG GTC CTG AAG AAT G-3′, M6 5′-AAG GCT CTG GCA CTA ACC CaC TTG ACC TTT TTC CTG-3′, M7 5′-GGT GCT GTG CTA ACC Cac CCC TTG ACC TTT TTC CTG GGC-3′. All reporter constructs were subcloned in a modified pXP1 Luciferase reporter construct (Tremblay & Viger 1999). The mouse KLF6 expression vector was generated by subcloning into pcDNA3 (Invitrogen) the mouse Klf6 cDNA was obtained by RT-PCR using the following primers: forward (XbaI cloning site underlined) 5′-GCT CTG CTA GAA TGA AAC TTT CAC CTG CGC TCC-3′; reverse (BamHI cloning site underlined) 5′-CGG GAT CCT CAG AGG TGC CTC TTC ATG TGC-3′. The mouse SF1 expression plasmid has been described previously (Tremblay & Viger 2001). Expression vector for NUR77 (Philips et al. 1997) was kindly provided by Dr Jacques Drouin (Laboratoire de Génétique Moléculaire, Institut de Recherches Cliniques de Montréal, Montréal, Canada).

All plasmids were verified by sequencing (Centre de génomique de Québec, CHUL Research Centre, Québec City, Canada).

Cell culture and transfections

The mouse Leydig cell lines MLTC-1, LC540 and TM3 were obtained from ATCC (Manassas, VA, USA). The mouse Sertoli cell line MSC-1 (McGuinness et al. 1994) was kindly provided by Dr Michael D Griswold (Washington State University, Pullman, WA, USA). Mouse MA-10 Leydig cells (Ascoli 1981), provided by Dr Mario Ascoli (University of Iowa, Iowa City, IA, USA), were grown in Waymouth’s medium supplemented with 15% horse serum. African green monkey kidney CV-1 fibroblast cells were obtained.

**Figure 2**

Fine mapping of the 40 bp element in the proximal human INSL3 promoter. Several cell lines (MA-10 and MLTC-1 Leydig cells, MSC-1 Sertoli cells, CV-1 fibroblast cells) were transfected with various −1137 bp human INSL3 promoter constructs: a wild-type promoter and a series of trinucleotide or dinucleotide mutated constructs (M1–M8; the mutations are in lowercase). The underlined sequence corresponds to the previously described NBRE/SF1 binding site (Robert et al. 2006, Tremblay & Robert 2005, Tremblay et al. 2009). The CA-rich sequence is boxed. Thick lines below the promoter diagrams indicate the position of the NBRE/SF1, SF1, COUP-TF and AR elements. Results are shown as % activity (± s.e.m.) relative to the activity of the −1137 bp wild-type reporter in MA-10 Leydig cells. Different letters indicate statistically significant differences.
from ATCC and grown in DMEM supplemented with 10% newborn calf serum. All transfections were done in 24-well plates using the calcium phosphate precipitation method as described previously (Tremblay & Viger 2001, Robert et al. 2006). The cells were then lysed and luciferase activities measured using the Dual Luciferase Assay System (Promega) and the Luminoskan Ascent luminometer (Thermo Scientific). Luciferase activities in MA-10, MLTC-1, MSC-1 and CV-1 cells were standardized to the activity of a RSV-Luciferase reporter. Data reported represent the average of at least four experiments (n ≥ 4) using different DNA preparations, each performed in duplicate.

RNA isolation, reverse transcription and PCR

Total RNA was isolated from MA-10 Leydig cells using the RNeasy Plus Extraction Kit (Qiagen). First-strand cDNAs were synthesized from a 5 µg aliquot of the various RNAs using the Superscript III Reverse Transcriptase System (Life Technologies). PCRs were performed using the following Klf6-specific primers: forward (XbaI cloning site underlined) 5’-GCT CTA GAT ATC TTC AGA GTG AGC CCT GCT AC-3’; reverse (KpnI cloning site underlined): 5’-GGG GTA CCA GCC CCA TAG TTG AGA AGA TTC C-3’. The primers for αTubulin (Tuba1a) were described previously (Moisan et al. 2008). Semi-quantitative PCRs for Klf6 and Tuba1a were performed on a Tgradient thermocycler (Biometra, Göttingen, Germany) using Vent DNA polymerase (New England Biolabs, Beverly, MA, USA) under the following conditions: 5 min at 95°C followed by 30 cycles of denaturation (45 s at 95°C), annealing (45 s at 54°C) and extension (45 s at 72°C) with a final extension step of 5 min at 72°C. PCR products were then analysed by agarose gel electrophoresis and ethidium bromide staining.

Protein preparation and western blottings

Nuclear extracts were prepared by the procedure outlined by Schreiber et al. (1989). Protein concentrations were estimated using standard Bradford assay. In knockdown experiments, mouse MA-10 Leydig cells were transfected using jetPRIME as recommended in the manufacturer’s instructions (PolyPlus-transfection, Illkirch, France), with 400 nM of siRNA (a mix of 133 nM of three different siRNAs) directed against KLF6 or scrambled siRNA as control (Life Technologies). Nuclear proteins (15 µg) were boiled 10 min in a denaturing loading buffer, fractionated by SDS–PAGE and transferred onto PVDF membrane (Millipore). Immunodetection was performed using an avidin–biotin approach, according to the manufacturer’s instructions (Vector Laboratories, Ontario, Canada). Detection of KLF6 was performed carried out using an anti-KLF6 rabbit polyclonal antiserum (R-173, 1:200 dilution, Santa Cruz Biotechnologies) and a secondary biotinylated anti-rabbit antibody (Life Technologies). Nuclear proteins (15 µg) were denatured and fractionated by SDS–PAGE and transferred onto PVDF membrane (Millipore). Immunodetection was performed using an avidin–biotin approach, according to the manufacturer’s instructions (Vector Laboratories, Ontario, Canada). Detection of LAMIN B (LMNB1) was performed using a goat polyclonal anti-LMNB1 antiserum (C-20, 1:500 dilution; Santa Cruz Biotechnologies) and a secondary peroxidase-conjugated anti-goat antibody (1:5000, Vector Laboratories, Ontario, Canada).

Electromobility shift assays

Recombinant KLF6 protein was in vitro translated using the T7 Quick Coupled TnT System (Promega). DNA binding assays were performed using either 3 µg of MA-10 nuclear extracts or 3 µl of in vitro-translated protein as described previously (Martin & Tremblay 2005). The 32P-labelled double-stranded oligonucleotides used as probe were (the KLF element is overlined): 5’-GCT CTA GAT ATC TTC AGA GTG AGC CCT GCT AC-3’. Binding of the protein was then challenged by increasing doses (black triangles; molar excesses of 2× and 5×) of unlabelled oligonucleotides corresponding to the wild-type −108 bp element (wt) or oligonucleotides that contain mutations (M3, M4; numbers correspond to the constructs used in Fig. 2) in and around the CA-rich element. n.s., non-specific binding.

Figure 3

A nuclear protein from MA-10 cells specifically binds to the CA-rich element. EMSA was used to assess the binding of nuclear extracts from MA-10 cells (MA-10 N.E.) to a double-stranded 32P-labelled oligonucleotide corresponding to the CA-rich element at −108 bp. Binding of the protein was then challenged by increasing doses (black triangles; molar excesses of 2× and 5×) of unlabelled oligonucleotides corresponding to the wild-type −108 bp element (wt) or oligonucleotides that contain mutations (M3, M4; numbers correspond to the constructs used in Fig. 2) in and around the CA-rich element. n.s., non-specific binding.
underlined): sense 5'-ACT AAC CCC ACC CTG GCC CT-3' and antisense 5'-AGG TCA AGG GTG GGG TTA GT-3'. For the competition experiments, 2- or 5-fold molar excess of double-stranded oligonucleotides (wild-type or mutated as described above for the promoter mutagenesis) were added to the reaction. For supershift/blocking experiments, 3 or 5 μg of normal rabbit IgG or a commercially available anti-KLF6 antiserum (R-173, Santa Cruz Biotechnologies) were added to the binding reaction.

**Immunohistochemistry**

Adult CD-1 mice (~40 day old) were obtained on site and killed by CO₂ inhalation. The testes were harvested and fixed with ice-cold 4% paraformaldehyde (w/v) for 24 h. Tissues were then dehydrated with ethanol, substituted with xylene, embedded in paraffin and cut into 5 μM sections. Following paraffin removal, tissues were blocked with 0.5% goat serum in PBS for 1 h at 25°C. Immunodetection was performed using an avidin–biotin approach according to the manufacturer's instructions for the Vectastain Elite ABC reagent (Vector Laboratories, Ontario, Canada). KLF6 protein localization was assessed using an anti-KLF6 rabbit polyclonal antiserum (R-173, 4 μg/mL; Santa Cruz Biotechnologies). Negative control corresponds to the same procedure except that the anti-KLF6 antibody was replaced with normal rabbit IgG (SC2027, 4 μg/mL, Santa Cruz Biotechnologies). Final detection was done using AEC (3-amino-9-ethylcarbazole from Sigma-Aldrich) as substrate and the sections were counterstained with hematoxylin Gill no.1 (VWR International, Mount-Royal, Québec, Canada). 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).

**Statistical analyses**

Statistical analyses were carried out using one-way ANOVA or ANOVA on ranks (when parameters were not met) followed by a Student–Newman–Keuls post test. For all statistical analyses, P < 0.05 was considered to be statistically significant. All statistical analyses were done using SigmaStat software package (Systat Software Inc, San Jose, CA, USA).

**Results**

**Mapping of an important regulatory element in the proximal human INSL3 promoter**

As there is currently no human Leydig cell line available, several mouse Leydig cell lines were used, including the well-characterized MA-10 cell line model. To locate

![Figure 4](http://dx.doi.org/10.1530/JME-15-0139)

KLF6 is expressed in testicular somatic cells. (A) Semi-quantitative RT-PCR using primers specific for Klf6 was used to detect the presence of Klf6 mRNA in Leydig (MA-10, MLTC-1, LC540, TM3) and Sertoli (MSC-1) cell lines; α-Tubulin (Tuba1a) as used as control. (B) For detection of KLF6 protein, western blottings were performed using nuclear extracts from various cell lines including Leydig (MA-10, MLTC-1, LC540, TM3), Sertoli (MSC-1), fibroblast (CV-1), as well as from MA-10 Leydig cells transfected with an empty expression vector (MA-10+Ctl) or an expression vector for KLF6 (MA-10+KLF6). LAMIN B (LMNB1) was used as a loading control. (C) Mouse MA-10 Leydig cells transfected with scrambled siRNA (siRNA Ctl) or siRNA against KLF6 (siRNA KLF6) and western blotting were used to confirm KLF6 protein levels in knockdown MA-10 cells; LAMIN B (LMNB1) was used as a loading control. (D) Within the testis, KLF6 is specifically expressed in somatic cells. Immunohistochemistry was performed on adult mouse testis sections using an anti-KLF6 antiserum. The nuclei of Leydig (arrows) and Sertoli (arrowheads) cells are labelled (red-orange staining). Omission of the primary antibody served as negative control (inset). Magnifications: 400×. A full colour version of this figure is available at http://dx.doi.org/10.1530/JME-15-0139.
important regulatory elements in the human INSL3 promoter, various 5′ progressive deletion constructs were transfected in mouse MA-10 Leydig cells and in CV-1 fibroblasts. As shown in Fig. 1, the activity of a −1137 bp reporter construct was higher in MA-10 Leydig cells compared with CV-1 fibroblast cells, which is consistent with the fact that MA-10 Leydig cells endogenously express Insl3 whereas CV-1 cells do not (Sadeghian et al. 2005) and our unpublished data). Deletion to −132 bp did not significantly affect promoter activity (Fig. 1). Further deletion to −93 bp, however, led to a decrease in human INSL3 promoter activity, with only 15–17% of activity remaining (Fig. 1). This indicates that important regulatory elements are located within a −40 bp region located between nucleotides −132 and −93 bp. This region contains a previously characterized NBRE/SF1 binding site at −95 bp for the nuclear receptor NUR77 that can also be recognized by SF1 (Tremblay & Robert 2005, Robert et al. 2006), as well as a DR0 site for the binding of the nuclear receptor COUP-TFII (−91/−103 bp) originally identified in the mouse Insl3 promoter (Mendoza-Villarroel et al. 2014). To test the possibility that the loss in promoter activity with the −93 bp construct might be due to removal of the NBRE/SF1 and/or COUP-TFII element, cells were transfected with a −1137 human INSL3 reporter construct that harbours a mutation at −97 bp known to abolish binding of NUR77 and SF1 (Tremblay & Robert 2005, Robert et al. 2006) as well as COUP-TFII (Mendoza-Villarroel et al. 2014). As shown in Fig. 1, this mutation led to a decrease of ~18–20% of promoter activity. This indicates that the majority of the activity within the −132 to −93 bp region is conferred by element(s) other than the binding site for the nuclear receptors.

To further delineate the sequences responsible for conferring nearly 80% of human INSL3 promoter activity within the −132 to −93 bp regions, a series of mutated reporter constructs in the context of the −1137 bp promoter were generated and transfected in two Leydig cell lines, MA-10 and MLTC-1, as well as in Sertoli MSC-1 cells and CV-1 fibroblasts. As shown in Fig. 2, activity of the human INSL3 promoter is significantly higher in both Leydig cell lines compared with Sertoli and fibroblast cells. In MA-10 and MLTC-1 Leydig cells, mutant M3 that changes three nucleotides in a CA-rich sequence resulted in a loss of ~75% in INSL3 promoter activity (Fig. 2). Mutations on either side of this CA-rich sequence either also decreased INSL3 promoter activity (M2 and M4) but to a lesser extent than M3 or did not reduce INSL3 promoter activity (M1 and M5) (Fig. 2). Additional mutations that change two nucleotides in the CA-rich region (M6, M7 and M8) also led to decreased human INSL3 promoter activity in Leydig cells, albeit less than the trinucleotide mutant M3 (Fig. 2). None of the mutations decreased INSL3 promoter activity

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**Figure 5**

KLF6 binds to the CA-rich element. (A) In vitro-produced KLF6 co-migrates with a protein in MA-10 nuclear extracts. EMSA was performed using a double-stranded, 32P-labelled oligonucleotide corresponding to the CA-rich element at −108 bp in the human INSL3 promoter along with in vitro-produced KLF6 (TNT KLF6), unprogrammed reticulocytes (TNT Ctrl) and nuclear extracts from MA-10 Leydig cells (MA-10 N.E.). (B) In vitro-produced KLF6 specifically binds to the CA-rich element. EMSA was used to assess the binding of in vitro-produced KLF6 to a double-stranded, 32P-labelled oligonucleotide corresponding to the CA-rich element at −108 bp in the human INSL3 promoter. KLF6 binding was then challenged by increasing doses (black triangles; molar excesses of 2x and 5x) of unlabelled oligonucleotides corresponding to the wild-type −108 bp element (wt) or two oligonucleotides that harbour mutations within (M3) and outside (M4) the CA-rich element. (C) KLF6 from MA-10 nuclear extracts binds to the CA-rich element. EMSA was used to determine the binding of the KLF6 protein present in MA-10 Leydig cells to the CA-rich element. The KLF6 binding was blocked by addition of 3 (+) and 5 (++) g of a KLF6 antisera (αKLF6). Normal rabbit IgG was used as control.
in Sertoli MSC-1 and CV-1 fibroblast cells (Fig. 2). All together, these results indicate that a CA-rich sequence located between −112 and −105 bp is important for maximal human INSL3 promoter activity in Leydig cells.

**Binding of a specific protein to the CA-rich sequence**

Next, we used EMSA to determine whether protein(s) from Leydig cell nuclear extracts could specifically bind to the CA-rich sequence in the human INSL3 promoter. As shown in Fig. 3, a binding was detected (Fig. 3, lane 2) that could be competed with increasing molar excess (2- and 5-fold) of unlabelled wild-type (WT) oligonucleotides (Fig. 3, lanes 3 and 4). Binding specificity was assessed by competition experiments using unlabelled oligonucleotides containing the same mutations as those shown in Fig. 2 for promoter activity. As shown in Fig. 3, oligonucleotides corresponding to mutant M3 (mutation within the CA-rich sequence that blunts promoter activity, Fig. 2) could not displace the binding complex (Fig. 3, lanes 5 and 6), whereas oligonucleotides corresponding to mutant M4 (mutation outside the CA-rich sequence which has limited effect on promoter activity, Fig. 2) could compete the binding (Fig. 3, lanes 7 and 8). Thus, a protein from MA-10 Leydig cell nuclear extracts specifically binds to the CA-rich sequence of the human INSL3 promoter.

**KLF6 is expressed in the mouse testis**

Sequence analysis revealed that the CA-rich element of the human INSL3 promoter (CTAACCCCACCCTTG) is strikingly similar to the CPE element (CTGACCCCACCCATG) previously characterized in the human placental-specific glycoprotein 5 (PSG5) promoter (Koritschoner et al. 1997). This element in the PSG5 promoter is recognized by members of the KLF family of transcription factors, and especially KLF6 (Koritschoner et al. 1997). As Klf6 has been detected in the whole testis by Northern blotting (Inuzuka et al. 1999), we sought to determine whether KLF6 was expressed in Leydig cells. RT-PCR using cDNAs from various Leydig cell lines (MA-10, MLTC-1, LC540 and TM3) and a Sertoli cell line (MSC-1) was first performed. As shown in Fig. 4A, Klf6 mRNA was detected in all cell lines. At the protein level, a band of 45 kDa corresponding to the KLF6 protein was detected by western blottings in both Leydig (MA-10, MLTC-1, LC540 and TM3) and Sertoli (MSC-1) cell lines but not in the CV-1 fibroblast cell line (Fig. 4B). MA-10 Leydig cells were also transfected with an empty expression vector or an expression vector encoding KLF6. As shown in Fig. 4B, a band of increased intensity was detected by western blotting with the anti-KLF6 antibody in the KLF6-overexpressing MA-10 cells (Fig. 4B). Conversely, transfection of siRNA directed against KLF6 in MA-10 cells lead to a significant reduction in KLF6 protein levels while control siRNA had no effect (Fig. 4C). Both the overexpression (Fig. 4B) and knockdown (Fig. 4C) data validate the specificity of the anti-KLF6 antibody. To further confirm KLF6 expression in Leydig cells in vivo, we performed immunohistochemistry on adult mouse testis sections. In agreement with the RT-PCR and western blotting results, the KLF6 protein was detected (red-orange staining) in the nuclei of Leydig (arrows in Fig. 4D) and Sertoli (arrowheads in Fig. 4D) cells. This labelling is specific since no signal was observed when the primary antibody was omitted (Fig. 4D, inset). Thus, within the mouse testis, KLF6 is present in the nuclei of somatic cells.
**KLF6 binds to the CA-rich sequence of the human INSL3 promoter**

We next tested whether *in vitro*-produced KLF6 could bind to the CA-rich sequence of the human INSL3 promoter by EMSA. As shown in Fig. 5A, *in vitro*-produced KLF6 co-migrates with a protein present in nuclear extracts from MA-10 Leydig cells. Furthermore, competition assays revealed that KLF6 binding was displaced by WT and M4 (mutation outside the CA-box) oligonucleotides, while the M3 oligonucleotide that contains a mutation in the CA-rich sequence was not as efficient at competing KLF6 binding (Fig. 5B). Furthermore, the complex present in MA-10 nuclear extracts could be competed by the addition of an anti-KLF6 antiserum (Fig. 5C, lanes 4 and 6). Thus endogenous KLF6 present in nuclear extracts from MA-10 Leydig cells can bind to the CA-rich element at −108 bp in the human INSL3 promoter.

**KLF6 activates the human INSL3 promoter**

To determine if KLF6 could directly regulate human INSL3 promoter activity, we performed transient transfections in MA-10 Leydig cells, CV-1 fibroblasts and MSC-1 Sertoli cells. As shown in Fig. 6, expression of KLF6 led to increased activity of the −1137 bp human INSL3 reporter construct in MA-10 Leydig and MSC-1 Sertoli cells but not in CV-1 fibroblasts. Consistent with the fact that KLF6 binds to the −108 bp CA-rich sequence that we now name the KLF element, a −1137 bp reporter construct harbouring a mutation in the KLF element was no longer significantly activated by KLF6 in MA-10 Leydig and MSC-1 Sertoli cells (Fig. 6). Thus, maximal activation of the human INSL3 promoter by KLF6 requires an intact KLF element at −108 bp.

**KLF6 transcriptionally cooperates with NUR77 and SF1 on the human INSL3 promoter**

The fact that KLF6 was unable to stimulate the human INSL3 promoter activity in heterologous CV-1 cells (Fig. 6) indicates that KLF6 likely requires other factor(s) that are common to both Leydig and Sertoli cells but absent from CV-1 fibroblasts. Because the KLF and NBRE/SF1 elements are adjacent (Fig. 2), and since NUR77 and SF1 are expressed in Leydig and Sertoli cells but not in CV-1 cells (Fig. 7A), we tested the possibility that these transcription factors might functionally cooperate with KLF6. As expected, both SF1 and NUR77 activated the human INSL3 promoter 2.2- and 3.7-fold, respectively, in
MA-10 Leydig cells (Fig. 7B). Combination of KLF6 with SF1 or with NUR77 led to a stronger activation (~7-fold) of the INSL3 promoter (Fig. 7B). Thus KLF6 and the nuclear receptors NUR77 and SF1 transcriptionally cooperate on the human INSL3 promoter.

**Discussion**

Depending on the species, the INSL3 gene is located either immediately downstream (human) or within the last intron (mouse) of the jak3 gene. Because of this unusual gene structure, the regulatory motifs driving INSL3 expression in Leydig cells are believed to be comprised within a relatively short 5′ flanking sequence. Using the MA-10 Leydig cell line as a model, the first 200 bp upstream of the transcription start site were found to be sufficient to confer activity to the mouse (Zimmermann et al. 1998, Koskimies et al. 2002) and rat (Sadeghian et al. 2005) Insl3 promoter. This region is also known to be activated by the nuclear receptors SF1 (NR5A1), NUR77 (NR4A1) and AR (NR3C1) (Zimmermann et al. 1998, Koskimies et al. 2002, Truong et al. 2003, Sadeghian et al. 2005, Tremblay & Robert 2005, Robert et al. 2006, Lagué & Tremblay 2008, Tremblay et al. 2009). In this study, we found that the Krüppel family member KLF6 is expressed in Leydig cells where it contributes to the activity of the human INSL3 promoter but not the mouse Insl3 promoter.

**A CA-rich sequence essential for INSL3 promoter activity**

Comparison of the activity of a ~1.1 kb human INSL3 promoter in a homologous (MA-10 Leydig cells) and a heterologous (CV-1 fibroblasts) cell lines revealed that this promoter fragment was more active by ~500% in Leydig cells. This indicates that the 1.1 kb human INSL3 promoter fragment contains regulatory elements conferring Leydig cell-specific activity. Deletion analyses revealed that the main regulatory elements of the human INSL3 promoter are located within the ~132 bp proximal region, more precisely between ~132 and ~93 bp. Mutation of 2 or 3 nt in this region in the context of the ~1.1 kb reporter uncovered the importance of a CA-rich sequence (CCCCACCC) and particularly the core CAC trinucleotides when analysed in two Leydig cell lines, MA-10 and MLTC-1. Mutations (M3 and M8) that increased the number of As (e.g. CAC to CAAC or CAACCA) resulted in a dramatic decrease in INSL3 promoter activity to ~25% of wild type in both Leydig cell lines, whereas mutations that preserved an intact CACC motif (M6 and M7) had minimal or no effect. None of the mutations reduced INSL3 promoter activity in cell lines that do not endogenously express Insl3 (MSC-1 Sertoli and CV-1 fibroblast cells). The integrity of the CACC motif is thus indispensable for human INSL3 promoter activity in Leydig cells. Although the human and mouse INSL3 promoters share conserved elements such as testosterone/AR responsiveness at ~118 to ~92 bp (Lagüé & Tremblay 2008), COUP-TFI at ~103 to ~91 bp (Mendoza-Villarroel et al. 2014), NBR/SF1 at ~95 bp (Zimmermann et al. 1998, Koskimies et al. 2002, Robert et al. 2006) and SF1 at ~45 bp (Zimmermann et al. 1998, Koskimies et al. 2002; and our unpublished observations), the CA-rich sequence (CCCCACCC) is unique to the human INSL3 promoter and was not found in the Insl3 promoter of all other species analysed including mouse, rat, porcine, bovine and canine (data not shown).

**KLF6 is a novel activator of the human INSL3 promoter**

The CCCCCACCC motif and surrounding sequences of the human INSL3 promoter (CTAACCCCCACCCCTTG) are nearly identical (13/15 identical nt; mismatches are underlined) to the CPE element identified in the human PSG5 promoter. The KLF family member KLF6 was found bound to this CA-rich motif in the CPE element (Koritschoner et al. 1997). Although some KLF factors have been detected in the testis (Sogawa et al. 1993, Anderson et al. 1995, Inuzuka et al. 1999, Scoby et al. 2000, Behr & Kaestner 2002, Godmann et al. 2008), none have been reported in Leydig cells. Because KLF6 binds to a sequence that is essentially identical to the critical regulatory region we have mapped in the human INSL3 promoter, we chose to further characterize this transcription factor. KLF6 was detected at the mRNA and protein level in all mouse Leydig and Sertoli cell lines tested. KLF6 was also detected in the nuclei of both Leydig and Sertoli cells in the adult mouse testis. In humans, data from the Human Protein Atlas project revealed the presence of KLF6 in the nucleus of Leydig cells, albeit with moderate intensity and low frequency (http://www.proteinatlas.org/ENSG00000067082-KLF6/tissue/testis). Furthermore, KLF6 can activate the human INSL3 promoter in mouse MA-10 Leydig cells, and this transactivation was abrogated when the CA-rich motif, to which KLF6 binds, was mutated. Apart from the implication of KLF6, we cannot formally exclude the possibility that other KLF family members could also be involved in INSL3 gene transcription in Leydig cells.

Although KLF6 can regulate a human INSL3 reporter construct in Leydig cell lines, its involvement in endogenous INSL3 transcription remains to be
demonstrated. Since this cannot be easily addressed in humans and since there are currently no human Leydig cell line available, we turned to the mouse system in order to determine whether KLF6 could regulate Insl3 transcription endogenously in mouse MA-10 Leydig cells. In KLF6-depleted (using siRNA) mouse MA-10 Leydig cells, Insl3 mRNA levels were not decreased (data not shown). Conversely, overexpression of KLF6 did not increase endogenous Insl3 mRNA levels in mouse MA-10 Leydig cells (data not shown). ChIP experiments were also performed but no recruitment of KLF6 was observed on the mouse Insl3 promoter region. Finally, KLF6 failed to activate the mouse Insl3 promoter (1.1 kb fragment) in MA-10 and MLTC1 Leydig cells (data not shown). All these results are consistent with the fact that the mouse Insl3 promoter lacks a CCCCCACCC motif for the binding of KLF6. Taken together, these data indicate that KLF6 is not involved in the regulation of mouse Insl3 gene expression but contributes to the activity of the human INSL3 promoter.

KLF6 functionally cooperates with the nuclear receptors NUR77 and SF1 on the human INSL3 promoter

In addition to MA-10 Leydig cells, KLF6 could also activate the human INSL3 promoter in the Sertoli cell line MSC-1 but not in CV-1 fibroblast cells. This indicates that in addition to binding to its element, KLF6 requires the presence of additional factor(s) that are common to Leydig and Sertoli cells but absent from fibroblasts. Transcription factors known to be present in both Sertoli and Leydig cells include the MADS-box factors MEF2 (Daems et al., 2014), the zinc finger factor GATA4 (reviewed in Viger et al., 2008) and the nuclear receptors SF1 (reviewed in Schimmer & White, 2010) and NUR77 (reviewed in Martin & Tremblay, 2010). Interestingly, the KLF element in the human INSL3 promoter is adjacent to a previously characterized binding site for the nuclear receptors SF1 and NUR77 (Robert et al., 2006). Consistent with this, we found that KLF6 functionally cooperates with both SF1 and NUR77 in regulating human INSL3 promoter activity. Despite this, other factor(s) present in Leydig cells but not in Sertoli cells must also be involved, as INSL3 is not expressed in Sertoli cells. This is supported by the fact that the activity of the human INSL3 promoter is significantly lower in Sertoli than in Leydig cells. More work is required to fully decipher the combinatorial code of transcription factors that directs human INSL3 expression exclusively in Leydig cells.

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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Author contribution
M A T, R E M V, N M R and F B performed the research. M A T, R E M V, N M R, F B and J J T analysed the data. J J T designed the research study and wrote the paper.

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