Transcription of platelet-derived growth factor receptor α in Leydig cells involves specificity protein 1 and 3

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Abstract

Platelet-derived growth factor (PDGF) A is secreted by Sertoli cells and acts on Leydig precursor cells, which express the receptor PDGFRA, triggering their differentiation into steroidogenically active Leydig cells. There is, however, no information regarding the molecular mechanisms that govern *Pdgfra* expression in Leydig cells. In this study, we isolated and characterized a 2.2 kb fragment of the rat *Pdgfra* 5'-flanking sequence in the TM3 Leydig cell line, which endogenously expresses *Pdgfra*. A series of 5' progressive deletions of the *Pdgfra* promoter was generated and transfected in TM3 cells. Using this approach, two regions (−183/−154 and −154/−105), each conferring 46% of *Pdgfra* promoter activity, were identified. To better define the regulatory elements, trinucleotide mutations spanning the −154/−105 region were introduced by site-directed mutagenesis in the context of the −2.2 kb *Pdgfra* promoter. Mutations that altered the TCCGAGGGAAAC sequence at −138 bp significantly decreased *Pdgfra* promoter activity in TM3 cells. Several proteins from TM3 nuclear extracts were found to bind to this G(C/A) motif in electromobility shift assay. Two of the proteins were identified as the transcription factors SP1 and SP3. Using transient transfections of TM3 Leydig cells, SP1 and SP3 were found to activate the *Pdgfra* promoter by threefold. The SP1/SP3-dependent activation of the *Pdgfra* promoter was severely blunted when the G(C/A) motif was mutated. Our study provides new insights into the regulatory mechanisms of *Pdgfra* transcription in Leydig cells, which includes a role for the transcription factors SP1 and SP3.

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Introduction

In mammalian males, there are two distinct populations of Leydig cells present in the testicular interstitium that are responsible for the production of testosterone: the fetal (FLC) and adult (ALC) Leydig cells (reviewed in Haider (2004)). During the embryonic period, the FLC produces androgens essential for masculinization of the male fetus (reviewed in Haider (2004)). Dysfunction or absence of the FLC leads to pseudohermaphroditism. During the early postnatal period, the FLC regress and are substituted by the ALC derived from undifferentiated stem cells (Ge *et al.* 2006). The ALC produce testosterone that is indispensable for the completion of sexual differentiation and reproductive function in males.

It has been suggested that FLC and ALC are derived from undifferentiated primordial stem cells. However, the origin of the primordial stem cells and consequently the Leydig cells remains unclear. Two sources have been proposed: the mesoderm (mesonephrogenic mesenchyme or coelomic epithelium) (Buehr *et al.* 1993, Karl & Capel 1998, Merchant-Larios & Moreno-Mendoza 1998, Schmahl *et al.* 2000) and ectoderm (neural crest) (Mayerhofer *et al.* 1992, Davidoff *et al.*

2004, Lobo *et al.* 2004). Both theories are consistent with the mesenchymal-like morphology of the Leydig stem cells. Differentiation of the primordial stem cells into Leydig cells is a crucial step of the male sex differentiation process. Several signaling molecules are known to initiate the differentiation of Leydig cells such as the platelet-derived growth factor A (PDGFA) secreted by Sertoli cells and its receptor PDGFRA present on Leydig cell progenitors (reviewed in Cool & Capel (2009)). Disruption of the *Pdgfa* (*Pdgfa*^{tm1Cbet}/*Pdgfa*^{tm1Cbet}) or *Pdgfra* (*Pdgfra*^{tm1Sor}/*Pdgfra*^{tm1Sor}) gene in mice (Gnessi *et al.* 2000, Brennan *et al.* 2003, Schmahl *et al.* 2008) was shown to impair the differentiation of Leydig cells (reviewed in Basciani *et al.* (2010)). Leydig precursor cells, or Leydig stem cells (Ge *et al.* 2006), are present but they fail to differentiate into steroidogenically active Leydig cells (reviewed in Basciani *et al.* (2010)). Despite the importance of PDGFRA in Leydig cell differentiation, there is no information regarding the molecular mechanisms that regulate its expression in Leydig cells. However, in vascular smooth muscle cells (VSMC), expression of the rat *Pdgfra* gene is under the positive regulatory influence of the transcription factor specificity protein 1 (SP1; Bonello & Khachigian 2004).

SP1 and SP3 are ubiquitously expressed transcription factors with a DNA-binding domain composed of three zinc fingers like all members of the SP family. SP1 and SP3 activate gene expression by binding to GA-, GT-, and GC-rich motifs in the regulatory region of target genes (reviewed in [Li et al. \(2004\)](#)). SP factors contribute to tissue- and cell-specific gene expression by functionally interacting with other tissue-restricted transcription factors ([Li et al. 2004](#)). In Leydig cells, SP1 was shown to regulate the transcription of genes encoding the LH receptor ([Nikula et al. 2001](#)), the nuclear receptor NR5A1 (SF1) ([Scherrer et al. 2002](#)), the angiogenic factor VEGF ([Schwarzenbach et al. 2004](#)), the acyl-CoA synthetase ([Sheng et al. 2005](#)), and the cholesterol transporter TSPO ([Giatzakis et al. 2007](#)).

In the present study, we characterized the rat *Pdgfra* promoter in Leydig cells. Transfections of TM3 Leydig cells revealed the presence of two regions critical for *Pdgfra* promoter activity. These regions were recognized by several nuclear proteins, two of which being SP1 and SP3. We report that SP1 and SP3 bind to and activate the *Pdgfra* promoter through a novel G(C/A) motif at -138 bp.

Materials and methods

Cell culture

The mouse (TM3, mLTC-1) and rat (LC-540) Leydig cell lines were obtained from American Type Culture Collection (Manassas, VA, USA) and grown as recommended by the vendor. The mouse MA-10 Leydig cell line was provided by Dr Mario Ascoli (University of Iowa, Iowa City, IA, USA) ([Ascoli 1981](#)) and cultured in Waymouth's MB752/1 media (Sigma-Aldrich Canada) supplemented with 15% horse serum (Invitrogen Canada). All cell lines were supplemented with gentamycin/streptomycin sulfate (50 mg/l) and incubated at 37 °C under 5% CO₂.

RNA isolation, reverse transcription, PCR, and real-time PCR

Total RNA from adult mouse testis and primary Leydig cells from immature rats (prepared as described in [Martin et al. \(2008\)](#)) was isolated using TRIzol (Invitrogen Canada) followed by phenol/chloroform extractions and isopropanol precipitation. The RNA pellet was then washed in 75% ethanol before it was dissolved in RNase-free water. All animal experiments were carried out in accordance with the rules and regulations set by the Canadian Council for Animal Care and the policies and procedures of the Laval University Institutional Animal Care Committee and were approved by the Animal Care and Ethics

Committee of Laval University (protocol # 06-059). Total RNA from Leydig cell lines, TM3, LC-540, mLTC-1, and MA-10, was extracted using the RNeasy Plus Kit (Qiagen) according to the manufacturer's recommendations. An aliquot of the total RNA (5 µg) was then used as a template to synthesize the first-strand cDNA using the Transcriptor Reverse Transcriptase (Roche Diagnostics). One tenth of the first-strand cDNA was used as a template in the real-time PCRs (qPCR). qPCRs were performed using a LightCycler 1.5 instrument and the LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics) according to the manufacturer's protocol. qPCRs were performed using the *Pdgfra*-specific primers ([Table 1](#)) and the previously described *Rpl19*-specific primers ([Guigon et al. 2005](#)). The PCRs were done using the following conditions: 10 min at 95 °C followed by 40 cycles of denaturation (5 s at 95 °C), annealing (5 s at 59 °C for *Pdgfra* and 62 °C for *Rpl19* cDNAs), and extension (10 s for *Pdgfra* and 20 s for *Rpl19* at 72 °C) with single acquisition of fluorescence at the end of each extension steps. The specificity of PCR products was confirmed by analysis of the melting curve, agarose gel electrophoresis, and by sequencing (Centre de génomique de Québec, CHUQ). Quantification of gene expression was performed using the Relative Quantification Software (Roche Diagnostics) and is expressed as a ratio of *Pdgfra* to *Rpl19* mRNA levels. Each amplification was performed in duplicate using a pool of three different preparations of first-strand cDNAs for each of the three different RNA extractions. The expression of HLF, RBPJ-K, HMX2, MYT1, and NFAT family members in a panel of Leydig cell lines was determined by PCR using oligonucleotides listed in [Table 1](#).

Plasmids

A 2185 bp fragment (-2170 to +14 bp) of the 5'-flanking sequence of the rat *Pdgfra* gene was generated from rat genomic DNA by PCR using the primers listed in [Table 1](#). A series of 5' progressive deletion constructs (-514, -243, -183, -154, -105, -71, and -50 bp) were generated using the primers listed in [Table 1](#) and the -2170 to +14 bp *Pdgfra* plasmid as template. All the *Pdgfra* promoter fragments were cloned into a modified pXP1 luciferase reporter plasmid ([Tremblay & Viger 1999](#)) and verified by sequencing and digestion with restriction enzymes. Site-directed serially arranged trinucleotide mutations were introduced between -154 and -105 bp of the -2170 to +14 bp promoter sequence of the rat *Pdgfra* construct using the QuikChange II XL mutagenesis kit (Stratagene, La Jolla, CA, USA) and the oligonucleotides listed in [Table 1](#). Expression vectors encoding rat HMX2 and RBPJ-K and the mouse HLF proteins were obtained by cloning the corresponding full-length cDNA, which

Table 1 Oligonucleotides used in the study of the rat *Pdgfra* promoter

Application	Description	Sequence ^a
Real-time PCR	<i>Pdgfra</i>	Forward: 5'-CAATCCAAAGATGTCCAGGTC-3' Reverse: 5'-ACCAAGTCAGGTCCCATTTAC-3'
	<i>Rpl19</i>	Forward: 5'-CTGAAGGTCAAAGGGAATGTG-3' Reverse: 5'-GGACAGAGTCTTGATGATCTC-3'
<i>Pdgfra</i> promoter	–2170 bp forward –514 bp forward –243 bp forward –183 bp forward –154 bp forward –105 bp forward –71 bp forward –50 bp forward +14 bp reverse	5'-GCTGGGTCCCAGCTCAGCGAGATCT-3' 5'-CGGGATCCGGCCATATTTCTAGCGAGGCC-3' 5'-CGGGATCCTTGTTGAAGTCTGGGGGTTGGG-3' 5'-GAAGATCTAGAGGTCTGAGCCTGAGAG-3' 5'-GAAGATCTCAAGGAGGAGTCCGAGGGAAAC-3' 5'-GAGGATCTGGGGACTTCATTTCTGACAGCTA-3' 5'-GAAGATCTGCAAAATGATTAGTTTTCGGAG-3' 5'-GAAGATCTGATGGACTATAACATTGAATC-3' 5'-ACGCGTCTGACTCAAGCTCCAACAGTAATGGGCTC-3'
Mutagenesis	M1 –155/–153 bp M2 –151/–149 bp M3 –147/–145 bp M4 –143/–141 bp M5 –139/–137 bp M6 –135/–133 bp M7 –131/–129 bp M8 –127/–125 bp M9 –123/–121 bp M10 –119/–117 bp M11 –115/–113 bp M12 –111/–109 bp	S: 5'-GCCTGAGAGGTTTCAGAGAtacAGGAGGAGTCCGAGGG-3' S: 5'-CCTGAGAGGTTTCAGAGAGCAAttcGAGTCCGAGGGA-3' S: 5'-GAGAGGTTTCAGAGAGCAAGGAGtctTCGAGGGAAACTT-3' S: 5'-GAGAGCAAGGAGGAGTaatAGGGAAACTTTTATTTTGAAGAGACC-3' S: 5'-GAGAGCAAGGAGGAGTCCGAtttAACTTTTATTTTGAAGAGACC-3' S: 5'-GCAAGGAGGAGTCCGAGGGAccctTTTATTTTGAAGAGACCGGG-3' S: 5'-GGAGTCCGAGGGAAACTgggATTTTGAAGAGACCAGGGG-3' S: 5'-GGAGTCCGAGGGAAACTTTTAgggTGAAGAGACCAGGGGGGGG-3' S: 5'-GTCCGAGGGAAACTTTTATTTTtccGAGACCAGGGGGGGG-3' S: 5'-GGGAAACTTTTATTTTGAAGctcCCAGGGGGGGGGGAC-3' S: 5'-GGGAAACTTTTATTTTGAAGAGAcctGGGGGGGGGACTTC-3' S: 5'-TTTGAAGAGACCAGGgtttGGGGGACTTCATTTCGACAG-3'
EMSA	WT1 –157/–127 bp WT2 –144/–112 bp M2 –151/–149 bp M3 –147/–145 bp M4 –143/–141 bp M5 –139/–137 bp M6 –135/–133 bp M7 –131/–129 bp M8 –127/–125 bp M9 –123/–121 bp M10 –119/–117 bp SP1/SP3 HLTF RBPJ-K E2F1 MYT1 CDX2 HMX2 NFAT	S: 5'-GAGCAAGGAGGAGTCCGAGGGAAACTTTTAT-3' S: 5'-TCCGAGGGAAACTTTTATTTTGAAGAGACCAGG-3' S: 5'-GAGCAaagGGAGTCCGAGGGAAACTTTTAT-3' S: 5'-GAGCAAGGAGtctTCCGAGGGAAACTTTTAT-3' S: 5'-GAGCAAGGAGGAGTaatAGGGAAACTTTTAT-3' S: 5'-GAGCAAGGAGGAGTCCGAtttAACTTTTAT-3' S: 5'-TCCGAGGGAAcctTTTATTTTGAAGAGACCAGG-3' S: 5'-TCCGAGGGAAACTgggATTTTGAAGAGACCAGG-3' S: 5'-TCCGAGGGAAACTTTTAgggTGAAGAGACCAGG-3' S: 5'-TCCGAGGGAAACTTTTATTTTaggGAGACCAGG-3' S: 5'-TCCGAGGGAAACTTTTATTTTGAAGctcCCAGG-3' S: 5'-CGGCGCAGGGCGGGGCGGGGCGAG-3' S: 5'-AGAAAAGGGAATATTACTTATCCCACAA-3' S: 5'-GGAACACGCCGTGGGAAAAAATTTGGG-3' S: 5'-CAACAGGCTCGCGCGGAGACAAAGGGGCTGCG-3' S: 5'-ATCAGTTGGAAGTTTCCAGGACATCTTC-3' S: 5'-GACTGACACACTATAAACGGCATGGATT-3' S: 5'-TTCAAGGGAACGTTTCGCGC-3' S: 5'-TGGAAATTTGTTTCATAGT-3'
ChIP	–184/+14 bp <i>Pdgfra</i> promoter	AS: 5'-CAAGCTCCAACAGTAATGGGC-3' S: 5'-GAGAGGTCTGAGCCTGAGAG-3'
cDNA/PCR and cloning	HLTF RBPJ-K MYT1 HMX2 NFATC1 NFATC2 NFATC3 NFATC4 NFAT5	AS: 5'-GGGGTACCATGTCTATACGTTCCAGAGG-3' S: 5'-GCTCTAGATTACAAGTCAATTAAAGTTCTG-3' AS: 5'-GCTCTAGAAAGATGGCGCCTGTTGTGACAG-3' S: 5'-CGGGATCCTTAGGACACCACGGTTGCTGTG-3' AS: 5'-GGGGTACCATGAGCTCAGAAAGTGATGAC-3' S: 5'-CGGAATTCCTAGACCTGAATGCCCTCAC-3' AS: 5'-GCTCTAGAAGGATGGGCAGCAAGGAAGATG-3' S: 5'-CGGGATCCTCAGTAGTCAAGCTTGTGTAC-3' AS: 5'-TATATGAGCCCATCCTTGCC-3' S: 5'-AACGGGATCTCCAGGACTTT-3' AS: 5'-ATGTGAGCAGGAGGAGAGGA-3' S: 5'-TCATCTGCTGTCCCAATGAA-3' AS: 5'-GCATTCTGGACAGCACTCAA-3' S: 5'-TGGCTTGGGAAACAGAAATC-3' AS: 5'-TCCTTATCTGTCAGAAGGCTTTGG-3' S: 5'-GTCTCGGCCAATGATCTCACTCAC-3' AS: 5'-GCTTTCTCAGCTTACCACGG-3' S: 5'-TGCAACACCACTGGTTTCATT-3'

S, sense; AS, antisense.

^aCloning sites are underlined while mutations are in lower caps.

were obtained by RT-PCR using the oligonucleotides listed in Table 1, in the pcDNA3 expression vector (Invitrogen). The mouse SP1 expression vector was obtained from Dr Robert Viger (CHUQ Research Centre, Laval University, Quebec, Canada), whereas the mouse SP3 expression vector was obtained from the Mammalian Gene Collection (Invitrogen).

Transfections and luciferase assays

Transient transfections of TM3 Leydig cells were performed in 24-well plates using the calcium phosphate co-precipitation method (Martin & Tremblay 2005). Briefly, on the day before transfection, 30 000 TM3 Leydig cells were seeded with 500 µl of media per well in 24-well plates. Four hours prior to transfection, the media were replaced with fresh media (500 µl/well). Cells were then transfected with 500 ng of rat *Pdgfra* reporter construct (Firefly Luciferase) along with 10 ng of phRL-TK (Renilla Luciferase) and 990 ng of pSP64 plasmid to adjust the total amount of DNA to 1.5 µg/well. The phRL-TK plasmid was used as an internal control for transfection efficiency. The media were replaced with fresh media (500 µl/well) 12–16 h after transfection. The TM3 Leydig cells were lysed 36–40 h following transfection, and the Dual-Luciferase Assay System (Promega Corporation) was used to measure luciferase activities (firefly and renilla) using a Luminoskan Ascent luminometer (Thermo Scientific, Waltham, MA, USA). Data reported represent an average of at least three experiments, each done in triplicate, using at least three different DNA preparations. When evaluating the effect of SP1, SP3, HLTF, RBPJ-K, and HMX2 on the *Pdgfra* promoter, the TM3 Leydig cells were co-transfected with 250 ng of either an expression vector encoding the corresponding full-length protein or an empty vector as control in addition to the *Pdgfra* reporters and phRL-TK internal control. The total amount of DNA was maintained at 1.5 µg/well by adding 740 ng pSP64 plasmid.

Electromobility shift assays

TM3 Leydig cells were scrapped off the Petri dish (100 mm) using 1.5 ml of PBS-EDTA (1 mM) and then centrifuged at 3500 *g* for 60 s at room temperature. Nuclear extracts were isolated according to the method described by Schreiber *et al.* (1989). For electromobility shift assay (EMSA), two probes were obtained by 5' labeling with ³²P double-stranded oligonucleotides corresponding to two regions (−157/−127 and −144/−112 bp) of the *Pdgfra* promoter (sequences provided in Table 1). Each probe (50 000 c.p.m.) was incubated with 5 µg of nuclear extracts from TM3 Leydig cells in 20 µl of 4 mM Tris-HCl (pH 8.0),

24 mM KCl, 0.5 mM EDTA (pH 8.0), 0.4 mM dithiothreitol, 5 mM MgCl₂, 6% glycerol, and 1 µg poly(dIdC) for 1 h on ice. In the competition experiments, the specific binding of nuclear protein extracts from TM3 cells to the probe was challenged with an excess (5× and 25× molar excess) of unlabeled oligonucleotides corresponding to wild-type (WT) sequences (*Pdgfra* promoter elements or consensus binding sequences for transcription factors) or to *Pdgfra* promoter mutants (M2–M10) as indicated. The sequences of the oligonucleotides used in the competition assays are indicated in Table 1. For the supershift experiments, 1.5 µg of goat polyclonal anti-SP1 antibody (sc-59x; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and/or rabbit anti-SP3 antibody (sc-644x; Santa Cruz Biotechnology) were added to the binding reaction mixture. Normal goat or rabbit IgGs were used as controls (sc-2028 and sc-2027; Santa Cruz Biotechnology).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed as described previously (Robert *et al.* 2006) with the following modification: TM3 cells were grown in 60-mm dishes until 80% confluence and sonicated with a Braun-Sonic 1510 Sonicator for eight cycles of 15 s pulses at two output control and constant duty cycle in order to obtain DNA fragments between 200 and 500 bp in size. Cross-linked DNA was immunoprecipitated overnight with rotation at 4 °C with anti-SP1 (sc-59x Santa Cruz Biotechnology and ab59267 Abcam, Cambridge, MA, USA), anti-SP3 (sc-644x Santa Cruz

Figure 1 *Pdgfra* is expressed in the adult mouse testis, primary Leydig cells, and several Leydig cell lines. qPCR was used to detect expression of *Pdgfra*. Total RNA was isolated from whole testis of adult mice, from primary Leydig cells from immature rats, and from various Leydig cell lines (TM3, LC-540, mLTC-1, and MA-10). Amplification of *Rpl19* was used to standardize the results.

Figure 2 Identification of two regions important for *Pdgfra* promoter activity in TM3 Leydig cells. TM3 Leydig cells were transfected with various 5' deletion constructs of the rat *Pdgfra* promoter (the 5'-end point of each construct is indicated on the left of the graph). Results are shown as relative activity over the 2.2 kb fragment promoter (mean $\pm$ S.E.M.). A different letter indicates a statistically significant difference.

Biotechnology and ab72594; Abcam), and anti-Histone H3 as positive control (ab1791 Abcam), and goat or rabbit IgGs (sc-2028, sc-2027 Santa Cruz Biotechnology) as negative controls. PCRs were done using 2 μ l of input chromatin sample and 5 μ l of immunoprecipitated chromatin with primers specific for the proximal region (−184 to +14 bp) of the rat *Pdgfra* promoter (sequences provided in Table 1). PCRs were carried out using Vent DNA Polymerase (New England Biolabs, Pickering, ON, Canada) at 94 °C for 5 min, followed by 32 cycles of 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 30 s, and completed by a final extension of 5 min at 72 °C. The PCR products were analyzed by electrophoresis on a 1.5% ethidium bromide-stained agarose gel.

In silico analysis

Analysis of potential regulatory elements in the proximal *Pdgfra* promoter region (−154/−108 bp) was performed using bioinformatics software tools MatInspector (<http://www.genomatrix.de/cgi-bin/matinspector/matinspector.pl>) and Evolutionary Conserved Regions ECR-Browser (<http://ecrbrowser.dcode.org>).

Statistical analyses

Transfection data were analyzed by a one-way ANOVA (SigmaStat Version 2.0, Jadel Corporation, San Rafael, CA, USA). Multiple range comparisons of paired means were then done using a Fisher's LSD test or the Newman–Keuls test. The level of significance was set at $P < 0.05$. Data are reported as mean $\pm$ S.E.M.

Results

Expression of *Pdgfra* in Leydig cell lines

To characterize the regulatory elements important for *Pdgfra* expression in Leydig cells, qPCR was first performed to identify a suitable Leydig cell line expressing this gene. *Pdgfra* mRNA was detected in all samples albeit at different levels (Fig. 1). *Pdgfra* mRNA was more abundant in primary Leydig cells and in the TM3 Leydig cell line. The TM3 Leydig cell line was chosen for further characterization of the *Pdgfra* promoter.

Localization of the regulatory elements of the rat *Pdgfra* promoter

A 2.2 kb (−2170 to +14 bp) fragment of the *Pdgfra* promoter was first isolated by PCR from rat genomic DNA. A 2.2 kb fragment was chosen because it sufficiently mimicked the endogenous *Pdgfra* expression profile in transgenic mice (Zhang *et al.* 1998). In order to locate the regulatory elements important for *Pdgfra* transcription in Leydig cells, a series of 5' progressive deletion constructs were generated from the 2.2 kb fragment and transiently transfected in TM3 cells. Activity of the rat *Pdgfra* promoter in TM3 Leydig cells was significantly increased with 5' deletion from −2170 to −514 bp

Figure 3 Fine mapping of the −154 to −108 bp region of the rat *Pdgfra* promoter. TM3 Leydig cells were transfected with either a wild-type (open bar) or a series of rat *Pdgfra* promoter constructs (solid bars) containing trinucleotide mutations in the −154/−108 bp region in the context of the −2170 bp. The wild-type *Pdgfra* promoter sequence is shown on top and the corresponding mutated nucleotides are shown underneath in lowercase. Results are shown as relative activity over the −2.2 kb fragment promoter (mean $\pm$ S.E.M.). A different letter indicates a statistically significant difference.

(Fig. 2). Subsequent 5' deletions from -514 to -183 bp had no effect on the *Pdgfra* promoter activity ($P > 0.05$; Fig. 2); however, deletions to -154 bp caused a significant decrease in promoter activity (Fig. 2). Further deletions to -105 bp abrogated *Pdgfra* promoter activity in TM3 Leydig cells (Fig. 2). There was no difference in *Pdgfra* promoter activity of either the -105 or -71 bp reporters as compared with that of the minimal -50 bp promoter (Fig. 2). These data indicate that *Pdgfra* promoter activity in TM3 Leydig cells is regulated mainly by two regions: $-183/-154$ and $-154/-105$ bp.

Fine mapping of the -154 to -105 region of the *Pdgfra* promoter

To better define the regulatory elements located within the $-154/-105$ bp region responsible for *Pdgfra* promoter activity, a linker scanning mutagenesis approach was used. Twelve mutants of the *Pdgfra* promoter, each harboring different trinucleotide mutations in the $-154/-108$ bp region, were generated in the context of the -2170 bp construct.

These reporter constructs were then transiently transfected in TM3 Leydig cells. As shown in Fig. 3, trinucleotide mutants M2–M9 decreased *Pdgfra* promoter activity, particularly mutants M6–M9 which encompass a 15-nt A/T-rich region between -135 and -121 bp.

Several proteins bind to the $-157/-112$ bp region of the *Pdgfra* promoter

To determine whether nuclear proteins bind to the $-157/-112$ bp region of the *Pdgfra* promoter, EMSAs were performed using nuclear extracts from TM3 Leydig cells and two overlapping radiolabeled probes: probe 1 spanned the region $-157/-127$ bp of the *Pdgfra* promoter (Fig. 4A) corresponding to the regions targeted by mutants M2–M5 (Table 1); and probe 2 encompassed the region $-144/-112$ bp of the *Pdgfra* promoter (Fig. 4B) corresponding to the sequence mutated in mutants M6–M10 in Fig. 3. Distinct complexes (identified as I, II, III, and IV for probe 1 and V, VI, and VII for probe 2) were observed (lane 2; Fig. 4A and B). Increasing the molar concentrations

Figure 4 Several proteins bind specifically to the -154 to -112 bp region of the *Pdgfra* promoter. EMSA was used to assess the binding of nuclear proteins ($5 \mu\text{g}$) from TM3 Leydig cells to two double-stranded ^{32}P -labeled oligonucleotide corresponding to the wild-type -157 to -127 bp (A) and -144 to -112 bp (B) regions of the rat *Pdgfra* promoter. The specific complexes are indicated on the left of each image (I–VII). Protein binding was challenged by increasing molar concentrations (black triangles; $5\times$ and $25\times$) of unlabeled oligonucleotides corresponding to the unlabeled probe and to various mutants (M2–M10) as indicated. The sequences of the oligonucleotides used are provided in Table 1. NS, non-specific bands correspond to bands that are not competed by the wild-type unlabeled oligonucleotides.

Figure 5 SP1 and SP3 bind to the *Pdgfra* promoter. (A) EMSA was used to assess the binding of endogenous SP1 and SP3 from TM3 Leydig cell nuclear extracts to a double-stranded ^{32}P -labeled oligonucleotide corresponding to the $-157/-127$ bp region of the *Pdgfra* promoter. Binding was challenged by an excess ($5\times$ and $25\times$) of unlabeled oligonucleotides corresponding to the unlabeled probe (lanes 3–4 and 11–12) or with oligonucleotides containing a consensus SP1/3 binding site (lanes 5 and 6; [Giatzakis & Papadopoulos 2004](#)). The SP1 and SP3 binding were supershifted with SP1 and SP3 antisera (αSP1 , αSP3). IgG: goat or rabbit IgGs. NS, non-specific bands correspond to bands that are not competed by the wild-type unlabeled oligonucleotides. (B) *In vivo* recruitment of SP1 and SP3 to the proximal *Pdgfra* promoter was determined by ChIP assays in TM3 Leydig cells. An aliquot of chromatin preparation before immunoprecipitation (input) was used as positive control. Chromatin was immunoprecipitated with antisera against SP1 (αSP1) and SP3 (αSP3). An anti-Histone H3 ($\alpha\text{Histone H3}$) antiserum was used as a positive control for the ChIP. Preimmune goat and rabbit serum (IgG) were used as negative controls. A 200-bp DNA fragment containing the SP1 and SP3 site within the rat *Pdgfra* promoter was amplified by PCR. A plasmid containing the rat *Pdgfra* promoter served as a positive control for the PCR while water served as a negative control.

($5\times$ and $25\times$) of unlabeled oligonucleotide corresponding to the respective probes displaced the protein–DNA complexes (lanes 3 and 4; [Fig. 4A](#) and B). Competition experiments were also performed

with oligonucleotides harboring trinucleotide mutations (same mutations that were used in the transfections; see [Fig. 3](#) for the sequences). As shown in [Fig. 4A](#) and B, oligonucleotides containing mutations M4, M8,

M9, and M10 were as efficient as the WT probe at competing protein binding. On the other hand, oligonucleotides harboring mutations M2, M3, and M6 could not compete with the probe in the binding assay (Fig. 4A and B). This indicated that mutations M2, M3, and M6 prevented binding of the transcription factors. Oligonucleotides for mutants M5 and M7 competed the formation of specific protein–DNA complexes; M5 blunted the formation of complexes III and IV (Fig. 4A, lanes 11 and 12), while M7 essentially competed complex V (Fig. 4B, lanes 7 and 8).

SP1 and SP3 bind to and activate the rat *Pdgfra* promoter

Since mutations M4, M5, and M6 decreased *Pdgfra* promoter activity (Fig. 3), while mutants M5 and M6 also abrogated some protein–DNA binding activities (Fig. 4), the sequence in this region was analyzed in more detail. Mutants M4, M5, and M6 alter the sequence CCGAGGGAAAC, a G(A/C)-rich sequence that constitutes a potential binding site for members of the ETS (Nye *et al.* 1992) and SP (Letovsky & Dynan 1989) families of transcription factors. We therefore tested whether SP1 and/or SP3 bind to the –157/–127 bp element of the *Pdgfra* promoter. Oligonucleotides containing a consensus SP1/3 binding site were as efficient as the WT probe at competing protein binding (compare lanes 5 and 6 with lanes 3 and 4 in Fig. 5A). Furthermore, as shown in lanes 8 and 16 of Fig. 5A, addition of an antiserum against the SP1 protein resulted in a supershift of complex I while having no effect on complexes II, III, and IV. Addition of an anti-SP3 antibody abrogated complex II (Fig. 5A, lane 14). Finally, addition of antisera for both SP1 and SP3 resulted in a supershift of complexes I and II. Taken together, these data indicate that these complexes I and II correspond to SP1 and SP3 (Fig. 5A, lane 18). Therefore, SP1 and SP3 are present in TM3 nuclear extracts that bind to the *Pdgfra* promoter.

To confirm the recruitment of SP1 and SP3 to the proximal *Pdgfra* promoter *in vivo*, ChIP assays were performed. ChIP was performed with two distinct antisera for each SP1 and SP3. As shown in Fig. 5B, a band of expected size (~200 bp) corresponding to the proximal *Pdgfra* promoter was detected in the samples immunoprecipitated with the anti-Histone H3 antibody (positive control for the immunoprecipitation; Fig. 5B, lane 6) as well as in the samples immunoprecipitated with all antisera against SP1 (Fig. 5B, lanes 7 and 9) and SP3 (Fig. 5B, lanes 8 and 10). This indicates that both SP1 and SP3 are associated with the proximal *Pdgfra* promoter in TM3 Leydig cells.

The role of SP1 and SP3 on *Pdgfra* promoter activity was assessed by transient transfections in TM3 Leydig cells. As shown in Fig. 6, the activity of the –2170 bp

Figure 6 The element at –138 bp is necessary and sufficient for SP1- and SP3-dependent activation of the *Pdgfra* promoter. TM3 Leydig cells were co-transfected with various 5' deletion constructs of the *Pdgfra* promoter (the 5'-end point of each construct is indicated on the left of the graph) or with a reporter harboring a trinucleotide mutation in the SP1/SP3 binding site at –138 bp (indicated by the large X) along with either an empty expression vector (open bars) or expression vectors for SP1 (grey bars) and SP3 (black bars). Results are shown as fold activation over control (mean $\pm$ S.E.M.). The asterisk indicates a statistically significant difference from control (empty expression vector).

Pdgfra promoter was increased about threefold in the presence of SP1 or SP3. Combination of SP1 and SP3 did not lead to further activation (data not shown). The SP1- and SP3-mediated transactivation was lost when the region between –154 and –105 bp was deleted, indicating that it contains an SP-responsive element. This –154/–105 bp contains the SP binding site at –138 bp (Fig. 5). A *Pdgfra* reporter harboring a mutation in this element (M5) known to prevent binding of SP factors (Figs 4 and 5) in the context of the –2170 bp promoter was only weakly activated by SP1 or SP3, and this activation was not statistically significant ($P > 0.05$). Altogether, these data indicate that SP1 and SP3 activate the *Pdgfra* promoter by binding to the G(C/A) motif at –138 bp.

Potential partners for SP1/3 in the regulation of *Pdgfra* transcription

We performed *in silico* analyses of the –154/–108 bp sequence to identify potential regulatory elements and transcription factors that could bind to this region (e.g. complexes III–VII) and act in concert with SP1/3 to regulate *Pdgfra* transcription in Leydig cells. Several potential transcription factor-binding sites were identified including RBPJ-K, E2F1, HLTF, NFAT, HMX2,

Figure 7 Putative transcription factors regulatory elements in the proximal region of the rat *Pdgfra* promoter. (A) Schematic representation of the promoter region of the rat *Pdgfra* gene (–2170/+14 bp) highlighting the –154 to –108 bp region (solid black rectangle) that was analyzed for potential regulatory elements using ECR-Browser and MatInspector. Putative regulatory elements identified by both methods include the recombination signal binding protein for immunoglobulin kappa J region (RBPJ-K), E2F transcription factor 1 (E2F1), helicase-like transcription factor (HLTF), H6 family homeobox 2 (HMX2), nuclear factor of activated T-cells (NFAT), myelin transcription factor 1 (MYT1), and caudal type homeobox 2 (CDX2). (B) EMSA was performed using nuclear proteins from TM3 Leydig cells to a double-stranded ³²P-labeled oligonucleotide corresponding to the –144/–112 bp region of the *Pdgfra* promoter (probe 2). Binding was then challenged by an excess (black triangles; 5× and 25×) of either unlabeled oligonucleotides corresponding to the –144/–112 bp *Pdgfra* promoter region (WT) or oligonucleotides harboring consensus sequences for the binding of the transcription factors HLTF (Hewetson *et al.* 2002), RBPJ-K (King *et al.* 2006), E2F1 (Zhang *et al.* 2008), MYT1 (Kim & Hudson 1992), CDX2 (Gendron *et al.* 2006), and HMX2 (Mennerich *et al.* 1999). The sequences of the oligonucleotides used are provided in Table 1. NS, non-specific.

MYT1, and CDX2 (Fig. 7A). Oligonucleotides containing consensus sequences for these transcription factors were then used in EMSA/competition experiments. As shown in Fig. 7B and summarized in Table 2, oligonucleotides harboring consensus binding sites for RBPJ-K, HLTF, HMX2, and MYT1 were as efficient as the WT *Pdgfra* probe at competing the formation of complexes V and VI but had no effect on complex VII.

Oligonucleotides harboring the consensus sequences for CDX2 and E2F1 could not compete any of the complexes (Fig. 7B and Table 2). Next, the presence of RBPJ-K, HLTF, NFAT, HMX2, and various NFAT family members (NFATc1, NFATc2, NFATc3, and NFATc5) were detected by RT-PCR in Leydig cell lines

Table 2 Summary of the results for the EMSA/competition assays, RT-PCR, *Pdgfra* promoter activation and cooperation with SP1/SP3 for the potential partners

Transcription factor	Consensus binding sequence ^b	References	Competes binding to complexes ^a			Present in Leydig cell lines ^a	Activates <i>Pdgfra</i> promoter ^a	Cooperates with SP1 or SP3 on <i>Pdgfra</i> promoter ^a
			V	VI	VII			
HLTF Helicase-like transcription factor	MCWTNK	Hewetson <i>et al.</i> (2002)	y	y	n	y	n	n
E2F1 E2F transcription factor 1	TTTSSCGC	Zheng <i>et al.</i> (1999)	n	n	n	ND	ND	ND
RBPJ-K Recombination signal binding protein for immunoglobulin kappa J region	TTCCCACG	Tun <i>et al.</i> (1994)	y	y	n	y	n	n
HMX2 H6 family homeobox 2	RGRSAAAC-GTTTC	Mennerich <i>et al.</i> (1999)	y	y	n	y	n	n
NFAT Nuclear factor of activated T-cells	WGGAAAAHN	Rao <i>et al.</i> (1997)	y	y	n	NFATc1 NFATc2 NFATc3 NFATc5	ND	ND
MYT1 Myelin transcription factor 1	AAAGTTT	Kim & Hudson (1992)	y	y	n	n	ND	ND
CDX2 Caudal type homeobox 2	MTTTATR	Margalit <i>et al.</i> (1993)	n	n	n	ND	ND	ND

^ay, yes; n, no; ND, not determined.
^bM: A or C; W: A or T; N: A, C, G, or T; K: G or T; S: C or G; R: G or A; H: A, T, or C.

(Table 2). However, none of the factors tested (RBPJ-K, HLTF, and HMX2) could activate the *Pdgfra* promoter either alone or in combination with SP1/SP3 when transiently transfected in TM3 Leydig cells (Table 2).

Discussion

In the rat, human, and mouse testis, FLC and ALC express PDGFRA (Gnessi *et al.* 1992, 1995, 2000, Schmahl *et al.* 2008). Using qPCR, we detected different levels of *Pdgfra* mRNA in the testis of the adult mouse, primary Leydig cells from immature rats, and in TM3, LC-540, mLTC-1, and MA-10 Leydig cell lines. The presence of the *Pdgfra* mRNA in the Leydig cell lines indicates that they possess the transcriptional regulators required for its expression. In the present study, we used the TM3 cell line as a model to decipher the transcriptional regulation of the *Pdgfra* gene in Leydig cells.

Our *Pdgfra* promoter analysis in TM3 Leydig cells revealed an increase in promoter activity when the 5' end of the -2170 *Pdgfra* promoter fragment was deleted to -514 bp. The increase in promoter activity may have been due to elimination of potential repressive sequences upstream of -514 bp. Kitami *et al.* (1995) reported a similar increase in *Pdgfra* promoter activity in VSMC following the deletion of the -1682 to -1428 bp region. They suggested that the

alternating d(TG)*n*-type purine-pyrimidine dinucleotide repeats contained in this region were responsible for the repression (Naylor & Clark 1990, Kitami *et al.* 1995). In addition, *in silico* analysis of the -2170 to -514 bp region of the *Pdgfra* promoter (MatInspector) revealed the presence of potential binding sites for several transcriptional repressors including ZNF219 (Sakai *et al.* 2003), BCL6 (Chang *et al.* 1996), GFI1 (Zweidler-Mckay *et al.* 1996), EN1 (engrailed homeobox 1; Jaynes & O'Farrell 1991), and Kaiso (Prokhortchouk *et al.* 2001). It is therefore likely that some of these transcriptional repressors along with the distal sequence d(TG)30 repeats present in the *Pdgfra* promoter act to ensure an inhibitory control of *Pdgfra* gene expression in the TM3 Leydig cells. Deletions between -514 and -183 bp had no impact on *Pdgfra* promoter activity, indicating that the proximal 197 bp (-183 to +14 bp) contains all the necessary regulatory elements for *Pdgfra* expression in TM3 Leydig cells. This is consistent with data for the human PDGFRA promoter where a proximal -464 bp fragment was found to be sufficient to confer maximal promoter activity in human teratocarcinoma and mouse 3T3 cells (Afink *et al.* 1995).

Our promoter deletion approach identified a first region, from -183 to -154 bp, as critical to maintain high *Pdgfra* promoter activity in TM3 Leydig cells indicating the presence of important regulatory elements. In a previous study, using DNase I footprinting and EMSA, the proximal promoter (-220 bp) was

found to contain an enhancer core sequence that is recognized by members of the C/EBP family of transcription factors (Fukuoka *et al.* 1999, Kitami *et al.* 1999, Yang *et al.* 2001, Afink *et al.* 2004). In VSMC, C/EBP δ was shown to up-regulate *Pdgfra* transcription, whereas C/EBP β repressed it (Fukuoka *et al.* 1999, Kitami *et al.* 1999, Yang *et al.* 2001, Afink *et al.* 2004). We obtained similar findings in TM3 Leydig cells where both C/EBP α and δ increased *Pdgfra* promoter activity, whereas C/EBP β acted as a repressor (data not shown). In testicular Leydig cells and in ovarian granulosa cells, both human chorionic gonadotropin (hCG) and LH increase C/EBP β levels (Sterneck *et al.* 1997, Nalbant *et al.* 1998). The same treatment, however, represses *PDGFRA* expression in human granulosa-luteal cells (Hwu *et al.* 2009) in agreement with a repressing role for C/EBP β in *Pdgfra* expression. Immature and mature Leydig cells isolated from 20- to 70-day-old rats exclusively express C/EBP β (Nalbant *et al.* 1998). However, no data are available regarding the expression of C/EBP family members in Leydig cells during the pre-natal period. Therefore, a role for other C/EBP family members (α , δ , and σ) in the activation of *Pdgfra* transcription in FLC *in utero* cannot be ruled out.

A second region, from -154 to -105 bp, was also found to be essential for *Pdgfra* promoter activity in TM3 Leydig cells. Site-directed mutagenesis of this region in the context of the -2170 bp construct revealed the importance of a G(C/A)-rich and a T/A-rich motif. Several protein complexes were found to be associated with these motifs. Mutations that altered the core sequence TCCGAGGGAAAC prevented binding of the nuclear proteins and significantly decreased *Pdgfra* promoter activity in TM3 cells. Interestingly, this motif is similar to binding sites for members of the ETS (Nye *et al.* 1992), SP (Letovsky & Dynan 1989), and NFAT (Rao *et al.* 1997) families of transcription factors. Our transactivation assays revealed that SP1 and SP3 activate the *Pdgfra* promoter by about threefold. This activation was lost with a deletion of the -154 to -105 bp region or with a mutation of the SP binding site at -138 bp. Binding of SP1 and SP3 to this element was indeed confirmed by the use of anti-SP1 and anti-SP3 antisera in EMSA, which led to supershift of two of the complexes. Both SP1 and SP3 have a $>90\%$ DNA sequence homology and are known to bind to the same DNA element with similar affinities (Li *et al.* 2004). Bonello & Khachigian (2004) also reported the implication of SP1 in *Pdgfra* promoter activity in VSMC. SP1-dependent activation of the *Pdgfra* promoter in these cells was found to involve an atypical recognition element located ~ 40 bp 3' of the SP1/3 element we have identified in TM3 Leydig cells (Fig. 8). This atypical element might in fact be responsible for the weak activation by SP1/3 of the full-length *Pdgfra* reporter harboring a mutation (M5) in the SP binding site at -138 bp. Altogether,

these findings indicate that the involvement of SP1 and SP3 might represent a conserved mechanism for *Pdgfra* expression in several tissues.

In addition to directly binding to and activating the *Pdgfra* promoter, the contribution of SP1 and SP3 in *Pdgfra* transcription is of interest since it could represent an alternative mechanism for the hormonal regulation of *Pdgfra* in Leydig cells. *Pdgfra* expression is known to be regulated by various hormones, including retinoic acid, estrogens, glucocorticoids, and cAMP in different cell types (Tsukamoto *et al.* 1994, Afink *et al.* 1995, Warshamana *et al.* 1998, Thuillier *et al.* 2003). Interestingly, SP1 and SP3 are known to interact with the receptors for these hormones and to act as their transcriptional effectors (Safe & Kim 2004).

Although SP1 and SP3 are ubiquitously expressed, they nonetheless contribute to tissue- and cell-specific gene expression through interactions and co-operations with cell-restricted transcription factors. Consistent with this, we found that SP1 and SP3 were responsible for only two of the four complexes detected with the probe containing the G(A/C)-rich motif. To identify potential partners that act in concert with SP1/3 in *Pdgfra* transcription in Leydig cells, we performed *in silico* analyses of the $-154/108$ bp sequence, EMSA/competition assays, and RT-PCR. We identified several potential candidates including RBPJ-K, HLTF, HMX2, and NFAT family members (NFATc1, NFATc2, NFATc3, and NFATc5). When tested in transactivation assays, RBPJ-K, HLTF, and HMX2 did not activate the *Pdgfra* promoter either alone or in concert with SP1/3 in these *in vitro* conditions. We cannot, however, exclude the

Figure 8 Schematic representation of regulatory elements identified in the proximal region of the *Pdgfra* promoter. In human, rat and mouse, *Pdgfra* transcription is modulated by C/EBP family members (Fukuoka *et al.* 1999, Yang *et al.* 2001, Afink *et al.* 2004), SP1 and SP3 (Bonello & Khachigian 2004; our present data), GLI1 (Xie *et al.* 2001), ETS1 (Bonello *et al.* 2005), PBX2 (Joosten *et al.* 2002), and PRX2 (Joosten *et al.* 2002) via regulatory elements present in the proximal promoter region (-214 to -24 bp). The transcription factors will bind to their respective response element causing either activation (solid arrows) or repression (blunted line) of *Pdgfra* expression. The GLI1 responsive element remains to be identified (question mark). VSMC, vascular smooth muscle cells; LC, Leydig cells; ECC, F9 embryonic carcinoma cells; C3H10T $\frac{1}{2}$, pre-adipocytes.

possibility that these factors might contribute to *Pdgfra* transcription in Leydig cells *in vivo*. In addition, members of the ETS and NFAT families are also interesting candidates since they are expressed in Leydig cells and can bind to GA-rich sequences (Rao *et al.* 1997, Giatzakis *et al.* 2007).

The transcriptional mechanisms regulating *Pdgfra* expression in Leydig cells had not been described, and, in other cell types, only a handful of transcription factors have been identified as regulators of *Pdgfra* expression (summarized in Fig. 8). Our study is the first to report a detailed analysis of the *Pdgfra* regulatory region in Leydig cells, which led to the identification of a role for the transcription factors SP1 and SP3. Both *Sp1* and *Sp3* genes have been inactivated in the mouse. SP1- or SP3-deficient mice die embryonically or at birth (reviewed in Zhao & Meng (2005)) impeding the assessment of a direct role for these transcriptional regulators in *Pdgfra* expression in Leydig cells *in vivo*. Our ChIP assays nonetheless confirmed that SP1 and SP3 are associated with the proximal *Pdgfra* promoter in a native chromatin context in TM3 Leydig cells thus supporting a role for these factors in *in vivo* *Pdgfra* transcription.

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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References

Afink GB, Nister M, Stassen BH, Joosten PH, Rademakers PJ, Bongcam-Rudloff E, Van Zoelen EJ & Mosselman S 1995 Molecular cloning and functional characterization of the human platelet-derived growth factor α receptor gene promoter. *Oncogene* **10** 1667–1672.

Afink G, Westermarck UK, Lammerts E & Nister M 2004 C/EBP is an essential component of PDGFRA transcription in MG-63 cells. *Biochemical and Biophysical Research Communications* **315** 313–318. (doi:10.1016/j.bbrc.2004.01.056)

Ascoli M 1981 Characterization of several clonal lines of cultured Leydig tumor cells: gonadotropin receptors and steroidogenic responses. *Endocrinology* **108** 88–95. (doi:10.1210/endo-108-1-88)

Basciani S, Mariani S, Spera G & Gnessi L 2010 Role of platelet-derived growth factors in the testis. *Endocrine Reviews* **31** 916–939. (doi:10.1210/er.2010-0004)

Bonello MR & Khachigian LM 2004 Fibroblast growth factor-2 represses platelet-derived growth factor receptor- α (PDGFR- α) transcription via ERK1/2-dependent Sp1 phosphorylation and an atypical cis-acting element in the proximal PDGFR- α promoter. *Journal of Biological Chemistry* **279** 2377–2382. (doi:10.1074/jbc.M308254200)

Bonello MR, Bobryshev YV & Khachigian LM 2005 Peroxide-inducible Ets-1 mediates platelet-derived growth factor receptor- α gene transcription in vascular smooth muscle cells. *American Journal of Pathology* **167** 1149–1159. (doi:10.1016/S0002-9440(10)61203-5)

Brennan J, Tilman C & Capel B 2003 Pdgfr- α mediates testis cord organization and fetal Leydig cell development in the XY gonad. *Genes and Development* **17** 800–810. (doi:10.1101/gad.1052503)

Buehr M, Gu S & McLaren A 1993 Mesonephric contribution to testis differentiation in the fetal mouse. *Development* **117** 273–281.

Chang CC, Ye BH, Chaganti RS & Dalla-Favera R 1996 BCL-6, a POZ/zinc-finger protein, is a sequence-specific transcriptional repressor. *PNAS* **93** 6947–6952. (doi:10.1073/pnas.93.14.6947)

Cool J & Capel B 2009 Mixed signals: development of the testis. *Seminars in Reproductive Medicine* **27** 5–13. (doi:10.1055/s-0028-1108005)

Davidoff MS, Middendorff R, Enikolopov G, Riethmacher D, Holstein AF & Müller D 2004 Progenitor cells of the testosterone-producing Leydig cells revealed. *Journal of Cell Biology* **167** 935–944. (doi:10.1083/jcb.200409107)

Fukuoka T, Kitami Y, Okura T & Hiwada K 1999 Transcriptional regulation of the platelet-derived growth factor α receptor gene via CCAAT/enhancer-binding protein-delta in vascular smooth muscle cells. *Journal of Biological Chemistry* **274** 25576–25582. (doi:10.1074/jbc.274.36.25576)

Ge RS, Dong Q, Sottas CM, Papadopoulos V, Zirkin BR & Hardy MP 2006 In search of rat stem Leydig cells: identification, isolation, and lineage-specific development. *PNAS* **103** 2719–2724. (doi:10.1073/pnas.0507692103)

Gendron FP, Mongrain S, Laprise P, McMahon S, Dubois CM, Blais M, Asselin C & Rivard N 2006 The CDX2 transcription factor regulates furin expression during intestinal epithelial cell differentiation. *American Journal of Physiology. Gastrointestinal and Liver Physiology* **290** G310–G318. (doi:10.1152/ajpgi.00217.2005)

Giatzakis C & Papadopoulos V 2004 Differential utilization of the promoter of peripheral-type benzodiazepine receptor by steroidogenic versus nonsteroidogenic cell lines and the role of Sp1 and Sp3 in the regulation of basal activity. *Endocrinology* **145** 1113–1123. (doi:10.1210/en.2003-1330)

Giatzakis C, Batarseh A, Dettin L & Papadopoulos V 2007 The role of Ets transcription factors in the basal transcription of the translocator protein (18 kDa). *Biochemistry* **46** 4763–4774. (doi:10.1021/bi062208o)

Gnessi L, Emidi A, Farini D, Scarpa S, Modesti A, Ciampani T, Silvestroni L & Spera G 1992 Rat Leydig cells bind platelet-derived growth factor through specific receptors and produce platelet-derived growth factor-like molecules. *Endocrinology* **130** 2219–2224. (doi:10.1210/en.130.4.2219)

Gnessi L, Emidi A, Jannini EA, Carosa E, Maroder M, Arizzi M, Ullisse S & Spera G 1995 Testicular development involves the spatiotemporal control of PDGFs and PDGF receptors gene expression and action. *Journal of Cell Biology* **131** 1105–1121. (doi:10.1083/jcb.131.4.1105)

Gnessi L, Basciani S, Mariani S, Arizzi M, Spera G, Wang C, Bondjers C, Karlsson L & Bethsholtz C 2000 Leydig cell loss and spermatogenic arrest in platelet-derived growth factor (PDGF)-A-deficient mice. *Journal of Cell Biology* **149** 1019–1026. (doi:10.1083/jcb.149.5.1019)

Guigon CJ, Coudouel N, Mazaud-Guitot S, Forest MG & Magre S 2005 Follicular cells acquire Sertoli cell characteristics after oocyte loss. *Endocrinology* **146** 2992–3004. (doi:10.1210/en.2005-0045)

- Haider SG 2004 Cell biology of Leydig cells in the testis. *International Review of Cytology* **233** 181–241. (doi:10.1016/S0074-7696(04)33005-6)
- Hewetson A, Hendrix EC, Mansharamani M, Lee VH & Chilton BS 2002 Identification of the RUSH consensus-binding site by cyclic amplification and selection of targets: demonstration that RUSH mediates the ability of prolactin to augment progesterone-dependent gene expression. *Molecular Endocrinology* **16** 2101–2112. (doi:10.1210/me.2002-0064)
- Hwu YM, Li SH, Lee RK, Lin MH, Tsai YH & Yeh TS 2009 Luteinizing hormone increases platelet-derived growth factor-D gene expression in human granulosa-luteal cells. *Fertility and Sterility* **92** 2065–2068. (doi:10.1016/j.fertnstert.2009.05.064)
- Jaynes JB & O'Farrell PH 1991 Active repression of transcription by the engrailed homeodomain protein. *EMBO Journal* **10** 1427–1433.
- Joosten PH, Toepoel M, van Oosterhout D, Afink GB & van Zoelen EJ 2002 A regulating element essential for PDGFRA transcription is recognized by neural tube defect-associated PRX homeobox transcription factors. *Biochimica et Biophysica Acta* **1588** 254–260.
- Karl J & Capel B 1998 Sertoli cells of the mouse testis originate from the coelomic epithelium. *Developmental Biology* **203** 323–333. (doi:10.1006/dbio.1998.9068)
- Kim JG & Hudson LD 1992 Novel member of the zinc finger superfamily: a C2-HC finger that recognizes a glia-specific gene. *Molecular and Cellular Biology* **12** 5632–5639.
- King IN, Kathiriyi IS, Murakami M, Nakagawa M, Gardner KA, Srivastava D & Nakagawa O 2006 Hrt and Hes negatively regulate Notch signaling through interactions with RBP-Jkappa. *Biochemical and Biophysical Research Communications* **345** 446–452. (doi:10.1016/j.bbrc.2006.04.097)
- Kitami Y, Inui H, Uno S & Inagami T 1995 Molecular structure and transcriptional regulation of the gene for the platelet-derived growth factor α receptor in cultured vascular smooth muscle cells. *Journal of Clinical Investigation* **96** 558–567. (doi:10.1172/JCI118068)
- Kitami Y, Fukuoka T, Hiwada K & Inagami T 1999 A high level of CCAAT-enhancer binding protein-delta expression is a major determinant for markedly elevated differential gene expression of the platelet-derived growth factor- α receptor in vascular smooth muscle cells of genetically hypertensive rats. *Circulation Research* **84** 64–73.
- Letovsky J & Dynan WS 1989 Measurement of the binding of transcription factor Sp1 to a single GC box recognition sequence. *Nucleic Acids Research* **17** 2639–2653. (doi:10.1093/nar/17.7.2639)
- Li L, He S, Sun JM & Davie JR 2004 Gene regulation by Sp1 and Sp3. *Biochemistry and Cell Biology* **82** 460–471. (doi:10.1139/o04-045)
- Lobo MV, Arenas MI, Alonso EJ, Gomez G, Bazan E, Paino CL, Fernandez E, Fraile B, Paniagua R, Moyano A *et al.* 2004 Nestin, a neuroectodermal stem cell marker molecule, is expressed in Leydig cells of the human testis and in some specific cell types from human testicular tumours. *Cell and Tissue Research* **316** 369–376. (doi:10.1007/s00441-003-0848-4)
- Margalit Y, Yarus S, Shapira E, Gruenbaum Y & Fainsod A 1993 Isolation and characterization of target sequences of the chicken CdxA homeobox gene. *Nucleic Acids Research* **21** 4915–4922. (doi:10.1093/nar/21.21.4915)
- Martin LJ & Tremblay JJ 2005 The human 3β -hydroxysteroid dehydrogenase/ Δ^2 - Δ^4 isomerase type 2 promoter is a novel target for the immediate early orphan nuclear receptor NUR77 in steroidogenic cells. *Endocrinology* **146** 861–869. (doi:10.1210/en.2004-0859)
- Martin LJ, Boucher N, Brousseau C & Tremblay JJ 2008 The orphan nuclear receptor NUR77 regulates hormone-induced STAR transcription in Leydig cells through a cooperation with CaMKI. *Molecular Endocrinology* **22** 2021–2037. (doi:10.1210/me.2007-0370)
- Mayerhofer A, Seidl K, Lahr G, Bitter-Suermann D, Christoph A, Barthels D, Wille W & Gatzl M 1992 Leydig cells express neural cell adhesion molecules *in vivo* and *in vitro*. *Biology of Reproduction* **47** 656–664. (doi:10.1095/biolreprod47.4.656)
- Mennerich D, Hoffmann S, Hadrys T, Arnold HH & Bober E 1999 Two highly related homeodomain proteins, Nkx5-1 and Nkx5-2, display different DNA binding specificities. *Biological Chemistry* **380** 1041–1048. (doi:10.1515/BC.1999.130)
- Merchant-Larios H & Moreno-Mendoza N 1998 Mesonephric stromal cells differentiate into Leydig cells in the mouse fetal testis. *Experimental Cell Research* **244** 230–238. (doi:10.1006/excr.1998.4215)
- Nalbant D, Williams SC, Stocco DM & Khan SA 1998 Luteinizing hormone-dependent gene regulation in Leydig cells may be mediated by CCAAT/enhancer-binding protein- β . *Endocrinology* **139** 272–279. (doi:10.1210/en.139.1.272)
- Naylor LH & Clark EM 1990 d(TG)n.d(CA)n sequences upstream of the rat prolactin gene form Z-DNA and inhibit gene transcription. *Nucleic Acids Research* **18** 1595–1601. (doi:10.1093/nar/18.6.1595)
- Nikula H, Koskimies P, El-Hefnawy T & Huhtaniemi I 2001 Functional characterization of the basal promoter of the murine LH receptor gene in immortalized mouse Leydig tumor cells. *Journal of Molecular Endocrinology* **26** 21–29. (doi:10.1677/jme.0.0260021)
- Nye JA, Petersen JM, Gunther CV, Jonsen MD & Graves BJ 1992 Interaction of murine ets-1 with GGA-binding sites establishes the ETS domain as a new DNA-binding motif. *Genes and Development* **6** 975–990. (doi:10.1101/gad.6.6.975)
- Prokhorchouk A, Hendrich B, Jorgensen H, Ruzov A, Wilm M, Georgiev G, Bird A & Prokhorchouk E 2001 The p120 catenin partner Kaiso is a DNA methylation-dependent transcriptional repressor. *Genes and Development* **15** 1613–1618. (doi:10.1101/gad.198501)
- Rao A, Luo C & Hogan PG 1997 Transcription factors of the NFAT family: regulation and function. *Annual Review of Immunology* **15** 707–747. (doi:10.1146/annurev.immunol.15.1.707)
- Robert NM, Martin LJ & Tremblay JJ 2006 The orphan nuclear receptor NR4A1 regulates *Insulin-like 3* gene transcription in Leydig cells. *Biology of Reproduction* **74** 322–330. (doi:10.1095/biolreprod.105.044560)
- Safe S & Kim K 2004 Nuclear receptor-mediated transactivation through interaction with Sp proteins. *Progress in Nucleic Acid Research and Molecular Biology* **77** 1–36. (doi:10.1016/S0079-6603(04)77001-4)
- Sakai T, Hino K, Wada S & Maeda H 2003 Identification of the DNA binding specificity of the human ZNF219 protein and its function as a transcriptional repressor. *DNA Research* **10** 155–165. (doi:10.1093/dnares/10.4.155)
- Scherrer SP, Rice DA & Heckert LL 2002 Expression of steroidogenic factor 1 in the testis requires an interactive array of elements within its proximal promoter. *Biology of Reproduction* **67** 1509–1521. (doi:10.1095/biolreprod.102.006932)
- Schmahl J, Eicher EM, Washburn LL & Capel B 2000 Sry induces cell proliferation in the mouse gonad. *Development* **127** 65–73.
- Schmahl J, Rizzolo K & Soriano P 2008 The PDGF signaling pathway controls multiple steroid-producing lineages. *Genes and Development* **22** 3255–3267. (doi:10.1101/gad.1723908)
- Schreiber E, Matthias P, Muller MM & Schaffner W 1989 Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Research* **17** 6419. (doi:10.1093/nar/17.15.6419)
- Schwarzenbach H, Chakrabarti G, Paust HJ & Mukhopadhyay AK 2004 Gonadotropin-mediated regulation of the murine VEGF expression in MA-10 Leydig cells. *Journal of Andrology* **25** 128–139.
- Sheng Y, Li J, Dufau ML & Tsai-Morris CH 2005 The gonadotropin-regulated long-chain acyl CoA synthetase gene: a novel downstream Sp1/Sp3 binding element critical for transcriptional promoter activity. *Gene* **360** 20–26. (doi:10.1016/j.gene.2005.07.006)
- Sterneck E, Tessarollo L & Johnson PF 1997 An essential role for C/EBP β in female reproduction. *Genes and Development* **11** 2153–2162. (doi:10.1101/gad.11.17.2153)
- Thuillier R, Wang Y & Culty M 2003 Prenatal exposure to estrogenic compounds alters the expression pattern of platelet-derived growth

- factor receptors α and β in neonatal rat testis: identification of gonocytes as targets of estrogen exposure. *Biology of Reproduction* **68** 867–880. (doi:10.1095/biolreprod.102.009605)
- Tremblay JJ & Viger RS 1999 Transcription factor GATA-4 enhances Müllerian inhibiting substance gene transcription through a direct interaction with the nuclear receptor SF-1. *Molecular Endocrinology* **13** 1388–1401. (doi:10.1210/me.13.8.1388)
- Tsukamoto T, Matsui T, Takaishi T, Ito M, Fukase M & Chihara K 1994 Retinoic acid differentially affects platelet-derived growth factor and epidermal growth factor-regulated cell growth of mouse osteoblast-like cells. *Cell Growth and Differentiation* **5** 207–212.
- Tun T, Hamaguchi Y, Matsunami N, Furukawa T, Honjo T & Kawaichi M 1994 Recognition sequence of a highly conserved DNA binding protein RBP-Jkappa. *Nucleic Acids Research* **22** 965–971. (doi:10.1093/nar/22.6.965)
- Warshamana GS, Martinez S, Lasky JA, Corti M & Brody AR 1998 Dexamethasone activates expression of the PDGF- α receptor and induces lung fibroblast proliferation. *American Journal of Physiology* **274** L499–L507.
- Xie J, Aszterbaum M, Zhang X, Bonifas JM, Zachary C, Epstein E & McCormick F 2001 A role of PDGFR α in basal cell carcinoma proliferation. *PNAS* **98** 9255–9259. (doi:10.1073/pnas.151173398)
- Yang ZH, Kitami Y, Takata Y, Okura T & Hiwada K 2001 Targeted overexpression of CCAAT/enhancer-binding protein-delta evokes enhanced gene transcription of platelet-derived growth factor α -receptor in vascular smooth muscle cells. *Circulation Research* **89** 503–508. (doi:10.1161/hh1801.096265)
- Zhang XQ, Afink GB, Svensson K, Jacobs JJ, Gunther T, Forsberg-Nilsson K, van Zoelen EJ, Westermarck B & Nister M 1998 Specific expression in mouse mesoderm- and neural crest-derived tissues of a human PDGFRA promoter/lacZ transgene. *Mechanisms of Development* **70** 167–180. (doi:10.1016/S0925-4773(97)00190-1)
- Zhang L, Sheppard OR, Shah AM & Brewer AC 2008 Positive regulation of the NADPH oxidase NOX4 promoter in vascular smooth muscle cells by E2F. *Free Radical Biology & Medicine* **45** 679–685. (doi:10.1016/j.freeradbiomed.2008.05.019)
- Zhao C & Meng A 2005 Sp1-like transcription factors are regulators of embryonic development in vertebrates. *Development, Growth and Differentiation* **47** 201–211. (doi:10.1111/j.1440-169X.2005.00797.x)
- Zheng N, Fraenkel E, Pabo CO & Pavletich NP 1999 Structural basis of DNA recognition by the heterodimeric cell cycle transcription factor E2F-DP. *Genes and Development* **13** 666–674. (doi:10.1101/gad.13.6.666)
- Zweidler-Mckay PA, Grimes HL, Flubacher MM & Tschlis PN 1996 Gfi-1 encodes a nuclear zinc finger protein that binds DNA and functions as a transcriptional repressor. *Molecular and Cellular Biology* **16** 4024–4034.

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