GATA4 knockdown in MA-10 Leydig cells identifies multiple target genes in the steroidogenic pathway

Francis Bergeron¹, Gabriel Nadeau¹ and Robert S Viger^{1,2}

¹Reproduction, Mother and Child Health, Room T3-67, Centre de Recherche du Centre Hospitalier Universitaire (CHU) de Québec, Centre de Recherche en Biologie de la Reproduction (CRBR), 2705 Laurier Boulevard, Quebec City, Quebec, Canada G1V 4G2 and ²Department of Obstetrics and Gynecology, Faculty of Medicine, Laval University, Quebec City, Quebec, Canada G1K 7P4

Correspondence should be addressed to R S Viger; Email: robert.viger@crchul.ulaval.ca

Abstract

GATA4 is an essential transcription factor required for the initiation of genital ridge formation, for normal testicular and ovarian differentiation at the time of sex determination, and for male and female fertility in adulthood. In spite of its crucial roles, the genes and/or gene networks that are ultimately regulated by GATA4 in gonadal tissues remain to be fully understood. This is particularly true for the steroidogenic lineages such as Leydig cells of the testis where many *in vitro* (promoter) studies have provided good circumstantial evidence that GATA4 is a key regulator of Leydig cell gene expression and steroidogenesis, but formal proof is still lacking. We therefore performed a microarray screening analysis of MA-10 Leydig cells in which *Gata4* expression was knocked down using an siRNA strategy. Analysis identified several GATA4-regulated pathways including cholesterol synthesis, cholesterol transport, and especially steroidogenesis. A decrease in GATA4 protein was associated with decreased expression of steroidogenic genes previously suspected to be GATA4 targets such as *Cyp11a1* and *Star*. *Gata4* knockdown also led to an important decrease in other novel steroidogenic targets including *Srd5a1*, *Gsta3*, *Hsd3b1*, and *Hsd3b6*, as well as genes known to participate in cholesterol metabolism such as *Scarb1*, *Ldlr*, *Soat1*, *Scap*, and *Cyp51*. Consistent with the decreased expression of these genes, a reduction in GATA4 protein compromised the ability of MA-10 cells to produce steroids both basally and under hormone stimulation. These data therefore provide strong evidence that GATA4 is an essential transcription factor that sits atop of the Leydig cell steroidogenic program.

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Introduction

GATA-binding protein 4 (GATA4) is a transcription factor belonging to the GATA family of zinc finger proteins. It is critically required for the development and function of multiple tissues derived from both mesoderm and endoderm (reviewed in Molkenin (2000), Viger *et al.* (2008) and Chlon & Crispino (2012)). This includes both the testis and ovary where GATA4 is one of the earliest markers of gonadal development. It is expressed in the coelomic epithelial layer of the genital ridge, starting at around E.10.0 in the mouse (Mazaud Guittot *et al.* 2007, Hu *et al.* 2013). Following gonadal differentiation, GATA4 remains highly expressed in all gonadal somatic lineages of both sexes throughout ontogeny (reviewed in LaVoie (2003), Viger *et al.* (2004, 2008) and Zaytouni *et al.* (2011)). In the ovary, this includes granulosa, theca, and luteal cells. In the testis, GATA4 is found in peritubular, Leydig, and Sertoli cells. The presence of GATA4 protein in Sertoli cells (Imai *et al.* 2004) and the activity of *Gata4* promoter regulatory sequences driving Sertoli cell expression (Mazaud Guittot *et al.* 2007) are

independent of the stage of germ cell maturation making this gene and its promoter a highly useful and frequently used marker for this cell type.

Several conditional and gonad-specific knockout mouse models have provided important insights into the *in vivo* roles played by GATA4 in gonadogenesis. Consistent with its very early expression at the stage of coelomic epithelial thickening, conditionally *Gata4*-deficient XX or XY embryos do not initiate the formation of a genital ridge (Hu *et al.* 2013). Elegant mouse models used by Tevosian *et al.*, where the *Gata4* gene was excised around the time of sex determination by gonad-specific Cre drivers, have shown that GATA4 is also essential for normal testicular and ovarian differentiation (reviewed in Tevosian (2014)). These crucial functions in early mammalian gonadogenesis necessitate not only GATA4 but also a functional cooperation between GATA4 and its transcriptional partner Friend of GATA 2 (FOG2/ZFPM2; Tevosian *et al.* 2002, Manuylov *et al.* 2008, 2011). The requirement for GATA4 protein in testis and ovary is maintained during later fetal development and into adulthood, as several different groups have

reported that loss of GATA4 at these stages is associated with disrupted cord formation and impaired spermatogenesis in the testis (Kyrönlahti *et al.* 2011a, Manuylov *et al.* 2011), and impaired granulosa cell proliferation, theca cell recruitment, and a failure of follicular development ultimately leading to reduced fertility in the adult female (Kyrönlahti *et al.* 2011b, Bennett *et al.* 2012, Efimenko *et al.* 2013).

Important insights into the mechanism of action of GATA4 in gonadal development and function have also come from the identification of some of its target genes. Some of the earliest genes acting downstream of GATA4 in the genital ridge and differentiating testis include steroidogenic factor 1/adrenal 4-binding protein (*Nr5a1*), LIM homeobox protein 9 (*Lhx9*), sex determining region of chromosome Y (*Sry*), SRY-box containing gene 9 (*Sox9*), and anti-Müllerian hormone *Amh* (Tevosian *et al.* 2002, Manuylov *et al.* 2007, Hu *et al.* 2013). *In vitro* and *in vivo* studies have suggested that some of these genes are direct targets for GATA4, acting in their 5' regulatory (promoter) regions (Viger *et al.* 1998, Tremblay & Viger 2001, Tevosian *et al.* 2002, Manuylov *et al.* 2007, Miyamoto *et al.* 2008, Hu *et al.* 2013). In the newly formed testis, conditional loss of *Gata4* expression highlighted the requirement for GATA4 in sustaining testis cord morphogenesis by promoting the expression of the *Dmrt1* gene (Manuylov *et al.* 2011), whose promoter also appears to be under direct GATA4 control (Lei & Heckert 2004).

Another class of potential GATA4-regulated gene targets in gonadal cells is genes involved in the steroidogenic pathway (reviewed in LaVoie (2003), Tremblay & Viger (2003a) and Viger *et al.* (2008)). Proposed steroidogenic targets for GATA4 identified through *in vitro* promoter characterization studies have included steroidogenic acute regulatory protein (*Star*), P450 cholesterol side chain cleavage (*Cyp11a1*), steroid 17 α -hydroxylase (*Cyp17a1*), 3 β -HSD type 2 (*HSD3B2*), and P450 aromatase (*Cyp19a1*) (reviewed in Viger *et al.* (2008), Shi *et al.* (2009) and King & LaVoie (2012)). Proof that these genes are indeed direct targets for GATA transcriptional regulation, however, has yet to be formally provided. While conditional knockout of *Gata4*, or of *Gata4* and *Gata6* in the ovary, is associated with decreased expression of some of the genes (*Cyp19a1*, *Cyp11a1*, and *Star*; Kyrönlahti *et al.* 2011b, Bennett *et al.* 2012), it remains unclear whether they are actual GATA4 transcriptional targets given the important disruption in folliculogenesis that is associated with the knockouts. In comparison to Sertoli cells of the testis, conditional *Gata4* knockout models have been less useful for studying steroidogenic (Leydig) cells, as reliable Leydig cell-specific Cre drivers, especially in the adult, are lacking. Moreover, GATA4 protein appears to be essential for the development and differentiation of steroidogenic cells in the fetal testis, which further compounds the problem (Tevosian *et al.* 2002,

Bielinska *et al.* 2007). To circumvent these difficulties and to identify novel GATA4-regulated gene targets in Leydig cells, we performed a microarray screening analysis of MA-10 Leydig cells, in which *Gata4* expression was knocked down using siRNAs directed against the *Gata4* coding sequence. Our results show that GATA4 is essential for steroid production in MA-10 cells by regulating the expression of several key genes required for steroidogenesis.

Materials and methods

GATA4 siRNA-mediated knockdown in MA-10 cells

MA-10 Leydig cells were plated in 60 mM petri dishes and transfected the next day at 50–60% confluence with 150 nM of predesigned Stealth RNAi molecules purchased from Invitrogen directed against the GATA4 coding sequence and Stealth RNAi Negative Control Duplexes (Invitrogen, cat. no. 12935-300) used as negative controls. Two different siRNA sequences against GATA4 were used in this study, both giving comparable results – GATA4MSS247223: 5'- CCAUUCUGAUAUGUUUGAUGACUU-3'; GATA4MSS247225: 5'- UCCAUCAGUCUGUCUGCUCUGAA-3'. The siRNA was transfected using 16 μ l of jetPRIME transfection reagent and 200 μ l of jetPRIME transfection buffer (Polyplus transfection, New York, NY, USA). The cell media was changed the day after transfection; 48 h later, half of the cells were collected for RNA extraction and the other half for total protein extraction. For the *Star* promoter luciferase assay, 500 ng/well of a WT or GATA mutated *Star* promoter construct was cotransfected with 150 nM of siRNA GATA4MSS247225 using 2.5 μ l/well of jetPRIME in MA-10 cells at 50–60% confluence. The media was changed 24 h after transfection and cells were assayed for luciferase activity as described previously.

RNA isolation

Total RNA was extracted by TRI reagent (Invitrogen) according to the manufacturer's instructions. RNA quantity and purity were determined by absorbance at 260/280 nm and RNA integrity by agarose gel electrophoresis. For the microarray experiment, total RNA was further purified using an RNeasy >t column (Qiagen), and the integrity of the RNA was assessed using an Agilent Bioanalyzer (Agilent Technologies Canada, Inc., Mississauga, Ontario, Canada).

RT and quantitative real-time PCR

First-strand cDNAs were synthesized from a 1 μ g aliquot of total RNA using random hexamer nucleotides and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Real-time qPCR was performed with 2 μ l of cDNA using a LightCycler 1.5 instrument and the LightCycler FastStart DNA Master SYBR Green I Kit (Roche Diagnostics Canada). The primers used for qPCR are given in Table 1. The following conditions were used for qPCR: 10 min at 95 °C followed by 40 cycles of denaturation (5 s at 95 °C), annealing (60–62 °C), and extension (20 s at 72 °C) with a single

Table 1 Oligonucleotide primers used for qPCR analysis.

Gene product	Sequence
<i>Gata4</i>	F: 5'-CTCTGGAGGCGAGATGGGAC-3' R: 5'-CGCATTGCAAGAGGCCTGGG-3'
<i>Amhr2</i>	F: 5'-CCCTCTGCCCTCTGGGCCTT-3' R: 5'-ACTGGCCATCCTGCCAACGC-3'
<i>Cyp11a1</i>	F: 5'-CACCAGTATTATCAGAGGCC-3' R: 5'-GATGAAGTCCTGAGCTACACC-3'
<i>Gsta3</i>	F: 5'-TGGCGGGGAAGCCAGTCTT-3' R: 5'-ACCTTGCCAGGTATCCCGAGT-3'
<i>Hsd3b1</i>	F: 5'-GGCTGGATGGAGCTGCCTGG-3' R: 5'-GCTCTCCTCAGGCACTGGGC-3'
<i>Hsd3b6</i>	F: 5'-TGTCACAGGTGTCATTCCCAGGCA-3' R: 5'-CTCTTCCTCGTTGCCATTACGATT-3'
<i>Inha</i>	F: 5'-CGAAGTGTCCGGGAGCTCGT-3' R: 5'-TGGCTGGTCTCAGAGGTGGC-3'
<i>Srd5a1</i>	F: 5'-TCTGATCCACTACGTGCAAAG-3' R: 5'-TCAAGTATCGGCTCTGCAAG-3'
<i>Star</i>	F: 5'-CAACTGGAAGCAACACTCA-3' R: 5'-CCTTGACATTGGGTTCCAC-3'
<i>Itgb8</i>	F: 5'-GCTTTCATTATGTGCGGCT-3' R: 5'-TGGCACAGGAGACCACTT-3'
<i>Vcan</i>	F: 5'-CGGACCTATGGATTCCGCTC-3' R: 5'-AGTTCTCCAACAGTCGCGAG-3'
<i>Sumo3</i>	F: 5'-GGGCAGAGAGCGTGACTC-3' R: 5'-TTGAAGTGTACCACCGAGCC-3'
<i>Egr1</i>	F: 5'-GAGCACCTGACCACAGAGTC-3' R: 5'-AAAGGGGTTTCAAGGCCACAA-3'
<i>Jun</i>	F: 5'-GCACCTCCGCGCAAGAAGT-3' R: 5'-AAGCCCTCCTGCTCGTGGT-3'
<i>Nr2f2</i>	F: 5'-TGGAGAAGCTCAAGGCACTG-3' R: 5'-AAGAGCTTCCGAACCGTGT-3'
<i>Rpl19</i>	F: 5'-CTGAAGGTCAAAGGAATGTG-3' R: 5'-GGACAGAGTCTTGATGATCTC-3'

F, forward primer; R, reverse primer.

acquisition of fluorescence levels at the end of each extension step. Each amplification was performed in duplicate using four different samples from each treatment: control siRNA or siRNA against GATA4 (three times with GATA4MSS247225 and one time with GATA4MSS247223). PCR products were confirmed by analysis of melting curve, agarose-gel electrophoresis, and sequencing. Differences in mRNA levels between samples were quantified using relative quantification with external standards as previously described (Boulende Sab *et al.* 2011). PCR DNA fragments containing the genes of interest were used to generate the standard curves. The amount of DNA for the target and reference (*Rpl19*) in the unknown samples was calculated by the LightCycler Software 3.5 (Roche Diagnostics Canada) using the respective dilution curves. Means of data are reported in arbitrary units as the ratio of the level of target gene to the *Rpl19* reference gene $\pm$ S.E.M.

Protein extraction and western blot

Total protein extracts were obtained 48 h after siRNA transfection of MA-10 cells. The cells were washed twice with cold PBS, scraped, and pelleted at 4 °C. The cells were lysed for 15 min on ice with 150 μ l of lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Igepal, and protease inhibitors). The cells were sonicated using a Branson sonifier 450 (Branson Ultrasonics, Danbury, CT, USA) for 30 s at two output control and constant duty cycle. The lysed cells were centrifuged for 10 min to pellet cellular debris.

The supernatants were collected and protein concentration was calculated by the Bradford method using Bio-Rad protein assay dye reagent (Bio-Rad). Western blotting was performed using MA-10 total cell extracts from both experimental conditions. The proteins were boiled for 10 min in a denaturing loading buffer and were separated by SDS-PAGE, transferred to Amersham Hybond-P membrane (GE Healthcare Life Sciences, Baie d'Urfe, Quebec, Canada), and immunodetected using antisera specific for GATA4 (goat polyclonal antibody, dilution 1:1000, cat. no. sc-1237x, Santa Cruz Biotechnologies), CYP11A1 (goat polyclonal antibody, 1:200 dilution, cat. no. sc-18043, Santa Cruz Biotechnologies), STAR (rabbit polyclonal antibody, 1:1000 dilution, cat. no. sc-25806, Santa Cruz Biotechnologies), or α -TUBULIN (monoclonal mouse antibody, 1:10 000 dilution, cat. no. #15168, Sigma-Aldrich). The membrane was blocked with 5% skimmed milk, and primary and secondary antibodies were incubated for 1 h in 1% skimmed milk (5% skimmed milk for α -TUBULIN and its secondary antibody). Immunodetection was performed using HRP secondary antibodies and ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) according to the manufacturer's instructions. The following secondary peroxidase antibodies were used: horse polyclonal anti-goat antibody (1:5000 dilution, cat. no. PI-9500, Vector Laboratories, Burlington, Ontario, Canada), goat polyclonal anti-rabbit antibody (1:10 000 dilution, cat. no. PI-1000, Vector Laboratories), and goat polyclonal anti-mouse antibody (1:20 000 dilution, cat. no. 115-035-003, Jackson Immuno-Research Laboratories, Inc., West Grove, PA, USA). Densitometry was performed using ImageJ Software as described (<http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blot-with-image-j/>).

Microarray analysis

DNA microarray analyses were carried out with Affymetrix Mouse Gene 1.0 ST arrays. The chips were processed according to the Affymetrix standard protocol. In brief, total RNA (200 ng/sample) was labeled using the Affymetrix GeneChip WT cDNA Synthesis and Amplification Kit protocol, and hybridized to the arrays as described by the manufacturer (Affymetrix, Santa Clara, CA, USA). The cDNA hybridization cocktail was incubated overnight at 45 °C with rotation in a hybridization oven. After 16 h of hybridization, the cocktail was removed and the arrays were washed and stained in an Affymetrix GeneChip fluidics station 450, according to the Affymetrix-recommended protocol (http://media.affymetrix.com/support/downloads/manuals/wt_sensetarget_label_manual.pdf). The arrays were scanned using the Affymetrix GCS 3000 7G and the Gene-Chip Operating Software (Affymetrix) to produce the intensity files. Microarray hybridization was carried out at the Microarray Facility of the Centre de recherche du CHU de Quebec. Three independent RNA samples treated with Stealth RNAi Negative Control and three with Stealth RNAi molecules against GATA4 (one sample with GATA4MSS247223 and two with GATA4MSS247225) were analyzed by microarray; two different GATA4 siRNAs were used to minimize off-target effects (Jackson & Linsley 2010). Analysis of microarray data and principal component analysis

(PCA; Ringner 2008) were performed using Partek Software version 6.6 (Partek Inc., St Louis, MO, USA). Robust multiarray analysis (RMA) background correction and a logarithmic transformation were applied to data arrays. Differentially expressed genes were analyzed by *t*-test paired two-tailed, two-sample equal variance comparison between the groups treated with control siRNA or siRNA against GATA4. The complete list of genes modulated in response to GATA4 knockdown in MA-10 cells is presented in [Supplementary File 1](#), see section on [supplementary data](#) given at the end of this article. Ingenuity interactive pathway analysis (IPA, Ingenuity Systems, www.ingenuity.com) was used to identify pathways and biological processes regulated by GATA4. The threshold value was fixed at 1.3-fold change, the *P* value <0.05, and a false discovery rate (FDR) <0.25 for IPA.

Plasmids

The –1.6 kb mice anti-Müllerian hormone receptor 2 (*Amhr2*)-luciferase promoter construct was generously provided by Dr José Teixeira (Teixeira *et al.* 1999). The minimal –33 bp mice *Amhr2*-luciferase promoter was amplified by PCR from the longer –1.6 kb sequence using the following primers – sense: 5'-CGCGAGCTCAGTTGGGGATTGAAGGCTTGG-3' and antisense: 5'-CGCAGATCTCGAGAAGGATGC-3'. The short –33 bp *Amhr2* promoter sequence was cloned into the XhoI/SacI sites of the same pGL3-Basic (Promega) luciferase backbone vector in which the longer –1.6 kb *Amhr2* promoter sequence was also cloned. *Amhr2* promoter constructs containing mutated GATA motifs were obtained by site-directed mutagenesis using PfuUltra (Agilent, Santa Clara, CA, USA) with the following primers (mutations are in lower case) – sense: 5'-CTGATGATTGTGAGCTAcCATGTGGTTGCTGG-3'; antisense: 5'-CCAGCAACCACATGgTAGCTCACAATCATCAG-3' and sense: 5'-GAGATTCGTAGTCAATTAcCACAGGGTCTCAGC-3'; antisense: 5'-GCTGAGAACCTGTGgTAATTGACTACGAAATCTC-3'. The –1.3 kb rat steroid 5 α -reductase 1 (*Srd5a1*) promoter sequence was amplified from rat genomic DNA and cloned into the BamHI/KpnI site of a modified pxP1-luciferase promoterless reporter plasmid (Tremblay & Viger 1999), with the following primers – sense: 5'-CGGGATCCCAACCCTGGCTGCTCTATAGC-3'; antisense: 5'-GGGGTACCGAGGTCGAGCAGTGTACTCC-3'. The *Srd5a1*-luciferase promoter construct containing a mutated GATA motif was obtained by site-directed mutagenesis using primers – sense: 5'-GATCTTGAGTTCCTGgTAGTAGTGCTCAAAGAGC-3'; antisense: 5'-GCTCTTTGAGCACTACTAcCAGGAACCTCAAGATC-3'. The minimal –32 bp *Srd5a1*-luciferase promoter construct (Seenundun & Robaire 2005) was generated by subcloning the following double-stranded oligonucleotide – sense: 5'-GATCCTTATATGAGGCTCCACTCGACACCCGGGCATG-GAGTACACTGCTCGACCTCGGTAC -3'; antisense: 5'-CGAGGTCGAGCAGTGTACTCCATGCCCGGTGTCCGAGTG-GAGCCTCATATAAG-3' – into the modified pxP1-luciferase reporter plasmid. Luciferase reporter constructs containing the mouse *Star* promoter (–902 bp, –902 bp containing a mutation of the GATA element at –63 bp, and the minimal –43 bp sequence) as well as the GATA4 expression plasmid have been described previously (Tremblay & Viger 2001, Martin *et al.* 2012).

Cell culture, transfections, and luciferase promoter assays

Mouse MA-10 Leydig cells were kindly provided by Dr Ascoli (1981). MA-10 cells were cultured in DMEM/F12 supplemented with 5% fetal bovine serum and 2.5% horse serum. The day before transfection 1.5×10^5 MA-10 cells were plated in 24-well plates by the calcium phosphate method (Jordan & Wurm 2004). The amount of promoter-luciferase reporter DNA was kept at 500 ng/well; GATA4 expression vector was used at 50 ng/well and pSP64 (Promega) was used as carrier to keep the final concentration of DNA at 1.5 μ g/well. The cell media was changed the day after the transfection and cells were lysed 1 day later by adding 50 μ l of lysis buffer (100 mM Tris-HCl (pH 8), 0.5% Igepal, and 5 mM dithiothreitol) directly to the cells. The lysate was collected and assayed for luciferase activity using a Luminoskan Ascent luminometer (Thermo, Milford, MA, USA). For all experiments, the data reported represent the fold change in promoter activity $\pm$ S.E.M. of at least four experiments, each done in triplicate.

Progesterone ELISA

A progesterone ELISA was performed using MA-10 growth media from both experimental conditions (treated with control siRNA or GATA4 siRNA). MA-10 cells were plated in 24-well plates and transfected (at 50–60% confluence) for 24 h with 150 nM siRNAs. Two days after transfection, cells were washed and cultivated for 4 h in the media without serum with vehicle (DMSO) or 10 μ M forskolin (Sigma) and 0.5 mM IBMX (Sigma). The media was collected and progesterone was measured using a progesterone EIA Kit (Cayman Chemical, cat. no. 582601, Ann Arbor, MI, USA) according to the manufacturer's instructions. The samples treated with DMSO were diluted 1:20 and forskolin/IBMX-stimulated samples were diluted 1:60.

Statistical analyses

A Student's *t*-test paired two-tailed comparison was used to detect significant differences between control and treated groups; *P*<0.05 was considered significant. All statistical analyses were done using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

To identify GATA4-regulated genes in the Leydig cell lineage, we performed a microarray screening analysis of MA-10 cells in which *Gata4* expression was decreased using an siRNA-mediated knockdown strategy. MA-10 cells are an ideal model to use because unlike other testicular somatic cells (reviewed in Viger *et al.* (2008)), they are known to contain almost exclusively GATA4 protein (Tremblay & Viger 2003b). MA-10 cells were transiently transfected with siRNAs that targeted two different regions of the *Gata4* coding sequence. In both cases, the siRNAs achieved significant inhibition at the level of *Gata4* mRNA (Fig. 1A) and

Figure 1 Knockdown of *Gata4* expression in MA-10 Leydig cells. MA-10 cells were transiently transfected with either control siRNA or siRNA against GATA4. (A) Global detection of *Gata4* mRNAs, using oligonucleotide primers corresponding to the *Gata4* coding region, was measured in four independent knockdown experiments ($n=4$) by real-time quantitative PCR (qPCR). Quantitative data were normalized using the housekeeping *Rpl19* gene and expressed as the mean $\pm$ S.E.M. An asterisk indicates a significant difference between the control and GATA4 siRNA groups ($P<0.05$). (B) Western blotting analysis was performed using MA-10 total cell extracts from both experimental conditions. Proteins were separated by SDS-PAGE, transferred to PVDF membrane, and immunodetected using antisera specific for GATA4 or α -TUBULIN (used as loading control).

GATA4 protein (Fig. 1B). α -tubulin was used as a control in the western blotting analysis to indicate that the GATA4 siRNA did not cause a general decrease in protein synthesis. Total cellular RNA was prepared from both GATA4 siRNA and control siRNA-treated MA-10 cells and then hybridized to Affymetrix Mouse Gene 1.0 ST arrays to identify global changes in genes under GATA4 control. RMA background correction and a logarithmic transformation were applied to data arrays, and differentially expressed genes were identified by *t*-test paired two-tailed two-sample equal variance comparisons. PCA of all normalized array data was performed to first analyze the RNA profiles of MA-10 cells treated with the control vs GATA4 siRNAs. As shown in Fig. 2, the control siRNA and GATA4 siRNA groups from three separate experiments clustered separately, indicating that the control and GATA4 siRNA treatments produced distinct, yet reproducible RNA populations. The complete list of MA-10 cell genes modulated in response to GATA4 knockdown, and expressed as fold change in comparison to the control siRNA, is provided in Supplementary File 1.

Our microarray analysis yielded 335 upregulated and 374 downregulated genes with a fold change of at least 1.3, a *P* value of <0.05 , and an FDR <0.25 . The data were analyzed using Ingenuity IPA (interactive pathway analysis of complex 'omics data' version 16542223) to identify key pathways dependent on GATA4. Analysis according to biological function (used to predict the effect of gene expression changes in the dataset on

biological processes) identified several processes that were modulated (z-score) in response to GATA4 knock-down in MA-10 cells. These included the conversion of hormone, metabolism of membrane lipid derivative, accumulation and transport of cholesterol ester, conversion of pregnenolone, synthesis of pregnenolone, secretion of steroid hormone, synthesis of androgen, and steroidogenesis (Supplementary File 2, see section on supplementary data given at the end of this article).

Table 2 gives a selected list of genes that were specifically upregulated in response to GATA4 knock-down in MA-10 cells. The two most highly upregulated genes were integrin beta 8 (*Itgb8*; 4.03-fold) and versican (*Vcan*, 3.32-fold), which encode for proteins involved in cell-cell and cell-extracellular matrix interactions. Significantly upregulated genes also included SMT3 suppressor of mif two homolog 3 (*Sumo3*, 1.87-fold) involved in protein posttranslational modification, and the immediate early genes early growth response 1 (*Egr1*, 1.63-fold) and JUN oncogene (*Jun*, 1.62-fold). The list of upregulated genes also identified several genes involved in cell signaling such as calcium/calmodulin-dependent protein kinase kinase 1 (*Camkk1*) and Smad family member 5 (*Smad5*, 1.53-fold), as well as several genes

Figure 2 RNA profile in MA-10 cells following GATA4 knockdown. Principal component analysis (PCA) of all normalized array data. Each point represents a hybridized sample: samples treated with siRNA against *Gata4* transcript sequence and samples treated with control siRNA. X-axis, first principal component (PC1); y-axis, second principal component (PC2). PCA was done using Partek Software 6.6.

Table 2 Selected list of genes upregulated in Affymetrix Gene 1.0 ST Array following GATA4 knockdown in MA-10 Leydig cells.

Transcript cluster ID	Gene symbol	Description	P value	False discovery rate	Fold change
10403229	<i>Itgb8</i>	Integrin beta 8	0.00037	0.074	4.03
10410931	<i>Vcan</i>	Versican	0.0014	0.112	3.32
10364287	<i>Sumo3</i>	SMT3 suppressor of mif two 3 homolog 3 (yeast)	0.0001	0.061	1.87
10378253	<i>Camkk1</i>	Calcium/calmodulin-dependent protein kinase kinase 1	0.0055	0.164	1.86
10454782	<i>Egr1</i>	Early growth response 1	0.0006	0.084	1.63
10514466	<i>Jun</i>	Jun oncogene	0.057	0.413	1.62
10564527	<i>Nr2f2</i>	Nuclear receptor subfamily 2, group F, member 2	0.0009	0.096	1.61
10490731	<i>Sox18</i>	SRY-box containing gene 18	0.0055	0.172	1.60
10405605	<i>Smad5</i>	Mothers against decapentaplegic homolog 5 (drosophila)	0.0016	0.112	1.53
10482772	<i>Nr4a2</i>	Nuclear receptor subfamily 4, group A, member 2	0.004	0.078	1.43

coding for transcription factors including COUPTFII (*Nr2f2*, 1.61-fold), NURR1 (*Nr4a2*, 1.43-fold), and SRY-box containing gene 18 (*Sox18*; 1.6-fold). A subset of the upregulated genes was validated by qPCR (Fig. 3).

Many genes crucial to MA-10 cell function were found to be downregulated in response to GATA4 depletion. These included *Gata4* itself (−1.86-fold), which was targeted in the siRNA knockdown and also *Gata1* (−1.99-fold), another GATA family member whose mRNA is known to be present in MA-10 cells (Zhang *et al.* 2002), and whose expression is regulated by GATA factors (reviewed in Kobayashi & Yamamoto (2007)). Interestingly, however, many downregulated genes were those that encode for multiple components of the cholesterol transport, cholesterol biosynthesis, and steroidogenic pathways (Table 3). Genes involved in cholesterol transport included scavenger receptor B1 (*Scarb1*, −1.74-fold) and LDL receptor (*Ldlr*, −1.48-fold), which code for proteins required for receptor-mediated uptake of cholesterol bound to HDL and LDL respectively. The effect on cholesterol metabolism was not limited to cholesterol transport/bioavailability as we observed equally important decreases in the expression of several genes involved in *de novo* cholesterol biosynthesis; these included sterol-C5-desaturase (*Sc5d*, −1.51-fold), lanosterol 14 α -demethylase (*Cyp51*, −1.32-fold), and mevalonate decarboxylase (*Mvd*, −1.31-fold). The genes encoding sterol O-acyltransferase (*Soat1*, −1.33-fold), which regulates the level of intracellular free cholesterol by promoting the formation of esterified cholesterol for storage in lipid droplets, and sterol regulatory element binding protein (SREBP) cleavage activating protein (*Scap*, −1.37-fold), an important sensor of cholesterol in the endoplasmic reticulum, were also significantly downregulated.

Gene set enrichment analysis (GSEA version 2.0.13) pathway analysis (using gene set c2.all.v4.0.) was performed on the array data. The reactome steroid hormones pathway was one of the most downregulated pathways (in the top ten) and therefore identifies steroid metabolism as a major process in MA-10 cells that is critically dependent on the presence of GATA4

(Supplementary Files 3 and 4, see section on supplementary data given at the end of this article). Of the genes identified, *Star* was at the top of the list of genes with reduced expression in GATA4-depleted MA-10 cells (−3.65-fold). Other important downregulated genes associated with the regulation of steroidogenesis included members of the 3 β -HSD family (*Hsd3b6* and *Hsd3b1*; −2.65- and −1.97-fold respectively), anti-Müllerian hormone type 2 receptor (*Amhr2*, −2.31-fold), glutathione S-transferase alpha 3 (*Gsta3*,

Figure 3 Quantitative PCR validation of upregulated target genes identified in the microarray analysis following GATA4 knockdown. Global detection of the different genes of interest, using oligonucleotide primers corresponding to the respective coding regions, was measured in four different independent experiments ($n=4$) by qPCR. Quantitative data were normalized using the housekeeping *Rpl19* gene and expressed as the mean $\pm$ s.e.m. Asterisks indicate significant differences between the control and GATA4 siRNA groups ($P<0.05$). *Itgb8*, integrin beta 8; *Vcan*, versican; *Sumo3*, g SMT3 suppressor of mif two 3 homolog 3; *Egr1*, early growth response 1; *Jun*, jun proto-oncogene; *Nr2f2*, nuclear receptor subfamily 2, group F, member 2.

Table 3 Selected list of genes downregulated in Affymetrix Gene 1.0 ST array following GATA4 knockdown in MA-10 Leydig cells.

Transcript cluster ID	Gene symbol	Description	P value	False discovery rate	Fold change
10345065	<i>Gsta3</i>	Glutathione S-transferase, alpha 3	0.0003	0.070	−3.96
10571054	<i>Star</i>	Steroidogenic acute regulatory protein	0.0002	0.067	−3.65
10500559	<i>Hsd3b6</i>	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 6	0.0019	0.121	−2.65
10589994	<i>Eomes</i>	Eomesodermin homolog (<i>Xenopus laevis</i>)	0.0005	0.081	−2.57
10427222	<i>Amhr2</i>	Anti-Müllerian hormone type 2 receptor	0.0013	0.107	−2.31
10410452	<i>Srd5a1</i>	Steroid 5 alpha-reductase 1	0.0036	0.155	−2.23
10460400	<i>Pcx</i>	Pyruvate carboxylase	0.0001	0.061	−2.05
10603417	<i>Gata1</i>	GATA binding protein 1	1.94×10^{-5}	0.060	−1.99
10500565	<i>Hsd3b1</i>	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	0.0010	0.098	−1.97
10420747	<i>Gata4</i>	GATA-binding protein 4	0.0013	0.109	−1.86
10347669	<i>Inha</i>	Inhibin alpha	6.94×10^{-5}	0.060	−1.76
10533929	<i>Scarb1</i>	Scavenger receptor class B, member 1	1.70×10^{-4}	0.065	−1.74
10366052	<i>Kitl</i>	Kit ligand	0.0119	0.223	−1.56
10592585	<i>Sc5d</i>	Sterol-C5-desaturase	0.0054	0.171	−1.51
10569319	<i>Ctsd</i>	Cathepsin D	4.16×10^{-5}	0.060	−1.49
10583732	<i>Ldlr</i>	LDL receptor	4.63×10^{-4}	0.078	−1.48
10589511	<i>Scap</i>	SREBF chaperone	0.0017	0.116	−1.37
10359161	<i>Soat1</i>	Sterol O-acyltransferase 1	0.0016	0.115	−1.33
10527920	<i>Cyp51</i>	Cytochrome P450, family 51	0.0034	0.152	−1.32
10582310	<i>Mvd</i>	Mevalonate (diphospho) decarboxylase	0.00017	0.066	−1.31
10403743	<i>Inhba</i>	Inhibin beta-A	0.0081	0.197	−1.30
10585794	<i>Cyp11a1</i>	Cytochrome P450, family 11, subfamily a, polypeptide 1	0.0226	0.289	−1.28

−3.96-fold), and steroid 5 α -reductase type 1 (*Srd5a1*, −2.23-fold). Other non-steroidogenic targets found to be significantly downregulated included the T-box transcription factor eomesodermin (*Eomes*, −2.57-fold), pyruvate carboxylase (*Pcx*, −2.05-fold) involved in energy metabolism, kit ligand (*Kitl*, −1.56-fold), the protease cathepsin D (*Ctsd*, −1.49-fold), and two subunits of the inhibin hormone family (*Inha* and *Inba*; −1.76 and −1.3-fold respectively).

A subset of genes involved in steroidogenesis that were found to be significantly downregulated from the microarray analysis was validated by qPCR (Fig. 4). All genes examined exhibited the same pattern of significantly reduced expression as was observed from the Affymetrix Gene Chip screen, indicating that these targets are very sensitive to modulation of GATA4 levels in the MA-10 cell line. The downregulation of gene expression in response to GATA4 was further confirmed at the protein level by western blotting analysis; two representative targets, CYP11A1 and STAR, are shown in Fig. 5. Quantitation of the western blots showed that the decreases observed at the mRNA level were extended to the protein level. The significant drop in STAR (Fig. 5B), and especially CYP11A1 (Fig. 5A) protein level, was likely an important contributing factor to the crippling effect that GATA4 knockdown had on the ability of MA-10 cells to produce progesterone. As previously mentioned, two different GATA4 siRNAs were used in the microarray and qPCR analyses to reduce the likelihood of the observed changes in gene expression being due to off-target effects. To further demonstrate that the GATA4 siRNAs were indeed targeting GATA4-dependent functions, we transfected MA-10 cells with

control siRNA or siRNA against GATA4 along with either a −902 bp *Star*-luciferase reporter, a highly GATA-sensitive reporter (Tremblay & Viger 2001), or a similar reporter harboring a mutated GATA (GATA to GGTA) responsive element. As shown in Fig. 6, only the WT *Star* promoter (containing an intact GATA motif) exhibited a significant decrease in activity in a context of siRNA-mediated *Gata4* inhibition. Given the dramatic reductions in gene expression at several levels of the steroidogenic pathway, we expected GATA4 knockdown to severely compromise the ability of MA-10 cells to produce progesterone, their main steroid product (Ascoli 1981). Indeed as shown in Fig. 7, transfection of MA-10 cells under the same conditions used to generate the microarray results, markedly reduced the capacity of the cells to produce progesterone. This decrease was observed under basal conditions and in response to forskolin treatment, which mimics gonadotropin-stimulation and dramatically upregulates the amount of progesterone produced by the cells.

Genes modulated in response to GATA4 knockdown may either be direct targets for GATA4 transcriptional regulation at the level of the corresponding promoter regulatory sequences or indirectly regulated by GATA4. For some of the genes we identified, such as *Star*, *Cyp11a1*, and *Inha*, direct transcriptional by GATA4 is very likely given that *in vitro* transcription studies have previously identified these genes as potential GATA4-regulation targets (Silverman *et al.* 1999, Wooton-Kee & Clark 2000, Tremblay & Viger 2001, Robert *et al.* 2006, Sher *et al.* 2007). Our microarray analysis, however, identified additional novel steroidogenic targets (*Srd5a1* and *Amhr2*) that may also be direct transcriptional

Figure 4 Quantitative PCR validation of steroidogenic target genes identified in the microarray analysis following GATA4 knockdown. Global detection of the different genes of interest, using oligonucleotide primers corresponding to the respective coding regions, was measured in four different independent experiments ($n=4$) by qPCR. Quantitative data were normalized using the housekeeping *Rpl19* gene and expressed as the mean $\pm$ S.E.M. Asterisks indicate significant differences between the control and GATA4 siRNA groups ($P<0.05$). *Amhr2*, anti-Müllerian hormone type 2 receptor; *Cyp11a1*, cholesterol side-chain cleavage enzyme; *Gsta3*, glutathione *S*-transferase alpha 3; *Hsd3b1*, hydroxy- Δ^5 -steroid dehydrogenase, 3 β - and steroid Δ^4 -isomerase 1; *Hsd3b6*, hydroxy- Δ^5 -steroid dehydrogenase, 3 β - and steroid Δ^4 -isomerase 6; *Inha*, inhibin alpha; *Srd5a1*, steroid 5 α -reductase 1; *Star*, steroidogenic acute regulatory protein.

targets for GATA4 in Leydig cells. To test this possibility, the -1.3 and -1.6 kb proximal promoter regions of the rat *Srd5a1* and mouse *Amhr2* genes, respectively, were transfected in MA-10 cells to assess their responsiveness to GATA4 stimulation (Fig. 8). The mouse *Amhr2* proximal promoter has two perfect GATA motifs at nucleotide positions $-1172/-1167$ bp and $-221/-216$ bp, whereas the rat *Srd5a1* promoter has a single consensus GATA element at position $-1079/-1074$ bp and two imperfect motifs at nucleotide positions $-1036/-1030$ bp and $-685/-679$ bp. In these experiments, the -902 bp mouse *Star* promoter (Fig. 8A), a previously described GATA4 target (Tremblay & Viger 2001), was used as a positive control. When transfected in MA-10 cells, the *Srd5a1* and *Amhr2* promoters both showed a significant activation in response to GATA4

transactivation in comparison to their respective minimal promoters lacking GATA-responsive elements (Fig. 8B and C), suggesting that they may also be direct targets for GATA4. This was assessed by testing the GATA responsiveness of the corresponding promoter constructs containing mutated GATA motifs. Mutation of the *Amhr2* promoter proximal GATA motif ($-1172/-1167$ bp) attenuated GATA-mediated activation indicating that the *Amhr2* gene can be a direct target for GATA4. However, activation of the *Srd5a1* promoter is most likely indirect or triggered by its imperfect GATA motifs because mutation of the lone consensus GATA binding motif had no significant effect.

Discussion

The GATA4 transcription factor plays a pivotal role in the development and function of multiple tissues originating from both mesoderm and endoderm. Among these

Figure 5 GATA4 knockdown in MA-10 cells decreases STAR and CYP11A protein levels. Western blotting analysis was performed using MA-10 total cell extracts from both experimental conditions as indicated. Proteins were separated by SDS-PAGE, transferred to PVDF membrane, and immunodetected using antisera specific for (A) CYP11A1 or (B) STAR. Alpha-TUBULIN was used as a loading control. Densitometry was performed using ImageJ Software. Quantitative data indicate the average of four experiments $\pm$ S.E.M. Asterisks indicate significant differences between the control and GATA4 siRNA groups ($P<0.05$).

Figure 6 Knockdown of endogenous *Gata4* expression in MA-10 cells diminishes *Star* promoter activity. MA-10 cells were transfected with the -902 bp mouse *Star* promoter construct with either intact GATA motifs or carrying a mutation in the GATA binding site (GATA to GGTA). Cells then received either control siRNA or siRNA specific for GATA4. Promoter activities are expressed as fold change relative to the control siRNA group. The results report the means of four experiments $\pm$ S.E.M. An asterisk indicates a significant difference between the control and GATA4 siRNA groups ($P < 0.05$). Note that GATA4 knockdown only affects the activity of the *Star* promoter construct containing an intact GATA motif.

tissues, *Gata4* is highly expressed in the somatic cell lineages of gonads in both sexes from the onset of gonadal morphogenesis through adulthood (reviewed in Viger *et al.* (2008) and Zaytouni *et al.* (2011)). Consistent with this expression pattern, different genetic mouse models have begun to illuminate the complex and essential roles played by this transcription factor in the very first steps of gonadal development and later, for male and female fertility (Kyrölahti *et al.* 2011a,b, Manuylov *et al.* 2011, Bennett *et al.* 2012, Efimenko *et al.* 2013, Hu *et al.* 2013, Tevosian 2014). While these studies clearly established the essential nature of GATA4 in reproduction, understanding how GATA4 accomplishes these tasks, i.e., identifying its target genes and thus its mechanism of action, remains incomplete. In this study, we took advantage of the MA-10 Leydig cell line and an siRNA knockdown strategy to generate cells that are deficient in GATA4. Microarray analysis of these cells allowed us to show that the essential role of GATA4 in male reproductive function is likely achieved in part by its important contribution to the expression of several key genes involved in Leydig cell cholesterol metabolism and steroidogenesis.

While GATA4 has long been proposed to be a key regulator of steroidogenesis, formal proof is still lacking. Early insights into this role arose from the *in vitro* characterization of promoters of steroidogenic genes that GATA4 has been proposed to regulate (reviewed in Tremblay & Viger (2003a), Viger *et al.* (2004 and 2008), King & LaVoie (2012), Bouchard *et al.* (2005), Kwintkiewicz *et al.* (2007), Sher *et al.* (2007), Shi *et al.* (2009) and Shih *et al.* (2011)). To date, some supporting evidence for the potential *in vivo* requirement of GATA4 in steroidogenesis has come from conditional *Gata4*-knockout mice studies in the ovary. Kyrölahti *et al.* (2011b) were the first to report that gonadotropin-stimulated ovaries from heterozygous mice carrying a

single *Gata4* null allele, or from mice in which *Gata4* was deleted using an *Amhr2*-Cre driver, expressed less *Star*, *Cyp11a1*, and *Cyp19a1* mRNA. It remained unclear, however, whether this decrease was due to down-regulated transcription of the corresponding genes or whether it was due to the presence of fewer maturing follicles. Moreover, this model did not provide insights into GATA4 in Leydig cells as the *Amhr2*-driven Cre did not eliminate *Gata4* expression in the testicular interstitium (Kyrölahti *et al.* 2011a). In another study, Bennett *et al.* (2012) showed that conditional inactivation of *Gata4*, and especially *Gata4* and *Gata6* in the ovary, leads to decreased *Cyp11a1* and *Cyp19a1* expression. But again, it was unclear whether these changes were a consequence of decreased gene expression or a reflection of disrupted folliculogenesis. By contrast, our present result using GATA4-depleted MA-10 cells confirms that GATA4 is a key regulator of *Star* and *Cyp11a1* expression in Leydig cells. Interestingly, our identification of genes modulated in response to GATA4 knockdown in MA-10 cells was not limited to these two steroidogenic targets. We also observed a significant decrease in other components of the steroidogenic pathway including two 3 β -HSD isoforms (*Hsd3b1* and *Hsd3b6*), as well as a dramatic decrease in expression of *Gsta3* that encodes a glutathione transferase that catalyzes obligatory double-bond isomerizations of steroid precursors, leading to both progesterone and testosterone formation in steroidogenic tissues (Johansson & Mannervik 2001, Raffalli-Mathieu *et al.* 2008, Matsumura *et al.* 2013). These results therefore support a role for GATA4, much like it has been described for NR5A1 (Parker *et al.* 2002), as a transcriptional regulator that sits at the top of the steroidogenic program in Leydig cells.

Mouse genetic models offering direct insights into the contribution of GATA4 to steroidogenic gene expression in the testis have been difficult to interpret because of the

Figure 7 GATA4 knockdown attenuates (A) basal and hormone-stimulated (B) steroidogenesis in MA-10 cells. Accumulation of progesterone in serum free media was measured by ELISA after 4 h treatment with or without forskolin (FSK). Results indicate the means of four independent experiments expressed as nano gram of progesterone per milliliter of media $\pm$ S.E.M. Asterisks indicate significant differences between the control and GATA4 siRNA groups ($P < 0.05$).

Figure 8 GATA4 directly activates the promoters of newly identified GATA4-regulated target genes in MA-10 Leydig cells. MA-10 cells were transfected with the (A) -902 bp *Star* promoter-luciferase reporter, a known GATA4 target (Tremblay & Viger 2001), as well promoter-luciferase sequences for (B) the mouse *Amhr2* (-1.6 kb), and (C) rat *Srd5a1* (-1.3 kb) genes, two new GATA4 targets identified through the GATA4 knockdown analysis. Matching minimal promoter sequences (lacking GATA motifs), or promoter sequences containing mutated GATA consensus motifs, were generated to evaluate the direct GATA responsiveness of the promoters. All transfections were done in the presence of 50 ng of a GATA4 expression plasmid. Promoter activities are reported as fold change elicited by the presence of GATA4; the dotted line indicates the baseline value of 1 (no change). Data report the means of four independent $\pm$ S.E.M. Asterisks indicate significant differences ($P < 0.05$).

lack of reliable Cre-drivers allowing specific *Gata4* gene deletion solely in Leydig cells, especially in the mature testis. Nonetheless, Manuylov *et al.* (2011) have described the testicular phenotype of mice where *Gata4*

was excised in both Leydig and Sertoli cells early during fetal development using Cre recombinase driven by *Nr5a1* regulatory sequences (GATA4^{SF} XY mice). While these mice exhibit profound defects in cord morphogenesis due to a disruption of Sertoli cell expression of *Dmrt1*, GATA4^{SF} XY mice were also described as being undervirilized males with partially descended testes, hypoplastic penis, mammary teats, and a relatively shorter anogenital distance compared with their control counterparts (Manuylov *et al.* 2011). Cumulatively, these features are suggestive of decreased androgen exposure or androgen action during fetal development. Our present data showing an important role for GATA4 in MA-10 cell Leydig cell gene expression and steroid production supports these observations.

In addition to *Star* and *Cyp11a1*, our microarray analysis of MA-10 cells with reduced *Gata4* expression identified other interesting downregulated targets associated with steroidogenesis. As mentioned previously, this included two 3 β -HSDs, *Hsd3b1* and *Hsd3b6*, that encode essential enzymes required for progesterone production in steroidogenic tissues (reviewed in Simard *et al.* (2005)). In agreement with this finding, we have previously shown the promoter of the *HSD3B2* gene, the human equivalent of the mouse *Hsd3b1* gene, to be highly upregulated in response to GATA4 transactivation, especially in cooperation with the nuclear receptor NR5A1 (Martin *et al.* 2005). GATA4 has also been reported to be required for placental-specific activity of the promoter of the *HSD3B1* gene (Peng *et al.* 2004), the human equivalent of the rodent *Hsd3b6* gene. Apart from classical regulators of steroidogenesis, GATA4-deficient MA-10 cells also expressed less *Amhr2* mRNA, encoding the type 2 receptor for anti-Müllerian hormone and *Srd5a1* mRNA, encoding one of the three 5 α -reductase isoforms involved in the production of 5 α -reduced C-19 and C-21 steroids (Azzouni *et al.* 2012). The AMH/AMHRII system has profound effects on Leydig cell physiology (Teixeira *et al.* 2001). Activation of this pathway blocks Leydig cell differentiation and represses steroidogenesis, especially at the level of CYP17 (Racine *et al.* 1998). We showed that the rat -1.6 *Amhr2* promoter sequence, which contains two consensus GATA motifs, can be activated by GATA4 in MA-10 cells (Fig. 8B). Others have reported that the *Amhr2* promoter is also regulated by NR5A1 and WT1 (Teixeira *et al.* 1999, Hossain & Saunders 2003, Klattig *et al.* 2007). Interestingly, we have shown these two factors synergize with GATA4 on multiple gonadal promoters including *Sry*, *Cyp19a1*, *HSD3B2*, and *Inha* (Tremblay & Viger 2001, Bouchard *et al.* 2005, Martin *et al.* 2005, Miyamoto *et al.* 2008). A similar regulation might therefore also exist at the level of the *Amhr2* promoter in Leydig cells. Steroid 5 α -reductase type 1 is most prominently expressed in prostate, skin, liver, and sebaceous glands where it converts testosterone to the more potent androgen, 5 α -dihydrotestosterone (DHT; Azzouni *et al.* 2012). It is also found in Leydig cells, especially fetal Leydig cells and

progenitor and immature adult Leydig cells in rodents (Viger & Robaire 1995). Interestingly, the *Srd5a1* gene was markedly downregulated in GATA4-deficient MA-10 cells (Fig. 4), and its proximal promoter region could be directly transactivated by GATA4 (Fig. 8). DHT has been reported to be important for the testosterone-dependent restoration of spermatogenesis in contexts of experimental androgen withdrawal (O'Donnell *et al.* 1996, 1999), and possibly for the initiation of male germ development at puberty (Killian *et al.* 2003). As a potential regulator of testicular *Srd5a1* expression, GATA4 might be an equally important participant in these processes. GATA-dependent regulation might also be important in the regulation of *Srd5a1* expression in extra-gonadal tissues where GATA factors and SRD5A1 are co-expressed such as the skin and prostate (Perez-Stable *et al.* 2000, Kaufman *et al.* 2003, Azzouni *et al.* 2012).

Steroid hormones are derived from cholesterol. The steroidogenic capacity of a cell is therefore governed not only by regulating the enzymes that act upon cholesterol to convert it into steroids but also by regulating the availability of cholesterol itself. Steroidogenic cells such as Leydig cells use different mechanisms to obtain the cholesterol required for steroidogenesis. This includes uptake of cholesterol bound to circulating lipoproteins, mobilization of cholesterol esters stored in lipid droplets, and *de novo* cholesterol synthesis (reviewed in Miller & Bose (2011)). Interestingly, in addition to steroidogenic genes, GATA4 appears to be an active participant in the control of Leydig cell cholesterol metabolism as several genes (*Scarb1*, *Ldlr*, *Soat1*, *Scap*, and *Cyp51*) controlling different aspects of cholesterol transport and synthesis were found to be downregulated in GATA4-depleted MA-10 cells (Table 3). Downregulated expression of these genes, along with the multiple steroidogenic targets, likely contribute to the significant decrease in MA-10 progesterone production observed in response to GATA4 knockdown (Fig. 7). Although we have yet to ascertain whether these novel cholesterol regulatory genes are direct GATA4 targets, the promoter region of at least one of these genes, *Soat1*, has been previously reported to be a target for the nuclear receptor NR5A1 (Ferraz-de-Souza *et al.* 2011), a factor known to cooperate with GATA4 on a variety of different steroidogenic promoters (Tremblay & Viger 1999).

The reduction in *Gata4* expression in MA-10 cells also led to a significant upregulation of several genes (Table 2). The most highly upregulated genes were *Itgb8* and *Vcan*, encoding for proteins involved in cell-cell interaction and the regulation of cell proliferation and migration. While the expression of one of the genes (*Itgb8*) has been recently shown to be highly enriched in fetal Leydig cells (McDowell *et al.* 2012), the physiological significance of these genes for Leydig cell function and their regulation by GATA4 remain unknown. More interestingly were upregulated genes encoding for transcription factors known for their more

conspicuous participation in steroidogenesis. These included the proto-oncogene JUN, and the nuclear receptors NR2F2 (COUP-TFII) and NR4A2 (NURR1). JUN is a well-characterized regulator of *Star* expression in Leydig cells (Stocco *et al.* 2005); a regulation that appears to involve a synergistic interaction between JUN and GATA4 itself (Martin *et al.* 2012). Homozygous male mice carrying a conditional *Nr2f2* deletion are infertile due to deficits in Leydig cell differentiation and reduced testosterone production (Qin *et al.* 2008). Whether NR2F2 directly targets steroidogenic genes in Leydig cells, however, remains to be shown (Martin & Tremblay 2010). NR4A2 belongs to the NR4A family of nuclear receptors that have been shown to activate several promoters active in Leydig cells including *Star*, *HSD3B2*, *Hsd3b1*, and *Cyp17a1* (Martin & Tremblay 2010). Interestingly, the targets putatively regulated by JUN and NR4A proteins were identified as downregulated targets in our screening of GATA4-deficient MA-10 cells. It is therefore tempting to speculate that upregulation of JUN, NR2F2, and NR4A2 is a compensatory response of MA-10 cells to offset the decrease in steroidogenic gene expression or to increase *Gata4* expression itself in MA-10 cells deficient in GATA4. The demonstration that JUN can directly activate *Gata4* transcription would support at least the latter possibility (Murray *et al.* 2013).

In conclusion, our microarray study of GATA4-depleted MA-10 cells identifies this factor as an essential contributor to Leydig cell steroidogenesis via the regulation of a network of genes intimately involved in cholesterol delivery and the steroidogenic program itself. Given this important role, GATA4 needs to be given close attention as a potential causative factor in human pathologies that are associated with reduced fertility and diminished steroid hormone synthesis.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-14-0369>.

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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