The effect of human *GATA4* gene mutations on the activity of target gonadal promoters

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Abstract

GATA transcription factors are crucial regulators of cell-specific gene expression in many tissues including the gonads. Although clinical cases of reproductive dysfunction have yet to be formally linked to *GATA* gene mutations, they have begun to be reported in other systems. Heterozygous *GATA4* mutations have been associated with cases of congenital heart defects. Little is known, however, about the effect of these mutations on gonadal gene transcription. Since individuals carrying these mutations do not appear to suffer from gross reproductive defects, we hypothesized that this might be due to the differential transcriptional properties of the mutant proteins on heart versus gonadal target genes. Five mutations (S52F, E215D, G295S, V266M, and E359X) were recreated in the rat *GATA4* protein. Several parameters were used to analyze the transcriptional properties of the mutants: activation of known gonadal target promoters (*Star*, *Cyp19a1*, and *Inha*), DNA binding, and interaction with *GATA4* transcriptional partners. Three mutations (S52F, G295S, and E359X) reduced *GATA4* transcriptional activity on the different gonadal promoters. With the exception of the G295S mutant, which showed a significant loss of DNA-binding affinity, the decrease in activity of the other *GATA4* mutants was not associated with a change in DNA binding. All *GATA4* mutants retained their ability to interact and cooperate with their major gonadal partners (NR5A1 and NR5A2) thereby compensating in part for the loss in intrinsic *GATA4* transcriptional activity. Thus, unlike the heart, where the *GATA4* mutations have deleterious effects, our data suggest that they would have a lesser impact on gonadal gene transcription and function.

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Introduction

GATA4 is a member of the *GATA* family of zinc finger DNA-binding proteins that recognizes the consensus DNA motif (A/T) GATA (A/G) in the promoter region of target genes (Molkentin 2000). In the reproductive tract, *GATA4* is prominently expressed in somatic cells of both the testis and ovary, where it has been shown and/or proposed to regulate the transcription of numerous target genes (Viger *et al.* 2008). These targets include genes coding for hormones or components of the steroidogenic pathway such as anti-Müllerian hormone (*Amh*), inhibin subunit α (*Inha*), steroidogenic acute regulatory protein (*Star*), P450 aromatase (*Cyp19a1*), P450 side chain cleavage (*Cyp11a1*), P450 17 α -hydroxylase/17,20 lyase (*Cyp17a1*), and 3 β -hydroxysteroid dehydrogenase/ Δ^5 – Δ^4 isomerase type 2 (*HSD3B2*; Viger *et al.* 2008). In gonadal cells, *GATA4* activity has been shown to be modulated via cooperative interactions with other transcriptional regulators and/or cofactors (Viger *et al.* 2008). These include the

nuclear receptors steroidogenic factor 1 (SF-1/NR5A1) and liver receptor homolog 1 (LRH-1/NR5A2), which cooperate with *GATA4* to enhance transcription from the promoters of many of the *GATA4* targets listed above (Viger *et al.* 2008). *GATA4* transcriptional activity can also be modulated by post-translational modifications such as phosphorylation (Viger *et al.* 2008). Activation of the cAMP/PKA signaling pathway is a major mechanism for conveying the hormone responsiveness of many gonadal genes (Richards 1994). *GATA4* can be directly phosphorylated by PKA resulting in a dramatic enhancement of its transactivation potential on many *GATA4*-dependent gonadal promoters (Tremblay *et al.* 2002, Tremblay & Viger 2003a).

While human *GATA* mutations affecting normal reproductive function have yet to be identified, *GATA* gene sequence variations have begun to be linked to human diseases in other systems. For example, *GATA1* mutations have been reported in inherited anemia and thrombocytopenia (Nichols *et al.* 2000), as well as in Down's syndrome-related acute leukemia (Wechsler

et al. 2002). Although no mutation of the *GATA2* gene has yet been demonstrated in hematological diseases, it has been reported that decreased *GATA2* expression is observed in CD34 positive cells in patients with aplastic anemia (Fujimaki *et al.* 2001). *GATA3* mutations have been associated with HDR syndrome, a genetic disease characterized by hypoparathyroidism, sensorineural deafness, and renal dysplasia (Van Esch *et al.* 2000). *GATA6* frameshift mutations in the DNA-binding domain and C-terminus have been reported to cause malignant astrocytomas (Kamnasaran *et al.* 2007).

There have now been reports of several *GATA4* gene mutations affecting cardiac development and function (Garg *et al.* 2003, Okubo *et al.* 2004, Hirayama-Yamada *et al.* 2005, Nemer *et al.* 2006, Tang *et al.* 2006). Most of these mutations occur in amino acid residues highly conserved across species ranging from zebrafish to human, underlying the relative importance of these residues for proper *GATA4* function (the characteristics of some of these *GATA4* mutations are summarized in Table 1). A missense mutation, G296S, causes a decrease in *GATA4* transcriptional activity by weakening its DNA-binding capacity and by disrupting the cooperative interaction with its transcriptional partner TBX5, ultimately leading to reduced expression of target genes in the developing heart (Garg *et al.* 2003). Another missense mutation, E216D, also causes a reduction in *GATA4* transcriptional activity although the DNA-binding ability of the protein and its physical (protein–protein) and functional (transcriptional) interaction with Friend of *GATA 2* (FOG2) on cardiac targets are not apparently affected (Nemer *et al.* 2006). A third mutant, S52F, has also been reported to reduce *GATA4* transactivation potential, although its DNA-binding capacity remains intact (Hirayama-Yamada *et al.* 2005). Similarly, two frameshift mutations, causing the loss of a large portion of the C-terminal end of *GATA4*, S358X (Okubo *et al.* 2004), and E359X (Garg *et al.* 2003), appear to be transcriptionally inactive although the molecular interactions with DNA and/or other proteins

have not been investigated. Additional *GATA4* mutations have been described: V267M and V380M amino acid substitutions of *GATA4* protein have also been related to heart defects (Tang *et al.* 2006), but the molecular basis for the cardiac phenotypes have yet to be established.

To date, human *GATA4* mutations have been identified based on their deleterious effects on heart development and function. The impact of these mutations on *GATA4* function outside of the cardiac system has not been investigated. This includes the reproductive tract where *GATA4* has been shown to play a critical role in early gonadal morphogenesis, at least in the mouse (Tevosian *et al.* 2002, Bouma *et al.* 2007, Manuylov *et al.* 2007). In the present study, we have recreated five human *GATA4* mutations (listed in Table 1), known to affect heart function, in the rodent protein and tested their functional properties – DNA-binding capacity, transactivation potential, physical and functional interaction with known regulatory partners – on a variety of *GATA4*-dependent gonadal promoters. Our results show that *GATA4*-dependent activation of different gonadal promoters can be significantly affected by some of these mutations. However, the different *GATA4* mutants retained their ability to interact and cooperate with their major gonadal partners thereby compensating in part for the loss in intrinsic *GATA4* transcriptional activity. Thus, unlike the heart where these *GATA4* mutations have major deleterious effects, our data suggest that they would have less dramatic consequences on gonadal gene transcription and function.

Materials and methods

Plasmids

The murine –902 bp (*Star*), –218 bp PII aromatase (*Cyp19a1*), and –679 bp inhibin α (*Inha*) promoter constructs have been described previously (Tremblay & Viger 2001). The wild-type *GATA4* expression vector has

Table 1 Characteristics of the *GATA4* mutations studied

	Rodent equivalent	Nucleotide change	Gene region	Protein region	Associated human defect(s)	References
Mutation						
S52F	S52F	c.155C>T	Exon 2	TAD1	ASD	Hirayama-Yamada <i>et al.</i> (2005)
E216D	E215D	c.648C>G	Exon 3	NZf region	TOF	Nemer <i>et al.</i> (2006)
V267M	V266M	c.799G>A	Exon 4	CZf region	CHD	Tang <i>et al.</i> (2006)
G296S	G295S	c.886G>A	Exon 4	CZf-NLS gap	ASD, AR, AVSD, MR, PDA, PS, PVS, VSD	Garg <i>et al.</i> (2003) and Sarkozy <i>et al.</i> (2005)
E359X (frame-shift truncation)	E359X (rat) E360X (mouse)	c.1075delG	Exon 6	C-ter	ASD, dextrocardia	Garg <i>et al.</i> (2003)

AR, aortic regurgitation; ASD, atrial septal defect; AVSD, atrioventricular septal defect; CHD, congenital heart defect; CZf, C-terminal zinc finger; MR, mitral regurgitation; NLS, nuclear localization signal; NZf, N-terminal zinc finger; PDA, patent ductus arteriosus; PS, pulmonary stenosis; PVS, pulmonary valve stenosis; VSD, ventricular septal defect; TAD1, transcriptional activation domain 1; TOF, tetralogy of Fallot.

also been reported previously (Tremblay *et al.* 2001). The different GATA4 human mutations were recreated using the rat GATA4 coding sequence by site-directed mutagenesis. The mutations were introduced according to the manufacturer's instructions using a QuikChange XL mutagenesis kit (Stratagene, La Jolla, CA, USA) and the oligonucleotide primers indicated in Table 2. Expression plasmids for mouse NR5A1 (Lala *et al.* 1992), NR5A2 (Galarneau *et al.* 1996), and PKA catalytic subunit (Mayr & Montminy 2001) were kindly provided by Drs Keith Parker (University of Texas Southwestern Medical Center, Dallas, TX, USA), Luc Bélanger (Centre de recherche en cancérologie, Centre de recherche du CHUQ, Université Laval, Québec City, Canada), and Marc Montminy (The Salk Institute for Biological Studies, La Jolla, CA, USA) respectively.

Cell culture and transfections

African green monkey kidney CV-1 cells and mouse TM3 Leydig cells were used for all transfection studies. As a heterologous line (not expressing GATA or NR5A factors), CV-1 cells are a very convenient line for comparing the transcriptional properties of the different GATA4 mutants presented herein. TM3 cells express endogenous GATA proteins and are representative of a gonadal somatic cell line (Tremblay & Viger 2001). CV-1 cells were grown in DMEM supplemented with 10% newborn calf serum. TM3 cells were cultured in a 1:1 mixture of Ham's F12 and DMEM containing 5% horse serum and 2.5% FBS. HeLa cells, used to overexpress the different GATA4 mutants for western analysis, were grown in DMEM/F12 supplemented with 10% fetal bovine serum. All transfections were done in 24-well plates using the calcium phosphate precipitation method (Chen & Okayama 1987). Cells were plated at an initial density of 2×10^4 cells/well and transfected 24 h later. Culture medium was changed 12–16 h after transfection, and the cells were finally harvested the following day by lysing the cells in 50 µl lysis buffer

(100 mM Tris-HCl (pH 7.9), 0.5% Igepal (Sigma-Aldrich Oakville, Canada), and 5 mM dithiothreitol). An aliquot of the lysate was then assayed for luciferase activity using a Luminoskan Ascent microplate luminometer (Fisher Scientific Limited, Ottawa, Canada) and luciferine (BD Biosciences, Mississauga, Canada) as substrate. In all experiments, the total amount of DNA was kept constant at 2 µg/well using Sp64 (Promega Madison, WI, USA) as carrier DNA. Data reported represent the average of at least three experiments (range of 3–15), each done in duplicate.

Nuclear extracts and western blot

Nuclear extracts were prepared by the procedure outlined by Schreiber *et al.* (1989). In western analyses, 30 µg aliquots of nuclear extracts from HeLa cells overexpressing GATA4 (wild-type or mutated) were separated by SDS-PAGE then electrotransferred to Hybond polyvinylidene difluoride membrane (GE Healthcare Life Sciences, Baie d'Urfé, Canada). GATA4 proteins were detected using a commercially available GATA4 polyclonal antibody (# sc-1237X, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a VECTASTAIN-ABC-Amp Western blot detection kit (Vector Laboratories Canada, Burlington, Canada).

Electrophoretic mobility shift assay

Recombinant GATA4 wild-type and mutated proteins were *in vitro* translated using a QuickCoupled TNT *in vitro* transcription and translation system (Promega). Electrophoretic mobility shift assays (EMSA; DNA-binding) were performed using a 32 P-labeled double-stranded oligonucleotide corresponding to the conserved GATA element of the proximal murine *Star* promoter (Tremblay & Viger 2001). The oligos used were (GATA site is italicized) – sense: 5'-GATC-CAC TTTT *TTATCTCAAGTGA*-3', antisense: 5'-GATCT-CAC TTGAGATAAAAAAGTG-3'. Binding reactions

Table 2 Primers used to generate the different GATA4 mutants

	Sense (S)/antisense (A)	Sequence ^a
Mutation		
S52F	S	5'-GTG CTG GGC CTG T TC TAC CTG CAG GGC G-3'
	A	5'-C GCC CTG CAG GTA GAA CAG GCC CAG CAC-3'
E215D	S	5'-C TCA GAA GGC AGA GAC TGT GTC AAC TGC GGG-3'
	A	5'-CCC GCA GTT GAC ACA GTC TCT GCC TTC TGA G-3'
V266M	S	5'-T GCC TCC CGC CGG ATG GGC CTC TCC TGT G-3'
	A	5'-C ACA GGA GAG GCC CAT CCG GCG GGA GGC A-3'
G295S	S	5'-CCT GTT TGC AAT GCC TGC AGC CTC TAC ATG AAG CTC-3'
	A	5'-GAG CTT CAT GTA GAG GCT GCA GGC ATT GCA AAC AGG-3'
E359X	S	5'-C AGC AGC AGC AGT *AAG AGA TGC GCC CCA T-3'
	A	5'-A TGG GGC GCA TCT CTT *ACT GCT GCT GCT GCT G-3'

The position of the deletion is indicated by an asterisk (*).

^aMutated nucleotides are italicized.

were done using 2 µl of *in vitro* translated protein in 20 µl buffer (4 mM Tris-HCl (pH 7.9), 24 mM KCl, 0.4 mM EDTA (pH 8.0), 0.4 mM dithiothreitol, 5 mM MgCl₂, 10% glycerol, and 100 ng polydeoxyinosinideoxycytidylic acid) for 1 h at 4 °C. GATA4-containing complexes were subsequently analyzed by electrophoresis through a 4% non-denaturing polyacrylamide gel in 0.5× Tris-borate-EDTA buffer at 200 V for 1.5 h at 4 °C followed by autoradiography.

Production of histidine fusion proteins

Recombinant histidine (HIS)-NR5A1 and HIS-NR5A2 fusion proteins were obtained by cloning the corresponding coding sequences in frame with HIS using the commercially available pRSETB fusion protein vector (Invitrogen, Burlington, Canada). The resulting constructs were introduced into the *Escherichia coli* strain BL21, and the fusion proteins were produced by inducing the bacterial cultures with isopropyl-1-thio-β-galactopyranoside. After the induction, the bacterial cultures were lysed by sonication and the fusion proteins were purified using a Talon metal affinity resin (BD Biosciences) according to instructions outlined by the manufacturer.

In vitro pull-down (protein-protein interaction) assay

Protein-protein interaction studies were done using ³⁵S-labeled *in vitro* translated wild-type or mutated GATA4 proteins, and the purified HIS-NR5A1, HIS-NR5A2, or HIS-β-galactosidase (HIS-LacZ) fusion proteins coupled to a Talon metal affinity resin (BD Biosciences). The ³⁵S-labeled GATA4 proteins were obtained using the TNT system from Promega. The pull-down procedure has been described previously (Martin *et al.* 2005).

Statistical analysis

Statistical comparisons between multiple groups (Figs 2A, 4A and B, and 5A and B) were analyzed by one-way ANOVA followed by a Student-Newman-Keuls test. Where normality and/or equal variance among groups was not met, data were analyzed with equivalent (Kruskal-Wallis ANOVA/Student-Newman-Keuls) non-parametric tests. *P* < 0.05 was considered significant. All statistical analyses were done with the aid of the SigmaStat 3.5 software package (Systat Software Inc., Point Richmond, CA, USA).

Results

The GATA4 mutants have reduced transcriptional activity on gonadal promoters

To investigate, the effect of human *GATA4* mutations reported to affect heart development and function on the

transcriptional regulation of gonadal target genes; we recreated five mutations in context of the highly conserved rat orthologue (Fig. 1 and Table 1). The different GATA4 mutants were first compared with the wild-type protein for their ability to transactivate three known GATA-responsive gonadal promoters (*Star*, *Cyp19a1*, and *Inha*) in heterologous CV-1 fibroblasts that lack endogenous GATA4 expression and TM3 mouse Leydig cells that express endogenous GATA proteins (Fig. 2A). Much like their action on heart-specific promoters (Garg *et al.* 2003, Okubo *et al.* 2004, Hirayama-Yamada *et al.* 2005, Nemer *et al.* 2006, Tang *et al.* 2006), the GATA4 G295S, E359X, and S52F mutations also markedly reduced the ability of the GATA4 protein to transactivate the three gonadal promoters in CV-1 cells (Fig. 2A). This effect was intrinsic to the mutant proteins since the mutations themselves did not affect the expression level or the cellular localization of the overexpressed proteins (Fig. 2B). In contrast to these three mutations however, the E215D and V266M mutants retained significant transcriptional activity (Fig. 2A). For the E215D mutant, its transactivation potential was equivalent to the wild-type GATA4 protein. Similar results were also observed in TM3 cells; transcriptional activities were correspondingly lower, however, due to the presence of existing GATA factors in these cells.

DNA-binding affinity of the GATA4 mutants

The reduced transcriptional activity observed for some of the GATA4 mutants suggested that the amino acid modifications involved might directly affect GATA4 DNA binding. To study this possibility, DNA binding of wild-type and mutant GATA4 proteins on the conserved GATA element of the murine *Star* promoter was compared by EMSA (Fig. 3). Consistent with the initial study characterizing the binding of the mouse orthologue of the human G296S GATA4 mutant protein on a heart-specific promoter GATA element (Garg *et al.* 2003), the rodent G295S mutant also showed markedly reduced binding compared with the wild-type GATA4 protein on a typical gonadal promoter GATA element (Fig. 3A, left panel). Weak binding of the G295S mutant was still observed even at a fivefold excess of protein (Fig. 3A, right panel), suggesting that impaired DNA binding contributes to the reduced transcriptional activity of this particular mutant. In contrast to the G295S variant, the other GATA4 mutants (E215D, S52F, V266M, and E359X) showed strong DNA binding reminiscent of the wild-type protein (Fig. 3B).

PKA-dependent enhancement of GATA4-mediated transactivation

We have previously reported that the transcriptional activity of GATA4 in gonadal cells is enhanced by

Figure 1 Amino acid positions of five known GATA4 heterozygous point mutations associated with human disease. (A) Structure and amino acid sequence of the human GATA4 protein. The locations of the various mutations recreated in this study (S52, E216, V267, G296, and E359) are indicated by black circles. (B) The mutated GATA4 amino acid residues (black boxes) are conserved between human and rodent species. The E359X mutation produces a frameshift truncation; the modified sequence is indicated in bold italic, above and downstream of the mutation. The rodent equivalents of the different GATA4 human mutations are provided in Table 1.

cAMP/PKA-mediated phosphorylation and that this enhancement is an important mechanism for up-regulating expression of certain hormone-dependent gonadal target genes (Tremblay *et al.* 2002, Tremblay & Viger 2003a). We therefore surmised that mutations in GATA4 might impair its ability to respond to PKA

stimulation. To test this possibility, PKA catalytic subunit was co-transfected with wild-type GATA4 or the different GATA4 mutants (Fig. 4). PKA enhancement of GATA4 activity was assessed on both the *Star* (Fig. 4A) and *Cyp19a1* (Fig. 4B) promoters. As expected, PKA markedly enhanced wild-type GATA4

Figure 2 The various *GATA4* mutations differentially affect the transcriptional properties of the *GATA4* protein on different gonadal promoters. (A) The ability of the wild-type (WT) and mutant *GATA4* proteins to transactivate the murine *Star*, aromatase (*Cyp19a1*), and inhibin α (*Inha*) promoters when overexpressed in CV-1 fibroblasts or TM3 mouse Leydig cells. For each cell line, cells were co-transfected with the indicated luciferase reporters along with either an empty expression vector (serving as control) or expression vectors (50 ng) for the different *GATA4* proteins as indicated. All promoter activities are reported as fold activation over control ($\pm$ s.e.m.). Groups with similar letters are not significantly different from one another ($P > 0.05$). (B) HeLa cells were transfected with expression vectors encoding WT *GATA4* or the different mutants. Western blot analysis shows that all proteins were similarly expressed in isolated nuclear extracts.

activity on both promoters in both CV-1 and TM3 cells. Interestingly, PKA overexpression still resulted in a significant enhancement of the activity of most, if not all, of all the *GATA4* mutant proteins (Fig. 4A and B). For some of the mutants, PKA-mediated enhancement was even greater than the wild-type *GATA4* protein. In CV-1 cells, this was particularly evident for the S52F, G295S, and E359X proteins where transcriptional activity in the absence of PKA was very low. Moreover, PKA was able to raise the activity of these three mutants to the level of the unstimulated wild-type *GATA4* protein.

Functional cooperation between *GATA4* and its transcriptional partners NR5A1 and NR5A2

The nuclear receptor NR5A1 has been shown to be an important transcriptional partner for *GATA* factors in enhancing the promoter activities of several genes normally expressed in endocrine cells (Viger *et al.* 2008). More recent studies have extended these findings to include the related factor NR5A2 (Bouchard *et al.* 2005, Martin *et al.* 2005, Robert *et al.* 2006). We therefore tested whether the different

Figure 3 DNA-binding properties of the GATA4 mutants. Electrophoretic mobility shift assays were used to assess the binding of *in vitro* produced WT or mutated GATA4 proteins to a double-stranded ^{32}P -labeled oligonucleotide corresponding to the consensus GATA element (nucleotide position –63 bp) of the murine *Star* promoter. (A) Left panel: the G295S mutation reduces DNA binding. Binding of GATA4 WT and G295S mutant proteins are competed with increasing doses (solid triangle) of unlabeled oligonucleotides (molar excesses of 2 $\times$, 5 $\times$, and 10 $\times$). Right panel: the GATA4 G295S mutant shows decreased DNA binding, even at a concentration that is five times higher than that of the WT GATA4 protein. (B) The other GATA4 mutants (E215D, S52F, V266M, and E359X) exhibit DNA-binding capacities similar to the WT GATA protein. The solid triangles indicate increasing amounts (2 $\times$, 5 $\times$, and 10 $\times$) of unlabeled (self) competitor oligonucleotides.

GATA4 mutations could disrupt the functional synergism between GATA4 and NR5A1 (Fig. 5A) or between GATA4 and NR5A2 (Fig. 5B) on the *Cyp19a1* promoter. The *Cyp19a1* promoter is a well-characterized target for GATA4 and NR5A1 or NR5A2 (Tremblay & Viger 2001, Clyne *et al.* 2002, 2004, Bouchard *et al.* 2005). As expected, NR5A1 and NR5A2 strongly synergized with wild-type GATA4 in both CV-1 and TM3 cells (Fig. 5A and B). In CV-1 cells, synergism was still observed with four out of the five GATA4 mutants with NR5A1 and all mutants with NR5A2. A similar pattern was observed in TM3 cells except for the S52F, G295S, and E359X mutants. Interestingly, the hG296S mutant that was originally shown to be unable to interact with its cardiac-specific partner TBX5 (Garg *et al.* 2003), maintained its ability to functionally cooperate with its partners in the context of gonadal gene transcription, at least in CV-1 cells.

The functional cooperation between GATA4 and NR5A factors requires that the proteins contact each other via a direct protein–protein interaction (Bouchard *et al.* 2005, Martin *et al.* 2005). *In vitro*

pull-down assays were used to assess whether the different GATA4 mutants could still physically interact with either NR5A1 or NR5A2 (Fig. 5C). In these experiments, NR5A1 and NR5A2 are expressed as HIS-tagged fusion proteins immobilized to a metal resin. The different GATA4 proteins are then expressed as ^{35}S -labeled proteins. Labeled protein retained on the resin indicates an interaction which is visualized on an acrylamide gel. As shown in Fig. 5C (upper panels), both the wild-type and G295S GATA4 proteins were retained by the NR5A fusion proteins. A HIS–LacZ fusion protein was used as a non-specific control. Similar experiments were carried out with the remaining GATA4 mutants that showed interactions similar to the wild-type protein (Fig. 5C, lower panel).

Discussion

Initial *in vitro* work characterizing GATA4 target genes in gonadal cells (LaVoie 2003, Tremblay & Viger 2003b, Viger *et al.* 2004), along with subsequent genetic studies in the mouse (Tevosian *et al.* 2002, Bouma *et al.* 2007, Manuylov *et al.* 2007), has provided strong evidence that the GATA4 transcription factor plays a central role in reproductive function. Despite the lack of direct genetic evidence, the expectation is that this critical role extends to human reproductive function. The paucity of data in the literature describing GATA4 mutations associated with human disease is not unexpected given the early developmental roles that have been ascribed to this factor (Kuo *et al.* 1997, Molkentin *et al.* 1997). This notwithstanding, heterozygous GATA4 mutations have been associated with several cases of congenital heart defects (Garg *et al.* 2003, Okubo *et al.* 2004, Hirayama-Yamada *et al.* 2005, Nemer *et al.* 2006, Tang *et al.* 2006). Little is known, however, about the molecular basis for these heart phenotypes and even further whether these mutations could compromise GATA4 function in a gonadal context. In the present study, we report the effect of five different human GATA4 mutations (S52F, E216D, V267, G296S, and E359X) known to affect heart function on the transcriptional properties of GATA4 on target gonadal promoters.

To study the different mutations, GATA4 mutants were recreated in the rat orthologous protein. At the outset, it is unlikely that these mutations have a major detrimental effect in the reproductive organs, as they do in the heart, since individuals carrying these mutations appear to be able to procreate (Garg *et al.* 2003, Okubo *et al.* 2004, Hirayama-Yamada *et al.* 2005, Nemer *et al.* 2006, Tang *et al.* 2006). Absence of gross phenotypic abnormalities, however, does not necessarily preclude an effect at the molecular level. Indeed, in our analysis of the mutations, three GATA4 mutants

Figure 4 The ability of PKA to enhance the transcriptional properties of the GATA4 mutants on different gonadal promoters. CV-1 and TM3 cells were co-transfected with either a *Star* (A) or *Cyp19a1* (B) luciferase reporter along with either an empty expression vector (control) or expression vectors (50 ng) for WT or mutated GATA4 in the presence or absence of PKA catalytic subunit (50 ng). All promoter activities are reported as fold activation over control ($\pm$ S.E.M). Promoter activity stimulated by PKA alone is indicated by the dashed line. For each GATA4 protein tested, an asterisk (*) denotes a statistically significant difference ($P < 0.05$) between the stimulated group (+ PKA) and both the unstimulated group (– PKA) and PKA alone.

(S52F, G295S, and E359X) exhibited markedly weak transcriptional activity, compared with the wild-type protein, on three separate gonadal promoters (Fig. 2A). This effect is similar to what has been demonstrated on cardiac-specific promoters (Garg *et al.* 2003, Schluterman *et al.* 2007). The drop in transactivation potential associated with these mutants was not simply due to the amount of protein present, or to impaired cellular localization, since the GATA4 mutants and wild-type protein were expressed at comparable levels in HeLa cell nuclear extracts (Fig. 2B). Interestingly, the G295S mutant was the only one to show a dramatic reduction in DNA-binding affinity on a gonadal GATA promoter element even when used at concentrations five times that of the wild-type protein (Fig. 3A). These findings are consistent with the previously reported data in the heart (Garg *et al.* 2003), and suggest that the G295S mutant would poorly activate gonadal promoters due to a decrease in DNA binding. The role of the E359X variant remains to be better defined since it is still unclear whether the protein is properly and stably translated in an *in vivo* context. Based on our *in vitro* data, we can assume that the 44 nonsense amino acids added to the C-terminal domain of the prematurely

truncated GATA4 protein adversely affects its function. The S52F mutant had decreased transcriptional activity (Fig. 2A), but fully retained its DNA-binding capacity (Fig. 3B). Similarly, Schluterman *et al.* (2007) recently showed that the S52F mutation had decreased transcriptional activity on the heart-specific *ANF* and α -MHC promoters. They suggested that the mutation at position 52 altered a serine residue within the GATA4 N-terminal activation domain thereby disrupting the function of this region by changing its structure. Thus, conformational changes induced by the mutations likely explain the reduced transcriptional activity of some of the mutant proteins on both cardiac and gonadal target promoters.

Interestingly, the GATA4 E215D mutant had the same level of activity as the wild-type protein on gonadal promoters (Fig. 2A). This is different from the heart where the same mutant had reduced transcriptional activity on the *ANF* promoter without changes in DNA-binding ability or cellular localization of the protein (Nemer *et al.* 2006). These results are not a complete surprise since an engineered GATA4 mutation (E215K), targeting the same amino acid and leading to the loss of interaction between GATA4 and FOG2, also retained

Figure 5 The ability of NR5A family members to transcriptionally cooperate and physically interact with the GATA4 mutants. (A) Cooperation with NR5A1. (B) Cooperation with NR5A2. CV-1 and TM3 cells were co-transfected with the *Cyp19a1* luciferase reporter (which contains binding sites for both GATA and NR5A proteins) along with either an empty expression vector (control) or different combinations of expression vectors for WT or mutated GATA4 (50 ng), NR5A1 (25 ng), and NR5A2 (100 ng) as indicated. All promoter activities are reported as fold activation over control. Promoter activity stimulated by NR5A1 or NR5A2 alone is indicated by the dashed line. For each GATA4 protein tested, an asterisk (*) denotes a statistically significant difference ($P < 0.05$) between the synergism group (gray bar; GATA4 and NR5A factors both present) and groups where the GATA4 (black bar) and NR5A (open bar) factors were used alone. (C) *In vitro* pull-down assays were used to demonstrate that a physical protein–protein interaction between NR5A factors and GATA4 is retained with the different GATA4 mutants. Input indicates 10% of the labeled protein used in the pull-down assay.

strong transcriptional activity on different gonadal promoters (Robert *et al.* 2002). The differential activation elicited by the E215D mutant is then most likely due to differences in promoter context. Differential promoter sensitivity was also observed in the present study where the V266M mutant fully activated the *Star* promoter but had reduced activity on the *Cyp19a1* and *Inha* promoters (Fig. 2A). Therefore, with respect to the E215D mutant, important differences in

the way the mutated protein behaves at a molecular (i.e., transcriptional) level might be the reason why the heart, from a phenotypic standpoint, is more sensitive to the E215D mutation.

The E216D, V266M, and G295S mutations are located in close vicinity to the GATA4 zinc finger domains that are essential for DNA binding (Fig. 1A). The zinc finger region of GATA proteins, including GATA4, also mediates important protein–protein

interactions with other transcriptional regulators that help define the proper spatiotemporal expression of target genes (Molkentin 2000, Viger *et al.* 2008). Indeed, in the heart, Garg *et al.* showed that the probable molecular basis for the pronounced cardiac phenotype associated with the G295S mutation involved a failed protein–protein interaction with TBX5, a transcription factor responsible for normal heart development and function (Garg *et al.* 2003). In endocrine cells, we and others have shown that the nuclear receptors NR5A1 and NR5A2 are important transcriptional partners for GATA4 (Viger *et al.* 2008). Although NR5A1 is expressed in the heart, at least at the transcript level (Ramayya *et al.* 1997, Nishimura *et al.* 2004), there is currently no evidence for a functional role for this receptor as a transcriptional partner for GATA4 in this tissue. Unlike TBX5 in the heart (Garg *et al.* 2003), the functional synergism between the G295S mutant and its gonadal partners NR5A1 and NR5A2 was not compromised (Fig. 5A and B). The G295S mutant likely retains its ability to transcriptionally cooperate with NR5A factors because it can still physically interact with the two receptors (Fig. 5C). Similar to the G295S mutant, the E215D variant could also transcriptionally cooperate with NR5A1 and NR5A2 (Fig. 5). Since this amino acid substitution does not impair the interaction of GATA4 with FOG2 (Nemer *et al.* 2006), the E215 residue is likely important for the recruitment of a yet unidentified transcriptional partner, essential for proper heart tube formation, but not necessarily required for gonadal development and function. Thus, again, the observation that the mutant GATA4 proteins partially retained some of their transcriptional properties on target gonadal promoters provides some molecular basis as to why these mutations do not appear to cause any pronounced phenotypic defects in the reproductive tract but do so in the heart. It also stresses the importance of specific protein–protein interactions for the regulation of GATA4 activity in different cellular contexts. In contrast to the gonads, the heart might simply be more sensitive to disruptions in GATA4 function.

Another possibility for the lack of reproductive phenotypes associated with reported *GATA4* mutations might be redundancy. Sertoli cells of the testis, for example, express multiple GATA factors including GATA1, GATA4, and GATA6 (Viger *et al.* 2004). In this case, loss of GATA4 function might be compensated by either GATA1 or GATA6. The likelihood of compensatory mechanisms for GATA function in the gonads is supported by at least one genetic study in the mouse which showed that disruption of GATA1 function in Sertoli cells produces no major gonadal defect, most likely due to compensation by other GATA family members (Lindeboom *et al.* 2003). Yet another possibility is the fact that although some of the

GATA4 mutants exhibited a significant reduction in transcriptional activity on different gonadal target (Fig. 2A), many of these proteins showed a marked enhancement of activity in the presence of PKA to levels approaching or even surpassing the unstimulated wild-type protein (Fig. 4).

Taken together, our data suggest that the human *GATA4* mutations that have been associated with severe congenital heart abnormalities would likely have less dramatic consequences on gonad-specific gene transcription, since the mutant proteins retain many of their transcriptional properties on different GATA4-dependent gonadal target promoters. During the course of this study, more germ line heterozygous *GATA4* mutations have since been reported (Rajagopal *et al.* 2007, Reamon-Buettner *et al.* 2007, Tomita-Mitchell *et al.* 2007). Interestingly, some of these occur in the GATA4 intronic sequences or the 3' non-translated region of the GATA4 mRNA. These specific mutations leave the coding sequence unaltered and have been predicted to affect RNA folding, probably leading to its decay (Reamon-Buettner *et al.* 2007). Thus, far, only fully penetrant germ line *GATA4* mutations have been reported as a result of screening large families where congenital heart defects are present in more than one generation. Recently, the screening of large cohorts of individuals presenting cardiac malformations uncovered the first non-fully penetrant *GATA4* mutations (Rajagopal *et al.* 2007, Tomita-Mitchell *et al.* 2007). Given the identification of these novel *GATA4* mutations, a similar screen of individuals presenting reproductive abnormalities, and especially that involving gonadal sex reversal is both warranted and needed.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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