Transgenic mice expressing small interfering RNA against *Gata4* point to a crucial role of *Gata4* in the heart and gonads

Boris Thurisch, Shermi Y Liang¹, Nanette Sarioglu², Lutz Schomburg³, Jörg Bungert^{1*} and Christof Dame*

Department of Neonatology, Charité-Universitätsmedizin Berlin, Campus Virchow-Klinikum, Augustenburger Platz 1, D-13353 Berlin, Germany

¹Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, Florida 32610, USA

²Institute of Pathology, Charité-Universitätsmedizin Berlin, D-10117 Berlin, Germany

³Institute for Experimental Endocrinology, Charité-Universitätsmedizin Berlin, D-13353 Berlin, Germany

(Correspondence should be addressed to C Dame; Email: christof.dame@charite.de)

*(C Dame and J Bungert designed the research work and contributed equally to this work)

Abstract

Homozygous deficiency of the transcription factor *Gata4* in mice causes lethality due to defects in ventral morphogenesis and heart tube formation. There is increasing evidence demonstrating that GATA4 function is also relevant for normal developed organ systems, including the heart and endocrinum. To analyze the implication of *Gata4* beyond development, we generated transgenic mice expressing inducible small interfering RNA against *Gata4*. In longitudinal analysis, efficient suppression of *Gata4* mRNA (down to 80% of wild-type levels) and protein expression in the heart was detected 38 days after induction of *Gata4* short hairpin RNA. Decreased *Gata4* expression was associated with reduction in the expression of known cardiac target genes, but the function of the heart remained unperturbed at 20–30% of normal *Gata4* levels. Interestingly, *Gata4* expression was almost abolished in the ovary and testis. This was accompanied in the testis by a significant reduction of GATA4 downstream target genes, such as the genes encoding Mullerian inhibiting substance (MIS) and steroidogenic acute regulatory (StAR) protein. By contrast, expression levels of *Mis* and *Star* were only slightly modified in the ovary, and concentrations of circulating FSH and LH were normal in female transgenic mice after induction of *Gata4* short hairpin RNA. However, inhibition of *Gata4* expression led to the formation of ovarian teratoma in 10% of females. Histology of the teratomas showed predominantly ectodermal and mesodermal structures. Our data demonstrate that *Gata4* is critically involved in the function and integrity of the gonads *in vivo*.

Journal of Molecular Endocrinology (2009) **43**, 157–169

Introduction

The transcription factor GATA4 belongs to a family of six highly conserved zinc finger proteins, which bind to regulatory DNA fragments containing a 'T/A GATA G/A' core sequence. GATA factors are critically involved in the development and function of various organs (for review (Lowry & Atchley 2000, Molkentin 2000)). Based on the phenotypes of *Gata*-deficient transgenic mice and subsequent analysis of downstream target genes, GATA4/5/6 were subgrouped as primary endodermal transcription factors (Molkentin 2000). Each factor exhibits a distinct developmental-stage and tissue-specific expression pattern. Cumulative data show that GATA4 is a critical regulator of cardiac gene expression, modulating cardiomyocyte differentiation and adaptive responses of the adult heart. GATA4 is also expressed in various endocrine organs. In the male and female gonads, GATA4 contributes to

transcription of multiple hormone-encoding genes (Viger *et al.* 2008).

In mice, homozygous *Gata4* deficiency (*Gata4*^{-/-}) results in embryonic lethality between 8.5 and 10.5 dpc due to defects in yolk sac vasculogenesis, ventral morphogenesis, and heart tube formation (Kuo *et al.* 1997, Molkentin *et al.* 1997). Mutant mice with cardiac-specific deletion of *Gata4* show hypoplasia of the right ventricle during embryonic development; at later stages this heart fails to react by hypertrophy in consequence of hypertension and shows reduced myocyte viability (Zeisberg *et al.* 2005, Oka *et al.* 2006). However, transgenic mice with heterozygous *Gata4* deficiency (*Gata4*^{+/-}) do not suffer from cardiac failure (Kuo *et al.* 1997, Pu *et al.* 2004). Other data from transgenic mice indicate that a critical *Gata4* expression level is required for normal cardiac development and function (Pu *et al.* 2004, Zeisberg *et al.* 2005, Bisping *et al.* 2006, Oka *et al.* 2006, Jay *et al.* 2007, Rajagopal *et al.* 2007).

Transgenic models that overcome early embryonic lethality associated with *Gata4* deficiency provided the first evidence for GATA4 function in endocrine organs. Mice homozygous for a *Gata4* variant (*Gata4*^{ki/ki}) that cannot interact with its cofactor *friend-of-GATA2* (FOG2) show abnormal morphology of the testis (Tevosian *et al.* 2002, Manuylov *et al.* 2007). Recently, chimeric mice, generated by injection of *Gata4*^{-/-} embryonic stem (ES) cells into *Rosa26* blastocytes, have shown that GATA4 is directly required for the differentiation of Leydig cells in the fetal murine testis (Bielinska *et al.* 2007). Although various putative GATA4 target genes have been identified in the testis and ovary by means of *in vitro* studies (for review see (Viger *et al.* 2008)), to our knowledge no *in vivo* genetic model is currently available that elucidates GATA4 function in the ovary.

The *in vivo* function of GATA4 in the heart and endocrine organs is of increasing interest, since *GATA4* haploinsufficiency has been identified in humans (Pehlivan *et al.* 1999, Garg *et al.* 2003). Although the number of patients suffering from *GATA4* mutations reported in the literature are still relatively low, evidence is given that septal heart defects, in particular atrial septal defects of the secundum type and ventricular septal defects, as well as tetralogy of Fallot are predominant phenotypes (Pehlivan *et al.* 1999, Garg *et al.* 2003, Okubo *et al.* 2004, Hirayama-Yamada *et al.* 2005, Sarkozy *et al.* 2005, Nemer *et al.* 2006, Tang *et al.* 2006, Rajagopal *et al.* 2007, Tomita-Mitchell *et al.* 2007). Data on non-cardiac phenotypes in individuals with reduced GATA4 expression are very limited, but such disorders may include delayed psychomotor development (Pehlivan *et al.* 1999) or endocrine disorders. In mice, GATA4 deficiency can be induced by the chemotherapeutic agent doxorubicin (Li *et al.* 2006). These findings underline that GATA4 is a clinically relevant transcription factor, and the question regarding dose- and/or time-dependent effects of reduced GATA4 expression becomes of increasing interest for clinical medicine (Rajagopal *et al.* 2007, Viger *et al.* 2008).

We generated a double-transgenic mouse expressing the tetracycline repressor (*TetR*) and an inducible small interfering RNA (siRNA) directed against *Gata4* in order to achieve dose- and/or time-dependent suppression of *Gata4* expression. *In vivo* RNA interference (RNAi) has recently been established, but the general experience with such mouse models is still limited (Hasuwa *et al.* 2002, Bantounas *et al.* 2004, Kühn *et al.* 2007, Seibler *et al.* 2007). Herein, we successfully generated a novel transgenic mouse line expressing inducible *Gata4* siRNAs (si*Gata4*) and analyzed the effects of *in vivo* GATA4 reduction on tissue-specific gene expression and organ function, in particular the heart and gonads.

Material and Methods

Cell culture

C2C12 myoblast cells (ATCC CRL-1772; Manassas, VA, USA) were grown in DMEM (Invitrogen) supplemented with 2 mM glutamine, 10% FCS (Biochrom, Berlin, Germany), and 5% antibiotics (penicillin/streptomycin; Biochrom).

In vitro RNA interference

The pTER+ plasmid was used to express *Gata4* siRNA in C2C12 cells (van de Wetering *et al.* 2003). After software-based exclusion of significant homology within the murine genome, oligonucleotides corresponding to the murine *Gata4* coding sequence (NCBI accession no. NM_008092; construct 1: nt +1214 to +1234; 5'-GATCCCTCTCGATATGTTTGATGA CTTCAAGA GA GTCATCAAACATATCGAGATTTTGGAAA-3'; construct 2: nt +1242 to +1262; 5'-GATCCCGGCAG-AGAGTGTGTCAATTTTCAAGAGAAATTGACACA-CTCTCTGCCTTTTGG AAA-3'; shScram: 5'-GATCCC GTTAACTTCTTAATTGTGG TTCAAGAGA CCA-CAATTAAGAAG TTAACTTTGGAAA-3', plus their corresponding 3'→5' sequences) were ligated and cloned into pTER+. Transfections were carried out using FuGENE 6 transfection reagent (Roche Diagnostics). Highest efficiency was reached using FuGENE 6 and DNA in a 3:1 ratio. Four hours after transfection FCS (final concentration of 10%) and antibiotics (penicillin/streptomycin, final concentration of 5%) were added.

Generation of transgenic mice

FvB mice were used to generate the transgenic H1:*G4/TetR* strain. The coding sequence for the mouse *Gata4* short hairpin RNA (shRNA; construct 1) was ligated downstream of the H1 promoter using the plasmid pTER+ (Fig. 1A). The H1 promoter contains an operator sequence (TO) for the tetracycline (Tet) repressor (TetR), which inactivates the promoter (Fig. 1B). Doxycycline antagonizes TetR function and induces expression mediated by the H1 promoter. Plasmid pcDNA6/TR (Invitrogen) was digested with *XhoI* and *SalI*; pTER:sh*Gata4* was digested with *ScaI* and *StuI*. Target plasmid fragments were purified from agarose gels and resuspended in injection buffer at a concentration of 2 ng/μl (Bungert *et al.* 1995). Transgenic mice were generated in the transgenic core facility of the University of Florida, where the study was approved by the Institutional Review Board (IRB protocol No. D669). Transgenic founders were

Figure 1 Design and *in vitro* analysis of an inducible shRNA directed against murine *Gata4*. (A) Localization of the *Gata4* siRNA sequence within the murine *Gata4* gene locus (NCBI NT_039606), the *Gata4* mRNA (transcription start nt +1; translation start nt 608; stop codon: TAA; NCBI NM_008092), and the coding mRNA (nt 608–1933). The sequence of the sense strand (nt 1214–1234) of the *Gata4* siRNA is shown in the lower part of the panel. (B) Vector map of the pTER: *shGata4* plasmid. Shown is the construct for the inducible doxycycline (Dox) regulated expression of short hairpin RNA (shRNA), initially described by [van de Wetering et al. \(2003\)](#). pTER: *shGata4* contains a H1 promoter, recognized by RNA polymerase III, and a doxycycline-regulatory element upstream of the coding region for the *Gata4* shRNA. TO, tetracycline operator; H1:TO-promoter; H1 promoter modified by the tetracycline operator; *AmpR*, ampicillin resistance gene; *ZeoR*, zeocin resistance gene. (C) Analysis of *in vitro* efficiency of *Gata4* shRNA. Murine C2C12 myoblasts were transfected with inducible *Gata4* shRNA and *TetR* expression plasmids and incubated with doxycycline in a dose- and time-dependent manner. Expression of GATA4, GATA2, and α-Tubulin was analyzed by western blot.

identified by Southern blotting and confirmed by PCR. Double transgenic mice were mated to generate mice that carried both transgenes.

DNA isolation, PCR screening and Southern blot analysis

The presence of both transgenes was determined by Southern blot. Ten micrograms of genomic DNA was digested with HindIII, size fractionated on 1.2% agarose gels and transferred onto nylon membranes. Membranes were subsequently probed with DNA fragments corresponding to regions of the transgenes as previously described (Bungert *et al.* 1999). The H1:*G4* probe is a 1143 bp *Stu*I-HindIII fragment derived from pTER:*shGata4* and corresponds to the H1:*G4* transgene. The *TetR* probe is a 1591 bp *Xho*I-HindIII fragment derived from pcDNA6/TR and corresponds to the *TetR* transgene. Transgene copy numbers were determined using the Phosphorimager system (Storage Phosphor Screen, Molecular Dynamics, Amersham Pharmacia Biotech) after hybridizing the nylon membranes for 14 h with a radioactive probe corresponding to the murine β -globin promoter (Li *et al.* 1998). Radioactive signals were quantified using a PhosphorImager (Storm 860) and the IMAGEQUANT 5.0 software (both Amersham Pharmacia Biotech). For PCR, the following primers directed against DNA fragments of the transgenes were used: 1) H1:*G4* transgene: sense 5'-GTCACTAGGCGGGAACACC-3', antisense 5'-ACGGGGGAGGGGCAAACAACAG-3' and 2) *TetR* transgene sense: 5'-TAGATAGGCACCATA CTCACCTTTT-3', antisense: 5'-GGACCCACTTTCACATTT-3'.

RT-PCR analysis of *Gata4*, *Gata6*, atrial natriuretic factor, bone morphogenic protein-4, α -myosin heavy chain, oligoadenylate synthetase-2, Mullerian inhibiting substance, steroidogenic acute regulatory, and β -actin expression

Total RNA was prepared as previously described (Dame *et al.* 2006). The RNA pellet was dissolved in diethyl pyrocarbonate-treated H₂O at a concentration of 1 μ g/ μ l. First, strand cDNA synthesis was performed with 2 μ g total RNA using oligo(dT) primers and superscript II reverse transcriptase (Invitrogen; Dame *et al.* 2006). Expression analysis of the genes encoding atrial natriuretic factor (*Anf*; E *et al.* 2006), α -myosin heavy chain (α -*Mhc*; E *et al.* 2006), Mullerian inhibiting substance (*Mis*; Wang *et al.* 2005), and steroidogenic acute regulatory protein (*Star*; Manna *et al.* 1999) was performed with primer sets as previously described. Expression analysis of the genes encoding *Gata4*, *Gata6*, bone morphogenic protein-4 (*Bmp4*), 2'-5'-oligoadenylate synthetase 2 (*Oas2*), β -actin, and *Gapdh* was

performed with the following primer sets: *Gata4*: sense 5'-GATGGGACGGGACACTACCTG-3', antisense 5'-ACCTGCTGGCGTCTTAGA TTT-3'; *Gata6*: sense 5'-TTAACACTGATTGCTGCAACG-3', antisense 5'-GTT CATCGT AACGTGGCTGA-3'; *Bmp4*: sense 5'-ACCTCAACTCAACCAACC-3', antisense 5'-GTGTGGTATGTGTAGGTGG-3'; *Oas2*: sense 5'-CAATAATGTGGGCAAA-GATGATGG-3', antisense 5'-ACTGGGAGCGGTTG-GTTTTTAGAG-3'; β -actin: sense 5'-ACTGCTCTG-GCTCCTAGCAC-3', antisense 5'-ACATCTGCTGGAAG-GTGGAC-3'; and *Gapdh*: sense 5'-CCAAGGTCATCCAT-GACAAC-3', antisense 5'-ATCAGCCACAGCTTTCC-3'. PCR was carried out under the following conditions: DNA denaturation at 94 °C; primer annealing at 60 °C (except for *Gata4*: 62 °C); and extension at 72 °C (30 cycles, except for *Gata6*: 27 cycles; 30 s each phase). cDNAs generated from the cell lines were amplified and PCR products were electrophoresed in 1.2% agarose gels and stained with ethidium bromide.

RNA isolation and real-time PCR

To induce the expression of si*Gata4*, animals harboring the H1:*G4* and *tetR* transgenes were fed with 20 mg doxycycline/ml drinking water (plus 2% sucrose). Animals were sacrificed at defined time points (after 14, 29, 38, or 70 days respectively), and total RNA was extracted from heart, ovary, and testis, and reverse transcribed (Dame *et al.* 2006). One-twentieth of the reaction volume was used for quantification with the iCycler iQ Optical Real-Time PCR Detection System (Bio-Rad). The fluorescence threshold value was calculated using the iCycle iQ Optical System software 3.1. For *Gata4* and β -actin, TaqMan Real-Time PCR was performed using commercially available primers (m*Gata4* Mm00484689_m1 and m β -actin Mm00607939_s1; Applied Biosystems, Foster City, CA, USA). *Mis* and *Star* expression was analyzed by quantitative real-time PCR using the MiQ (Bio-Rad) using previously published primer sets (Wang *et al.* 2005, Rivera *et al.* 2009). Serial fivefold dilutions (100–0.16 ng) of cDNA of wild type tissue were used for standard curve calculations. All real-time PCR reactions were performed in triplicate in a 20 μ l mixture containing 1 μ l cDNA, 10 μ l TaqMan, 2 μ l PCR Master Mix (Applied Biosystems), 1 μ l primer, and 8 μ l H₂O. The transcript levels in the tissues of wild-type and transgenic mice were compared on the basis of standard curves.

Western blot analysis

Approximately, 20 μ g of whole protein extract from C2C12 myoblasts and 50 μ g of whole protein extract from tissue specimens respectively, were electrophoresed on 10% Ready Gels (Bio-Rad) under

denaturing conditions. Transfer was performed using the Mini Protean apparatus (Bio-Rad). Incubation with antibodies (anti-GATA2 (H116), anti-GATA4 (C-20), anti-GATA4 (H-112), anti- α -Tubulin (TU-02), or anti- β -Actin (AC-15); all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) and later detection of signals with appropriate HRP conjugated secondary antibodies were performed as previously described (Dame *et al.* 2004).

Analysis of hormone concentrations

Serum levels of LH and FSH were determined using a commercially available multiplex bead assay system (MILLIPLEX MAP rat pituitary panel, #RPT86K) according to the manufacturer's instructions (Millipore, Amsterdam, The Netherlands). Briefly, serum samples and commercial controls were diluted 1:3 with serum matrix, and serial dilutions of supplied standards were prepared. In addition, serum samples from control and gonadectomized rats and mice were analyzed in parallel to verify the suitability of the assay to measure both murine and rat LH and FSH. Diluted samples were incubated over night at 4 °C with antibody-immobilized fluorescent beads in a 96-well plate format. Biotinylated detection antibodies were added, samples were washed, incubated with streptavidin-phycoerythrin complexes and signals were recorded and analyzed using a Luminex 200 system (Luminex Corp., Austin, TX, USA).

Histopathological analysis of tissue specimens

Tissue was harvested and fixed at 4 °C in 4% paraformaldehyde for at least 6 h. Six micrometer thick sections were cut from the paraffin-embedded tissue and transferred onto glass slides. After H&E staining, histology was examined under an epifluorescence microscope (AxioPlan 2 Imaging System, Carl Zeiss, Jena, Germany), and photographs were taken with a connected digital camera (AxioCAM MRc) using the AxioVision 4.2 software (both Carl Zeiss).

Statistical analysis

Student's *t*-test or ANOVA was performed as indicated to reveal statistical significances. A $P < 0.05$ was considered to be statistically significant.

Results

In vitro efficacy of *Gata4* RNAi

The goal of this study was to generate a transgenic mouse expressing short hairpin RNA (shRNA) directed against *Gata4* and to then examine the consequence of

reduced GATA4 expression on the function of organs known to express this transcription factor. We first tested two different *Gata4* shRNA constructs in murine C2C12 myoblasts. These cells express GATA4 and were transfected with the pcDNA6/TR and the pTER:sh-*Gata4* plasmid. Double-transfected cells were treated without or with 1, 2.5, or 5 μ g/ml doxycycline for 3 or 7 days respectively. Three days after application of 2.5 μ g doxycycline per ml medium or alternatively 7 days after treatment with 5 μ g doxycycline per ml medium, most efficient reduction in Gata4 protein expression was detected (Fig. 1C). The *Gata4* siRNA sequence (TCTCGATATGTTTGATGAC) is almost identical to a human *GATA4* siRNA (NCBI accession no. L34357; nt 852–870 relative to the transcription start site: TCTCGATATGTTTGACGAC) that has previously been shown to efficiently reduce GATA4 expression in human hepatoma Hep3B cells (Dame *et al.* 2004). Normal expression levels of *Gata2* mRNA demonstrated that *Gata4* siRNA is specific. A control siRNA that was not homologous to the *Gata4* sequence (scrambled sequence) did not reduce *Gata4* expression (data not shown). These results show that *Gata4* siRNA can be expressed in an inducible manner and that it specifically reduces GATA4 expression (Fig. 1C).

Generation of transgenic mice

We next generated transgenic mice in which expression of *Gata4* can be repressed using inducible siRNA. Multiple coinjections of the *TetR* and the H1:*G4* constructs into fertilized oocytes resulted in a total of 494 transfected oocytes and 121 offsprings. One male animal of the transgenic founder lines contained both H1:*G4* and *TetR*. We also generated a line only harboring the *TetR* construct. However, H1:*G4* single transgenic animals could not be generated, possibly due to embryonic lethality. Thus, the presence of the *TetR* may be required for the survival of embryos carrying the *Gata4* shRNA transgene. Mice were then subsequently bred to generate a colony of mice harboring both transgenes (H1:*G4*/*TetR*). The integration pattern of this construct is somewhat complex and suggests that in addition to single copy integration the transgenes also integrated as multiple tandem repeats (Fig. 2). Based on the comparison of band intensities of transgene-derived fragments with a fragment from the murine β -globin gene in Southern blots, we estimated that the copy numbers of the transgenic constructs were between 10 and 15. The fact that the pattern of DNA fragments in Southern blot analysis remained the same after several generations for both the H1:*G4* and *TetR* transgene (si*Gata4*) suggests that the two constructs co-integrated into the same single locus.

Figure 2 Generation of H1:G4/*TetR* double-transgenic mice (*siGata4*). (A) *Gata4* shRNA (H1:G4 (1.9 kb)) and *TetR* expressing construct (*TetR*; 2.5 kb) were used to generate transgenic mice. After HindIII digestion of genomic DNA, the H1:G4 and *TetR* transgenes were identified by Southern blot and expected sizes for multi-copy integrations were observed, for the H1:G4 transgene 1.9 kb and for the *TetR* transgene 2.5 kb. Other bands are due to the end fragments of the transgenic constructs. (B) Southern blot analysis of parental founder (P) and F1/F2 offspring. The integration patterns of the H1:G4 and *TetR* transgenes are shown. The sizes of the bands depend on single integration (S) or various tandem integrations (T) of the transgene. In case of a single integration, the expected size for the H1:G4 transgene was >1.1 kb. In the case of the *TetR* transgene the expected size was >1.6 kb. Multiple tandem integrations in 5'→3' configurations resulted in a 1.9 kb signal with respect to the H1:G4 transgene and a 2.5 kb signal with respect to the *TetR* transgene. A 3'→5' tandem integration configuration is expected to yield a 2.2 kb signal for the H1:G4 and a 3.2 kb signal for the *TetR* transgene respectively.

Transgenic mice harboring the H1:G4 and the *TetR* expressions constructs appeared to be clinically normal, but breeding resulted only incidentally in pregnancy. However, some transgenic females (7 out of a total of 40 females) died within a period of 6 month without exhibiting a pathophysiological phenotype. Since *siGata4* transgenic mice not treated with doxycycline revealed up to 25% reduction of *Gata4* mRNA levels in the heart, we speculate that the increase in mortality of females is likely due to leaky expression of the *Gata4* siRNA in any specific organ system, but unlikely due to heart failure as shown in experiments described below. Of note, application of doxycycline to wild-type mice

did not affect the expression of *Gata4* mRNA or *Gata4* downstream targets in heart, demonstrating that doxycycline itself does not modulate *Gata4* expression. Mice expressing only the *TetR* construct were phenotypically normal and showed normal fertility, as previously described in the literature (Corbel & Rossi 2002, Szulc *et al.* 2006, Seibler *et al.* 2007). *TetR* transgenic mice did not reveal reductions in cardiac *Gata4* expression. There is also no other evidence for changes in gene expression patterns in *TetR* transgenic mouse lines (Corbel & Rossi 2002, Szulc *et al.* 2006, Seibler *et al.* 2007).

In vivo suppression of *Gata4* by shRNA in the heart

Adult wild-type and transgenic *siGata4* mice were treated with 20 mg doxycycline per ml drinking water for different time periods (14–70 days). In the heart, we observed a time-dependent reduction of *Gata4* mRNA expression, which reached significance 5 weeks after induction of *Gata4* shRNA. Suppression of GATA4 mRNA and protein (Fig. 3A and B) was associated with reduced expression of the known cardiac GATA4 target genes *Anf* and *Bmp4* (Fig. 3C; Grepin *et al.* 1994, Nemer & Nemer 2003). Surprisingly, the expression of the α -Mhc gene, another known GATA4 downstream target (Molkentin *et al.* 1994, Charron *et al.* 1999), was not affected. Most importantly, however, transgenic animals with induced *Gata4* shRNA did not show cardiac failure. Cardiac function was monitored by electrocardiography (in cooperation with Dr H Kasahara, University of Florida), which was normal (data not shown), and furthermore, histological examination did not reveal morphological changes (Fig. 3D). Thus, reduction of *Gata4* expression to levels <30% of wild type (as analyzed up to day 70) did not impair the function of non-stress exposed hearts. Unexpectedly, the overall expression of α -Mhc increased both in wild-type and transgenic *siGata4* mice during doxycycline treatment. It appears unlikely that this effect is mediated by up-regulation of *Gata6*, since *Gata6* mRNA levels were normal in transgenic mice 29 days after induction of *Gata4* shRNA (Fig. 3E). We consistently found that induction of *Gata4* shRNA expression was associated with a significant increase in *Oas2* gene expression (Fig. 3B). This is consistent with previous studies demonstrating that expression of shRNAs can activate the interferon response (Bridge *et al.* 2003, Sledz *et al.* 2003). However, the scientific impact of transgenic mice expressing shRNA against a gene of interest is supported by the recent finding that endogenous microRNA pathways are preserved in such models (Sasaguri *et al.* 2009).

Figure 3 *In vivo* cardiac GATA4 suppression by shRNA. Wild-type (WT) and si*Gata4* transgenic (TG) mice were treated with doxycycline for 14, 29, 38, and 70 d respectively. (A) Quantitative PCR analysis of *Gata4* mRNA expression (means $\pm$ s.d., * $P < 0.05$). (B) GATA4 suppression in the heart taken 38 days after induction of *Gata4* shRNA was confirmed by western blot. (C) Representative conventional RT-PCR analysis of *Anf*, α -*Mhc*, and *Oas2* expression in heart tissue specimens, obtained from WT and si*Gata4* TG mice treated with doxycycline for 14, 29, or 38 days respectively. (D) H&E staining of the myocardial tissue specimens from si*Gata4* transgenic versus wild-type mice after treatment with doxycycline for 70 days. (E) Analysis of *Gata6* mRNA expression in si*Gata4* TG and wild-type mice treated with doxycycline for 29 days.

Effects of *Gata4* shRNA on *Gata4* downstream target genes in normally developed female and male gonads

Next, we examined whether induction of *Gata4* shRNA affect expression of GATA4 downstream targets in the gonads. As shown in Fig. 4, activation of the H1:*G4* transgene resulted in strong reduction of *Gata4* mRNA in the ovary and testis at day 38. GATA4 protein expression in the testis was almost abolished (Fig. 4A). As in the heart, we also observed an induction of the interferon response gene *Oas-2* both in the testis and ovary (Fig. 3A and C). In the testis, *Gata4* mRNA reduction was associated with reduced *Mis* and *Star* gene expression, two previously identified GATA4 target genes (Tremblay & Viger 2001). By contrast, *Gata4* mRNA reduction in the ovary was associated only with moderate changes in *Star* and *Mis* gene expression (Fig. 4B and C). Although *Gata6* expression was not changed, this finding itself does not allow conclusions as to whether GATA6 functionally compensates the disturbance of GATA4 function in the ovary. However, this finding argues against global

down-regulation of gene expression by off-target effects. To elucidate consequences on steroidogenesis, blood samples were analyzed for hormone concentrations. The analysis revealed similar concentrations of FSH in transgenic females with induced *Gata4* shRNA (mean 15 472 pg/ml) versus those without doxycycline (mean 14 828 pg/ml) versus wild-types (15 136 pg/ml). Similarly, circulating LH concentrations did not differ between transgenic females with induced *Gata4* shRNA (mean 415 pg/ml) versus those without doxycycline (mean 668 pg/ml) versus wild-types (347 pg/ml). Because the effect of reduced GATA4 expression on target genes was more pronounced in the testis, reduced fertility in transgenic mice, in which the *Gata4* siRNA was not induced by doxycycline, may be caused rather by 'leaky' expression in males.

Development of ovarian tumors (teratoma) in doxycycline induced *Gata4* shRNA transgenic females

Of note, 2 out of 20 si*Gata4* transgenic females treated with doxycycline to induce *Gata4* shRNA developed ovarian teratoma (Fig. 5). One well-differentiated teratoma was found 14 days after activation of the H1:*G4* transgene. This teratoma was composed of structures predominantly derived from meso- and ectoderm (Fig. 5A–G). Another teratoma was obtained from a transgenic female mouse treated with doxycycline for 21 days. This teratoma was less differentiated and contained large areas of necrosis (Fig. 5H–K). No tumorigenesis was observed in wild-type mice (with or without doxycycline treatment).

Taken together, *in vivo* *Gata4* shRNA expression showed differential effects of GATA4 on its downstream hormone-encoding genes in male versus female gonads and caused an increased incidence of teratoma in the ovary.

Discussion

Transgenic mouse with inducible *Gata4* shRNA

Herein, we describe the first *in vivo* model of inducible *Gata4* shRNA. The transgenic mouse line is characterized by a stably integrated shRNA construct directed against *Gata4* that is under the control of a *TetR* repressor and inducible by doxycycline. Our decision to develop a mouse model with inducible shRNA against *Gata4* allowing for dose- and/or time-dependent suppression of *Gata4* expression without restriction to a specific organ system, was based on several considerations: first, we expected that a critical level of GATA4 is required for normal cardiac function (Pu *et al.* 2004, Zeisberg *et al.* 2005, Bisping *et al.* 2006, Oka *et al.* 2006,

Figure 4 Effects of *in vivo* *Gata4* shRNA expression on GATA4 target genes in the reproductive organs. WT and si*Gata4* TG mice were treated with doxycycline-containing water to induce H1:G4 over a time period of 38 days. (A) Expression of *Gata4* and GATA4 target genes in the testis. Quantitative PCR analysis of *Gata4* mRNA expression (left panel; means $\pm$ s.d., * P < 0.01) and conventional RT-PCR (right panel) for expression analysis of *Mis*, *Star*, *Oas2*, and β -actin in WT versus si*Gata4* TG mice. Western blot analysis of GATA4 expression after 38 days of H1:G4 induction (lower right panel). (B) Expression of *Gata4* in the ovary measured by quantitative PCR – indicates highly significant reduction of *Gata4* mRNA expression in si*Gata4* TG versus WT mice (left panel; means $\pm$ s.d., * P < 0.01). Conventional RT-PCR analysis indicates no changes in *Gata6* mRNA expression levels after 38 days of doxycycline treatment in TG versus WT mice (right panel). (C) Conventional RT-PCR analysis (left panel) for *Mis*, *Star*, *Oas2*, and β -actin expression in si*Gata4* TG versus WT mice. Real-time PCR analysis excluded significant changes in *Star* or *Mis* mRNA expression after 38 or 70 days of induction of *Gata4* shRNA (ANOVA).

Jay *et al.* 2007); and second, regarding the identification of GATA4 mutations in humans (Pehlivan *et al.* 1999, Garg *et al.* 2003, Nemer *et al.* 2006, Schluterman *et al.* 2007, Tomita-Mitchell *et al.* 2007), we were interested

whether *Gata4* levels of <50% of normal would be associated with non-cardiac disorders, in particular in the endocrine system.

In our initial experiments, we identified a murine *Gata4* siRNA sequence that significantly and specifically reduced GATA4 mRNA and protein expression in murine myoblasts. We also demonstrated that the *TetR* expression vector is tightly regulated by doxycycline in a dose-dependent manner (Fig. 1). The murine *Gata4* siRNA sequence is almost identical to a human GATA4 siRNA sequence, which effectively reduced GATA4 in human hepatoma cells (Dame *et al.* 2004).

Adult mice harboring the H1G4/*TetR* or the *TetR* transgenes were phenotypically normal. However, transgenic si*Gata4* female mice older than 3 months revealed an increased rate of mortality compared with wild-type mice. However, the reason for increased mortality remains unclear at this point. Since *Gata4* is expressed in a variety of organs, we attribute these observations to leaky expression of the H1G4 transgene, which in fact is suggested by reduced *Gata4* mRNA levels in the heart. Leaky expression of the transgene could also contribute to the observed breeding problems. Importantly, however, *Gata4* shRNA could be successfully induced in a time-dependent manner in animals after subjecting transgenic mice to doxycycline-containing drinking water (Figs 3 and 4). The maximum suppression of endogenous *Gata4* expression showed tissue-specific differences, e.g. reproductive organs revealed a more dramatic reduction in *Gata4* mRNA expression than the heart. The differences in *Gata4* reduction could be due to lower expression levels of *Gata4* in the male and female gonads versus the heart, to tissue-specific differences in *in vivo* shRNA expression (Oberdoerffer *et al.* 2005, Seibler *et al.* 2007, Sasaguri *et al.* 2009), or to differences in the uptake of doxycycline in the gonads. The time course experiment revealed the most dramatic effect on *Gata4* expression after 38 days of shRNA induction in transgenic mice. The reason for this relatively long time period is unclear at the moment, but could in part be due to slow turnover of cells combined with a long half-life of GATA4 mRNA and protein in certain tissues.

Reduction of *Gata4* expression by 80% does not impair the function of non-stress induced adult heart

We first analyzed the effect of *Gata4* shRNA in the heart. Even at 80% reduction of *Gata4* mRNA expression (after 38 days) and after long-term *Gata4* shRNA expression over a period of 70 days, transgenic mice did not show cardiac failure and revealed normal electrocardiography and histology of the myocardial tissue (Fig. 4D). This observation is consistent with a recent report by Oka *et al.* (2006) describing the phenotype of *Gata4-loxP*-targeted mice crossed with

Figure 5 Ovarian teratoma from female si*Gata4* transgenic mice with induced *Gata4* shRNA. (A) Macroscopy of a mature teratoma (I) harvested 14 days after induction of *Gata4* shRNA. (B) and (C) H&E overviews show characteristic solid and cystic structures of all three germ layers. (D) Structures derived from the ectoderm include cysts that are lined with weakly keratinized squamous epithelium (1), adjoined integumentary system with perspiratory glands (2), and neuronal tissue (3). Among mesodermal structures, fatty tissue (4) and connective tissue were found. (E) At higher magnification some clearly under-represented areas show structures derived from the endoderm, such as columnar surface epithelium (1) and short crypts (2). (F) Differentiated neuronal cells (1) are located beside glia cells and matrix (2). (G) As indicated by the arrow, a cyst of ectodermal origin shows intense cornification. (H) Macroscopy of an immature teratoma (II) harvested 21 days after induction of *Gata4* shRNA. (I) This teratoma contains connective tissue that shows only a few small vessels and exhibits focal necrosis. (J) The tumor consists of a dense, homogenous micro- and dark-cellular tissue without further differentiation. (K) The arrow indicates an area of phagocytic reaction with foam cells and multinuclear giant cells with incorporated cholesterol spicules.

α -*Mhc* promoter driven *Cre* transgenes (*Gata4**fl/fl* α -*Mhc*-*Cre*) to achieve conditional cardiac *Gata4* deletion. The heart of *Gata4**fl/fl* α -*Mhc*-*Cre* mice developed morphologically normal and showed a 70% suppression of cardiac GATA4 levels, which is therefore most appropriate for comparison with our si*Gata4* transgenic mouse. Other models with heterozygous *Gata4* insufficiency show disorders in cardiac development (Bisping *et al.* 2006, Jay *et al.* 2007). The difference, however, is that in the haploinsufficiency studies the phenotype due to reduction in *Gata4* expression could mainly result from developmental defects, while in transgenic mice and in *Gata4**fl/fl* α -*Mhc*-*Cre* mice expression of *Gata4* during development was normal. Our si*Gata4* transgenic mice demonstrate that under normal conditions the depletion of up to 80% of *Gata4* does not result in cardiac failure in a morphologically normal developed heart. These results may have high clinical impact, since GATA4 mRNA and protein levels vary by approximately twofold between normal and failing human hearts (Hall *et al.* 2004), and doxorubicin, which is broadly used for cancer treatment, causes cardiotoxic heart failure due to GATA4 depletion in wild-type mice (Aries *et al.* 2004). Of note, in models of pressure overload or exercise

stimulation GATA4 depletion of about 70% (*Gata4**fl/fl* α -*Mhc*-*Cre* mice) results in cardiac decompensation and dilatation (Oka *et al.* 2006). Another transgenic mouse strain analyzed by Oka *et al.* (2006), in which *Gata4*-*loxP*-targeted mice were crossed with the β -*Mhc* promoter driven *Cre*, revealed a 90–95% depletion of GATA4 protein and exhibited cardiac insufficiency even under normal (non-stress induced) conditions.

To further elucidate molecular consequences of *in vivo* *Gata4* suppression, we examined three heart-specific genes that were previously identified to be regulated by GATA4. Among these genes, *Bmp4* and *Anf* were expressed at lower levels in transgenic mice compared with wild-type mice, while the α -*Mhc* gene was expressed at similar levels (Fig. 3). It is possible that suppression of *Anf* and *Bmp4* requires lower concentrations of GATA4 compared with α -*Mhc*. This may also be due to differences in the affinity of GATA4 to regulatory elements of these genes. Alternatively, other activities may compensate for decreased levels of *Gata4* in α -*Mhc* gene expression. GATA6 is a candidate factor that has been discussed to compensate for GATA4 deficiency in various experimental models (Kuo *et al.* 1997, Morrissey *et al.* 1998, Bisping *et al.* 2006, Oka *et al.* 2006). Our data indicate that *Gata6* expression

is unaltered in si*Gata4* transgenic mice (Fig. 3E). In conclusion, our transgenic model together with previous studies provides evidence that a critical level of *Gata4* is required for the function of the non-stressed adult heart and that 20% of normal *Gata4* levels is sufficient for heart function (Bisping *et al.* 2006, Oka *et al.* 2006).

***In vivo* evidence for a role of *Gata4* in reproduction**

Our observation of a reduced breeding efficacy in the si*Gata4* transgenic colony and previous observations of *Gata4* mRNA expression in the gonads of developing and adult mice (Arceci *et al.* 1993, Morrissey *et al.* 1996) led us to examine the expression of *Gata4* and putative target genes in the testis and ovary. Of note, reduction of *Gata4* expression was more pronounced in the gonads compared with heart (Fig. 4).

Induced expression of *Gata4* shRNA resulted in a significant reduction of *STAR* and *MIS* gene expression in the testis. Very recently, *Gata4* has been found to be required for sex steroidogenic cell development in the fetal mouse (Bielinska *et al.* 2007). In the developing and adult male gonad of mouse and human, GATA4 is expressed in somatic cells, including testicular, Leydig, and Sertoli cells. *Gata4* mRNA levels were found to decrease during postnatal development in Sertoli, but not in Leydig cells (Viger *et al.* 1998, Ketola *et al.* 2000, 2002). Additionally, GATA4 expression has been found – although somewhat inconsistently – in human and murine fetal germ cells and human spermatogonia (Arceci *et al.* 1993, Viger *et al.* 1998, Ketola *et al.* 2000). In human germ cells, however, GATA4 expression is down-regulated after puberty (Ketola *et al.* 2000). Owing to embryonic lethality of *Gata4*^{-/-} mice (Kuo *et al.* 1997, Molkentin *et al.* 1997) and gonadal sex reversal in mice with only a single functional allele of *Gata4* (*Gata4*^{ki/+} XY^{AKL} mice that develop ovaries or ovotestes; Bouma *et al.* 2007), data on the *in vivo* implication of GATA4 in the normal developed gonads are limited (Kuo *et al.* 1997, Molkentin *et al.* 1997). However, it has been shown that interactions between GATA4 and its co-factor FOG2 and correct dosage of both factors are necessary for normal testicular development (Tevosian *et al.* 2002, Bouma *et al.* 2007). Most recent data indicate that *Gata4* is required in a cell autonomous fashion for proper differentiation of Leydig cells, independently from Sertoli cell-derived factors (Bielinska *et al.* 2007). Since normal GATA4 expression in Leydig cells increases when puberty starts and Leydig cells produce androgens necessary for spermatogenesis (Ketola *et al.* 2000), reduced fertility in the si*Gata4* transgenes may be caused by a defect in Leydig cell function. This is supported by the observation that GATA4 downstream target genes involved in gonadal somatic cell function and

steroidogenesis, including *Mis* or *Star* (Tremblay & Viger 1999, Hiroi *et al.* 2004), were down-regulated in si*Gata4* induced mice. An additional plausible mechanism for disorders in spermatogenesis is a disturbed activation of the reproductive homeobox on the X chromosome gene by *Gata4* deficiency in Sertoli cells (Bhardwaj *et al.* 2008). In humans, data on disorders in the development or function of the male gonads have not been associated with GATA4 yet, but appear likely, since some mutations affect the activity of target gonadal promoters *in vitro* (Bouchard *et al.* 2009). Of note, the currently known GATA4 mutations seem to retain their ability to interact and to cooperate with major gonadal partners (SF1-NR5A1 and LRH-1/NR5A2), thereby partially compensating the loss of intrinsic GATA4 transcriptional activity (Bouchard *et al.* 2009). Moreover, in androgen resistance, GATA4 expression in Sertoli and germ cells is weak or totally absent (Ketola *et al.* 2000).

In the murine ovary, *Gata4* expression has been detected in granulosa and thecal cells (Heikinheimo *et al.* 1997), where it is regulated by gonadotrophins (Kwintkiewicz *et al.* 2007). Interestingly, FSH increases transcription of the *Gata4* gene (Kwintkiewicz *et al.* 2007). While *Gata4* is abundantly expressed in granulosa cells of primary and early antral follicles during the period of active proliferation, it is rapidly down-regulated when granulosa cell proliferation ceases at ovulation, atresia, or luteinization (Heikinheimo *et al.* 1997, Laitinen *et al.* 2000). Low levels of *Gata4* mRNA are also expressed in the germinal epithelium and interstitial cells (Heikinheimo *et al.* 1997). We observed only minor changes in *Star* and *Mis* mRNA levels in the ovaries of mice expressing *Gata4* siRNA (Fig. 4C). The lack of *Mis* suppression in the ovary (Fig. 4C) may confirm previous *in vitro* data indicating that GATA4 and FOG2 play a distinctive role in the ovary. FOG2 is able to repress the GATA4 mediated *in vitro* transactivation of the *Mis* promoter (Anttonen *et al.* 2003). Thus, *Gata4* siRNA may not affect *Mis* gene expression in the adult ovary. Although GATA4 has been shown to control genes that are involved in the maturation and/or maintenance of granulosa cells within early follicles (Heikinheimo *et al.* 1997), normal levels of FSH and LH make it rather unlikely that female fertility is affected by *Gata4* suppression.

Ovarian teratoma in female si*Gata4* transgenic mice indicate a role of *Gata4* as tumor suppressor

The data presented here demonstrate for the first time that *Gata4* deficiency may play a role in tumorigenesis of the ovary. A total of 10% doxycycline induced *Gata4* shRNA transgenic female mice developed ovarian teratoma (Fig. 5), while no tumor formation was observed in non-transgenic mice, non-transgenic mice

treated with doxycycline, or in transgenic mice not exposed to doxycycline. There is currently no evidence from more than 16 transgenic mouse models of stable, inducible, or conditional RNAi for the development of teratoma as a non-specific side effect (Hasuwa *et al.* 2002, Oberdoerffer *et al.* 2005, Seibler *et al.* 2005) for review: Kühn *et al.* (2007). Although the mature and immature teratomas in *Gata4* shRNA transgenic mice contained structures from all three germ layers, the contribution from the endoderm was rather low. The teratomas observed here are similar to those formed in nude mice after injection of *Gata4* deficient ES cells (Soudais *et al.* 1995). GATA4 expression has been described in pediatric yolk sac tumors originating from either gonadal or extragonadal sites, but not in mature teratomas (Siltanen *et al.* 1999). The role of GATA4 in tumorigenesis is not well understood and may vary depending on the tissue from which the tumor originates. On the one hand, GATA4 has been suggested as a tumor suppressor gene because epigenetic silencing of the *GATA4* gene resulted in the down-regulation of anti-tumor target genes. For example, in gastrointestinal tumor cell lines it became evident that silencing of anti-tumor genes (*trefoil* factors, *inhibin- α* and *disabled-2*) due to GATA4 downregulation, methylation, or histone modifications is involved in tumor cell proliferation (Akiyama *et al.* 2003, Wakana *et al.* 2006, Viger *et al.* 2008). On the other hand, GATA4 also exhibits anti-apoptotic activity (Grepin *et al.* 1997, Laitinen *et al.* 2000, Aries *et al.* 2004). Nonetheless, the si*Gata4* transgenic animals studied here provide the first direct *in vivo* evidence for an increased risk of ovarian teratoma formation due to reduced expression of GATA4 or one of its target genes. In human females with Ala189Val FSH receptor mutations, a complete block of FSH action is associated with a strong reduction of GATA4 protein expression in the ovary. In these patients an increased risk for teratoma formation (or generation of other ovary associated tumors) should be considered (Vaskivuo *et al.* 2002).

In conclusion, our study demonstrates that transgenic mice expressing shRNA represents a useful model system to study the effect of reduced transcription factor expression in adult mice. We provide *in vivo* evidence that *Gata4* exhibits a dose-dependent effect on cardiac function. Of note, GATA4 differentially affects downstream targets in the male versus female gonads. Finally, our results suggest a higher risk of developing ovary-associated teratomas in *Gata4* deficiency. These findings should be considered in counseling patients with inherited or acquired GATA4 deficiency.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Funding

American Heart Association and the National Institutes of Health (RO1-DK052356 to J B); Studienstiftung des Deutschen Volkes (Fellowship to B T); Deutscher Akademischer Austauschdienst (Fellowship to B T); Fritz Thyssen Stiftung (AZ. 10-05-2-162 to C D).

Acknowledgements

We thank Dr van der Wetering for providing the pTER+ plasmid, and Mike Rule and the UF transgenic core facility for generating the transgenic mice. We also thank Dr Hideko Kasahara of UF for performing the electrocardiography. We also appreciate the discussion of the data and the manuscript by the members of our laboratories, in particular Hannes Sallmon and Martin Klar.

References

- Akiyama Y, Watkins N, Suzuki H, Jair KW, van Engeland M, Esteller M, Sakai H, Ren CY, Yuasa Y, Herman JG *et al.* 2003 GATA-4 and GATA-5 transcription factor genes and potential downstream antitumor target genes are epigenetically silenced in colorectal and gastric cancer. *Molecular and Cellular Biology* **23** 8429–8439.
- Anttonen M, Ketola I, Parviainen H, Pusa AK & Heikinheimo M 2003 FOG-2 and GATA-4 are coexpressed in the mouse ovary and can modulate Mullerian-inhibiting substance expression. *Biology of Reproduction* **68** 1333–1340.
- Arceci RJ, King AA, Simon MC, Orkin SH & Wilson DB 1993 Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. *Molecular and Cellular Biology* **13** 2235–2246.
- Aries A, Paradis P, Lefebvre C, Schwartz RJ & Nemer M 2004 Essential role of GATA-4 in cell survival and drug-induced cardiotoxicity. *PNAS* **101** 6975–6980.
- Bantounas I, Phylactou LA & Uney JB 2004 RNA interference and the use of small interfering RNA to study gene function in mammalian systems. *Journal of Molecular Endocrinology* **33** 545–557.
- Bhardwaj A, Rao MK, Kaur R, Buttigieg MR & Wilkinson MF 2008 GATA factors and androgen receptor collaborate to transcriptionally activate the *Rhox5* homeobox gene in Sertoli cells. *Molecular and Cellular Biology* **28** 2138–2153.
- Bielinska M, Seehra A, Toppa J, Heikinheimo M & Wilson DB 2007 GATA-4 is required for sex steroidogenic cell development in the fetal mouse. *Developmental Dynamics* **236** 203–213.
- Bisping E, Ikeda S, Kong SW, Tarnavski O, Bodyak N, McMullen JR, Rajagopal S, Son JK, Ma Q, Springer Z *et al.* 2006 Gata4 is required for maintenance of postnatal cardiac function and protection from pressure overload-induced heart failure. *PNAS* **103** 14471–14476.
- Bouchard MF, Taniguchi H & Viger RS 2009 The effect of human GATA4 gene mutations on the activity of target gonadal promoters. *Journal of Molecular Endocrinology* **42** 149–160.
- Bouma GJ, Washburn LL, Albrecht KH & Eicher EM 2007 Correct dosage of *Fog2* and *Gata4* transcription factors is critical for fetal testis development in mice. *PNAS* **104** 14994–14999.
- Bridge AJ, Pebernard S, Ducraux A, Nicoulaz AL & Iggo R 2003 Induction of an interferon response by RNAi vectors in mammalian cells. *Nature Genetics* **34** 263–264.
- Bungert J, Dave U, Lim KC, Lieuw KH, Shavit JA, Liu Q & Engel JD 1995 Synergistic regulation of human beta-globin gene switching by locus control region elements HS3 and HS4. *Genes and Development* **9** 3083–3096.
- Bungert J, Tanimoto K, Patel S, Liu Q, Fear M & Engel JD 1999 Hypersensitive site 2 specifies a unique function within the human beta-globin locus control region to stimulate globin gene transcription. *Molecular and Cellular Biology* **19** 3062–3072.

- Charron F, Paradis P, Bronchain O, Nemer G & Nemer M 1999 Cooperative interaction between GATA-4 and GATA-6 regulates myocardial gene expression. *Molecular and Cellular Biology* **19** 4355–4365.
- Corbel SY & Rossi FM 2002 Latest developments and *in vivo* use of the Tet system: *ex vivo* and *in vivo* delivery of tetracycline-regulated genes. *Current Opinion in Biotechnology* **13** 448–452.
- Dame C, Sola MC, Lim KC, Leach KM, Fandrey J, Ma Y, Knopfle G, Engel JD & Bungert J 2004 Hepatic erythropoietin gene regulation by GATA-4. *Journal of Biological Chemistry* **279** 2955–2961.
- Dame C, Kirschner KM, Bartz KV, Wallach T, Hussels CS & Scholz H 2006 Wilms tumor suppressor, Wt1, is a transcriptional activator of the erythropoietin gene. *Blood* **107** 4282–4290.
- E LL, Zhao YS, Guo XM, Wang CY, Jiang H, Li J, Duan CM & Song Y 2006 Enrichment of cardiomyocytes derived from mouse embryonic stem cells. *Journal of Heart and Lung Transplantation* **25** 664–674.
- Garg V, Kathiraya IS, Barnes R, Schluterman MK, King IN, Butler CA, Rothrock CR, Eapen RS, Hirayama-Yamada K, Joo K *et al.* 2003 GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5. *Nature* **424** 443–447.
- Grepin C, Dagnino L, Robitaille L, Haberstroh L, Antakly T & Nemer M 1994 A hormone-encoding gene identifies a pathway for cardiac but not skeletal muscle gene transcription. *Molecular and Cellular Biology* **14** 3115–3129.
- Grepin C, Nemer G & Nemer M 1997 Enhanced cardiogenesis in embryonic stem cells overexpressing the GATA-4 transcription factor. *Development* **124** 2387–2395.
- Hall JL, Grindle S, Han X, Fermin D, Park S, Chen Y, Bache RJ, Mariash A, Guan Z, Ormaza S *et al.* 2004 Genomic profiling of the human heart before and after mechanical support with a ventricular assist device reveals alterations in vascular signaling networks. *Physiological Genomics* **17** 283–291.
- Hasuwa H, Kaseda K, Einarsson T & Okabe M 2002 Small interfering RNA and gene silencing in transgenic mice and rats. *FEBS Letters* **532** 227–230.
- Heikinheimo M, Ermolaeva M, Bielinska M, Rahman NA, Narita N, Huhtaniemi IT, Tapanainen JS & Wilson DB 1997 Expression and hormonal regulation of transcription factors GATA-4 and GATA-6 in the mouse ovary. *Endocrinology* **138** 3505–3514.
- Hirayama-Yamada K, Kamisago M, Akimoto K, Aotsuka H, Nakamura Y, Tomita H, Furutani M, Inamura S, Takao A, Nakazawa M *et al.* 2005 Phenotypes with GATA4 or NKX2.5 mutations in familial atrial septal defect. *American Journal of Medical Genetics. Part A* **135** 47–52.
- Hiroi H, Christenson LK & Strauss JF III 2004 Regulation of transcription of the steroidogenic acute regulatory protein (StAR) gene: temporal and spatial changes in transcription factor binding and histone modification. *Molecular and Cellular Endocrinology* **215** 119–126.
- Jay PY, Bielinska M, Erlich JM, Mannisto S, Pu WT, Heikinheimo M & Wilson DB 2007 Impaired mesenchymal cell function in Gata4 mutant mice leads to diaphragmatic hernias and primary lung defects. *Developmental Biology* **301** 602–614.
- Ketola I, Pentikainen V, Vaskivuo T, Ilvesmaki V, Herva R, Dunkel L, Tapanainen JS, Toppari J & Heikinheimo M 2000 Expression of transcription factor GATA-4 during human testicular development and disease. *Journal of Clinical Endocrinology and Metabolism* **85** 3925–3931.
- Ketola I, Anttonen M, Vaskivuo T, Tapanainen JS, Toppari J & Heikinheimo M 2002 Developmental expression and spermatogenic stage specificity of transcription factors GATA-1 and GATA-4 and their cofactors FOG-1 and FOG-2 in the mouse testis. *European Journal of Endocrinology* **147** 397–406.
- Kühn R, Streif S & Wurst W 2007 RNA interference in mice. *Handbook of Experimental Pharmacology* **178** 147–174.
- Kuo CT, Morrissey EE, Anandappa R, Sigrist K, Lu MM, Parmacek MS, Soudais C & Leiden JM 1997 GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes and Development* **11** 1048–1060.
- Kwintkiewicz J, Cai Z & Stocco C 2007 Follicle-stimulating hormone-induced activation of Gata4 contributes in the up-regulation of Cyp19 expression in rat granulosa cells. *Molecular Endocrinology* **21** 933–947.
- Laitinen MP, Anttonen M, Ketola I, Wilson DB, Ritvos O, Butzow R & Heikinheimo M 2000 Transcription factors GATA-4 and GATA-6 and a GATA family cofactor, FOG-2, are expressed in human ovary and sex cord-derived ovarian tumors. *Journal of Clinical Endocrinology and Metabolism* **85** 3476–3483.
- Li G, Lim KC, Engel JD & Bungert J 1998 Individual LCR hypersensitive sites cooperate to generate an open chromatin domain spanning the human beta-globin locus. *Genes to Cells* **3** 415–429.
- Li L, Takemura G, Li Y, Miyata S, Esaki M, Okada H, Kanamori H, Khai NC, Maruyama R, Ogino A *et al.* 2006 Preventive effect of erythropoietin on cardiac dysfunction in doxorubicin-induced cardiomyopathy. *Circulation* **113** 535–543.
- Lowry JA & Atchley WR 2000 Molecular evolution of the GATA family of transcription factors: conservation within the DNA-binding domain. *Journal of Molecular Evolution* **50** 103–115.
- Manna PR, Pakarinen P, El-Hefnawy T & Huhtaniemi IT 1999 Functional assessment of the calcium messenger system in cultured mouse Leydig tumor cells: regulation of human chorionic gonadotropin-induced expression of the steroidogenic acute regulatory protein. *Endocrinology* **140** 1739–1751.
- Manuylov NL, Fujiwara Y, Adameyko II, Poulat F & Tevosian SG 2007 The regulation of Sox9 gene expression by the GATA4/FOG2 transcriptional complex in dominant XX sex reversal mouse models. *Developmental Biology* **307** 356–367.
- Molkentin JD 2000 The zinc finger-containing transcription factors GATA-4, -5, and -6. Ubiquitously expressed regulators of tissue-specific gene expression. *Journal of Biological Chemistry* **275** 38949–38952.
- Molkentin JD, Kalvakolanu DV & Markham BE 1994 Transcription factor GATA-4 regulates cardiac muscle-specific expression of the alpha-myosin heavy-chain gene. *Molecular and Cellular Biology* **14** 4947–4957.
- Molkentin JD, Lin Q, Duncan SA & Olson EN 1997 Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes and Development* **11** 1061–1072.
- Morrissey EE, Ip HS, Lu MM & Parmacek MS 1996 GATA-6: a zinc finger transcription factor that is expressed in multiple cell lineages derived from lateral mesoderm. *Developmental Biology* **177** 309–322.
- Morrissey EE, Tang Z, Sigrist K, Lu MM, Jiang F, Ip HS & Parmacek MS 1998 GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. *Genes and Development* **12** 3579–3590.
- Nemer G & Nemer M 2003 Transcriptional activation of BMP-4 and regulation of mammalian organogenesis by GATA-4 and -6. *Developmental Biology* **254** 131–148.
- Nemer G, Fadlalah F, Usta J, Nemer M, Dbaibo G, Obeid M & Bitar F 2006 A novel mutation in the GATA4 gene in patients with tetralogy of Fallot. *Human Mutation* **27** 293–294.
- Oberdoerffer P, Kanellopoulou C, Heissmeyer V, Paeper C, Borowski C, Aifantis I, Rao A & Rajewsky K 2005 Efficiency of RNA interference in the mouse hematopoietic system varies between cell types and developmental stages. *Molecular and Cellular Biology* **25** 3896–3905.
- Oka T, Maillet M, Watt AJ, Schwartz RJ, Aronow BJ, Duncan SA & Molkentin JD 2006 Cardiac-specific deletion of Gata4 reveals its requirement for hypertrophy, compensation, and myocyte viability. *Circulation Research* **98** 837–845.
- Okubo A, Miyoshi O, Baba K, Takagi M, Tsukamoto K, Kinoshita A, Yoshiura K, Kishino T, Ohta T, Niikawa N *et al.* 2004 A novel GATA4 mutation completely segregated with atrial septal defect in a large Japanese family. *Journal of Medical Genetics* **41** e97.
- Pehlivan T, Pober BR, Brueckner M, Garrett S, Slaugh R, Van Rheaden R, Wilson DB, Watson MS & Hing AV 1999 GATA4

- haploinsufficiency in patients with interstitial deletion of chromosome region 8p23.1 and congenital heart disease. *American Journal of Medical Genetics* **83** 201–206.
- Pu WT, Ishiwata T, Juraszek AL, Ma Q & Izumo S 2004 GATA4 is a dosage-sensitive regulator of cardiac morphogenesis. *Developmental Biology* **275** 235–244.
- Rajagopal SK, Ma Q, Obler D, Shen J, Manichaikul A, Tomita-Mitchell A, Boardman K, Briggs C, Garg V, Srivastava D *et al.* 2007 Spectrum of heart disease associated with murine and human GATA4 mutation. *Journal of Molecular and Cellular Cardiology* **43** 677–685.
- Rivera Z, Christian PJ, Marion SL, Brooks HL & Hoyer PB 2009 Steroidogenic capacity of residual ovarian tissue in 4-vinylcyclohexene diepoxide-treated mice. *Biology of Reproduction* **80** 328–336.
- Sarkozy A, Conti E, Neri C, D'Agostino R, Digilio MC, Esposito G, Toscano A, Marino B, Pizzuti A & Dallapiccola B 2005 Spectrum of atrial septal defects associated with mutations of NKX2.5 and GATA4 transcription factors. *Journal of Medical Genetics* **42** e16.
- Sasaguri H, Mitani T, Anzai M, Kubodera T, Saito Y, Yamada H, Mizusawa H & Yokota T 2009 Silencing efficiency differs among tissues and endogenous microRNA pathway is preserved in short hairpin RNA transgenic mice. *FEBS Letters* **583** 213–218.
- Schluterman MK, Krysiak AE, Kathiriyi IS, Abate N, Chandalia M, Srivastava D & Garg V 2007 Screening and biochemical analysis of GATA4 sequence variations identified in patients with congenital heart disease. *American Journal of Medical Genetics. Part A* **143** 817–823.
- Seibler J, Kuter-Luks B, Kern H, Streu S, Plum L, Mauer J, Kuhn R, Bruning JC & Schwenk F 2005 Single copy shRNA configuration for ubiquitous gene knockdown in mice. *Nucleic Acids Research* **33** e67.
- Seibler J, Kleinriders A, Kuter-Luks B, Niehaves S, Bruning JC & Schwenk F 2007 Reversible gene knockdown in mice using a tight, inducible shRNA expression system. *Nucleic Acids Research* **35** e54.
- Siltanen S, Anttonen M, Heikkilä P, Narita N, Laitinen M, Ritvos O, Wilson DB & Heikinheimo M 1999 Transcription factor GATA-4 is expressed in pediatric yolk sac tumors. *American Journal of Pathology* **155** 1823–1829.
- Sledz CA, Holko M, de Veer MJ, Silverman RH & Williams BR 2003 Activation of the interferon system by short-interfering RNAs. *Nature Cell Biology* **5** 834–839.
- Soudais C, Bielinska M, Heikinheimo M, MacArthur CA, Narita N, Saffitz JE, Simon MC, Leiden JM & Wilson DB 1995 Targeted mutagenesis of the transcription factor GATA-4 gene in mouse embryonic stem cells disrupts visceral endoderm differentiation *in vitro*. *Development* **121** 3877–3888.
- Szulc J, Wiznerowicz M, Sauvain MO, Trono D & Aebischer P 2006 A versatile tool for conditional gene expression and knockdown. *Nature Methods* **3** 109–116.
- Tang ZH, Xia L, Chang W, Li H, Shen F, Liu JY, Wang Q & Liu MG 2006 Two novel missense mutations of GATA4 gene in Chinese patients with sporadic congenital heart defects. *Zhonghua Yi Xue Yi Chuan Xue Za Zhi* **23** 134–137.
- Tevosian SG, Albrecht KH, Crispino JD, Fujiwara Y, Eicher EM & Orkin SH 2002 Gonadal differentiation, sex determination and normal Sry expression in mice require direct interaction between transcription partners GATA4 and FOG2. *Development* **129** 4627–4634.
- Tomita-Mitchell A, Maslen CL, Morris CD, Garg V & Goldmuntz E 2007 GATA4 sequence variants in patients with congenital heart disease. *Journal of Medical Genetics* **44** 779–783.
- Tremblay JJ & Viger RS 1999 Transcription factor GATA-4 enhances Mullerian inhibiting substance gene transcription through a direct interaction with the nuclear receptor SF-1. *Molecular Endocrinology* **13** 1388–1401.
- Tremblay JJ & Viger RS 2001 GATA factors differentially activate multiple gonadal promoters through conserved GATA regulatory elements. *Endocrinology* **142** 977–986.
- Vaskivuo TE, Aittomäki K, Anttonen M, Ruokonen A, Herva R, Osawa Y, Heikinheimo M, Huhtaniemi I & Tapanainen JS 2002 Effects of follicle-stimulating hormone (FSH) and human chorionic gonadotropin in individuals with an inactivating mutation of the FSH receptor. *Fertility and Sterility* **78** 108–113.
- Viger RS, Mertineit C, Trasler JM & Nemer M 1998 Transcription factor GATA-4 is expressed in a sexually dimorphic pattern during mouse gonadal development and is a potent activator of the Mullerian inhibiting substance promoter. *Development* **125** 2665–2675.
- Viger RS, Guittot SM, Anttonen M, Wilson DB & Heikinheimo M 2008 Role of the GATA family of transcription factors in endocrine development, function, and disease. *Molecular Endocrinology* **22** 781–798.
- Wakana K, Akiyama Y, Aso T & Yuasa Y 2006 Involvement of GATA-4/-5 transcription factors in ovarian carcinogenesis. *Cancer Letters* **241** 281–288.
- Wang PY, Koishi K, McGeachie AB, Kimber M, MacLaughlin DT, Donahoe PK & McLennan IS 2005 Mullerian inhibiting substance acts as a motor neuron survival factor *in vitro*. *PNAS* **102** 16421–16425.
- van de Wetering M, Oving I, Pon Fong MT, Brantjes H, van Leenen D, Holstege FC, Brummelkamp TR, Agami R & Clevers H 2003 Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector. *EMBO Reports* **4** 609–615.
- Zeisberg EM, Ma Q, Juraszek AL, Moses K, Schwartz RJ, Izumo S & Pu WT 2005 Morphogenesis of the right ventricle requires myocardial expression of Gata4. *Journal of Clinical Investigation* **115** 1522–1531.

Received in final form 6 May 2009

Accepted 1 June 2009

Made available online as an Accepted Preprint 2 June 2009