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 Bungert1* 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.

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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^{−/−}* kr) 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 (siGata4) 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’-GATCCCCCTCTCGATATGGTTGTGGATGATGA 3’; shGata4-1; construct 2: nt +1242 to +1262; 5’-GATCCGGGCGAGAGATGTGTCATATTTCAAGAAGAATGTGAATCTCTGCCTTTTGGAAA-3’; shScram: 5’-GATCCCGGT TTCAAGAG TTAACCTTTAAATGTGGA 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:shGata4 was digested with SalI 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

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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 Stul-HindIII fragment derived from pTER:shGata4 and corresponds to the H1:G4 transgene. The TetR probe is a 1591 bp XhoI-HindIII fragment derived from pcDNA6/TR and corresponds to the TetR transgene. Transgene copy numbers were determined using the PhosphoImager system (Storage Phosphor Screen, Molecular Dynamics, Amersham Pharmacia Biotech) after hybridizing the nylon membranes for 14 h with a radioactive probe corresponding to the transgene. 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 Stul-HindIII fragment derived from pTER:shGata4 and corresponds to the H1:G4 transgene. The TetR probe is a 1591 bp XhoI-HindIII fragment derived from pcDNA6/TR and corresponds to the TetR transgene. Transgene copy numbers were determined using the PhosphoImager 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 Phospho-Imager (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'-GTCTAGCTGGGACACTGCTG-3', antisense 5'-GATGGGACGGGACACTACCTG-3', 2) TetR transgene sense 5'-GGGCAGACCTTACATT-3', antisense 5'-GGCAACGACTTTCGACTTT-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 μg/μl. First, strand cDNA synthesis was performed with 2 μg 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 (Msi; 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'-GATGGGACGGGACACTGCTG-3', antisense 5'-ACCTGCTGGGCTTCTTAGA TTT-3'; Gata6: sense 5'-TTAACAATGTGCTGCAACGG-3', antisense 5'-GTT CACGCTGAAGCGCTGA-3'; Oas2: sense 5'-CAATAATTGCGGAAA GTGATGG-3', antisense 5'-ACTGGAGCGGTGTTGTTTTTAGAG-3'; β-actin: sense 5'-ACTGCTCTGCTGCTTACAGGAC-3', antisense 5'-ACATCTGGTGAAGGTGGAC-3'; and Gapdh: sense 5'-CCAAGGTCATCCCATGAACAAT-3', antisense 5'-ATACGGGAGCGCTTTCC-3'.

**RNA isolation and real-time PCR**

To induce the expression of sGata4, 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 iCycler iQ Optical System software 3.1. For Gata4 and β-actin, TaqMan Real-Time PCR was performed using commercially available primers (mGata4 Mm00484689_m1 and mβ-actin Mm00484689_m1; 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 μl mixture containing 1 μl cDNA, 10 μl TaqMan, 2 μl PCR Master Mix (Applied Biosystems), 1 μl primer, and 8 μl 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 μg of whole protein extract from C2C12 myoblasts and 50 μg 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-phycerythrin 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-shGata4 plasmid. Double-transfected cells were treated without or with 1, 2.5, or 5 μg/ml doxycycline for 3 or 7 days respectively. Three days after application of 2.5 μg doxycycline per ml medium or alternatively 7 days after treatment with 5 μg doxycycline per ml medium, most efficient reduction in Gata4 protein expression was detected (Fig. 1C). The Gata4 siRNA sequence (TCTCGATATGTTTGA) is almost identical to a human GATA4 siRNA (NCBI accession no. L34357; nt 852–870 relative to the transcription start site: TCTCGATATGTTTGA) 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 (siGata4) suggests that the two constructs co-integrated into the same single locus.
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. 2003, Sledz et al. 2006), 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).
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 Oas2 in both 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.

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

Of note, 2 out of 20 siGata4 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 tumorogenesis 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,
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 siGata4 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 4 Effects of in vivo Gata4 shRNA expression on GATA4 target genes in the reproductive organs.** WT and siGata4 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 ± s.d., *P<0.01) and conventional RT-PCR (right panel) for expression analysis of Mis, Star, Oas2, and β-actin in WT versus siGata4 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 siGata4 TG versus WT mice (left panel; means ± 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 siGata4 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...
α-Mhc promoter driven Cre transgenes (Gata4fl/ftα-Mhc-Cre) to achieve conditional cardiac Gata4 deletion. The heart of Gata4fl/ftα-Mhc-Cre mice developed morphologically normal and showed a 70% suppression of cardiac GATA4 levels, which is therefore most appropriate for comparison with our siGata4 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 Gata4fl/ftα-Mhc-Cre mice expression of Gata4 during development was normal. Our siGata4 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% (Gata4fl/ftα-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, Morrisey et al. 1998, Bisping et al. 2006, Oka et al. 2006). Our data indicate that Gata6 expression...
is unaltered in siGata4 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 siGata4 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+/-XaKI 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 siGata4 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 siGata4 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 siGata4 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-2 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 siGata4 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 in GATA4 transcription factor genes and potential downstream antitumor target genes are epigenetically silenced in colorectal and gastric cancer. Molecular and Cellular Biology 23 8429–8439.


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