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A tissue-specific knockout reveals that Gata1 is not essential for Sertoli cell function in the mouse

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ABSTRACT

The transcription factor Gata1 is essential for the development of erythroid cells. Consequently, Gata1 null mutants die *in utero* due to severe anaemia. Outside the haematopoietic system, Gata1 is only expressed in the Sertoli cells of the testis. To elucidate the function of Gata1 in the testis, we made a Sertoli cell-specific knockout of the *Gata1* gene in the mouse. We deleted a normally functioning 'floxed' *Gata1* gene in pre-Sertoli cells *in vivo* through the expression of Cre from a transgene driven by the *Desert Hedgehog* promoter. Surprisingly, Gata1 null testes developed to be morphologically normal, spermatogenesis was not obviously affected and expression levels of putative Gata1 target genes, and other Gata factors, were not altered. We conclude that expression of Gata1 in Sertoli cells is not essential for testis development or spermatogenesis in the mouse.

INTRODUCTION

The Gata transcription factor family consists of six members (Gata1–6). The family is divided into two groups, the haematopoietic (Gata1, 2 and 3) (1) and the heart and gut group (Gata4, 5 and 6) (2). The members share their highest homology in the Zn-finger DNA-binding domain and they all bind to the consensus site (T/A)GATA(A/G). Outside the DNA-binding domain, they are quite different. Most members are important for the proliferation and differentiation of specific cell types. Gata factors are thought to be important transcriptional regulators of Sertoli cell-specific gene expression (3,4). Many Sertoli cell-specific genes have Gata-binding sites in their promoters (4), and these Gata sites are essential for optimal or appropriate expression of reporter genes (4). Sertoli cells are known to express Gata1, 4 and 6 (5,6).

Gata1 is a tissue-specific transcription factor expressed in erythrocytes, megakaryocytes, eosinophils and mast cells (7–11). The major haematopoietic role of Gata1 is its requirement for the progression of erythroid precursor cells beyond the proerythroblast stage (12). Outside the haematopoietic system, Gata1 is exclusively expressed in the Sertoli cells of the testis (6,13). In the mouse and the rat gene, a haematopoietic and a testis-specific promoter have been described (13). Based on expression analysis, it has been suggested that Gata1 is a developmental stage- and spermatogenic cycle-specific regulator of gene expression in Sertoli cells (5). Although conclusive evidence for Gata1 target genes in Sertoli cells is still lacking, it has been proposed that the gene for anti-Mullerian hormone (Amh), also known as Mullerian-inhibiting substance (Mis), is a Gata target gene (14). The model implies that Gata4 acts as a positive regulator of Amh expression in late fetal/early postnatal pre-Sertoli cells, while Gata1 acts as a repressor of Amh expression at later developmental stages (14,15). The spatio-temporal expression patterns of the factors conform to this model (15,16), and two perfect Gata-binding sites are essential for the function of the Amh promoter in tissue culture cells (15). Furthermore, it has been reported that two human males harbouring a mutation in the *GATA1* gene displayed cryptorchidism (17). Cryptorchidism is also observed in mice overexpressing Amh under the metallothionein-1 promoter (18). However, cryptorchidism has not been reported for X-linked-thrombocytopenic males with different GATA1 mutations (19,20). Other Sertoli cell-specific candidate target genes of Gata1 are the follicle-stimulating hormone receptor (Fshr), inhibin α-subunit and inhibin/activin β-B-subunit. For Fshr, this notion is based on the presence of a conserved Gata site in the core promoter of the gene. This site can bind Gata1 *in vitro*, and is occupied in Sertoli cells as revealed by *in vivo* footprinting (21). The potential role of Gata1 in the expression of the inhibin α-subunit and inhibin/activin β-B-subunit genes is deduced from transient transfection assays with a bacterial reporter gene driven by the corresponding promoters in the rat testicular Leydig tumour cell line MA-10 and the mouse Sertoli cell line MSC-1 (22,23). In these studies, the Gata sites were essential for expression. In addition to this, the expression of the inhibin α-subunit and the inhibin/activin β-B-subunit occurs in a spatio-temporal pattern similar to that of Gata1 (24–26). However, these functional experiments were performed in transiently transfected cell lines, and the results may therefore not reflect the *in vivo* roles of Gata1 appropriately.

The Gata1 null mutation is embryonic lethal due to a block in erythropoiesis at the proerythroblast stage (12). To

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overcome this early embryonic lethality, we created a conditional knockout allele of the *Gata1* gene employing the Cre-loxP system (27). To delete the floxed *Gata1* gene in pre-Sertoli cells, we established transgenic mouse lines expressing Cre recombinase under the control of the *Desert Hedgehog* promoter. This promoter is active in the nervous system and Sertoli cell precursors early in testis development (28). Since it is not active in haematopoietic cells, the floxed *Gata1* gene remains active in erythroid cells of *Gata1*$^{floxed:y}$::*Dhh-Cre* compound animals, thus allowing us to study the role of Gata1 in testis during development and adulthood.

MATERIALS AND METHODS

Construction of the floxed *Gata1* locus

The 3′ homologous region is an EcoRI fragment 3′ from the last exon isolated from the *Gata1* locus-containing cosmid (pTCF 3′m*Gata1*). A BamHI–SalI fragment containing all the coding exons was cloned into a low copy number plasmid (pSC3Z m*Gata1* genomic). In this construct, the 5′ lox site was inserted in an EcoRI site in the intron between the first erythroid non-coding exon (IE) and the second exon (II).

In plasmid pGT1.8 IRES βgeo (29), the IRES and the *lacZ* gene were replaced (SacI–XhoI/blunt) with a green fluorescent protein (GFP) gene from the plasmid phGFP-S65T (SacI–BamHI/blunt). The cassette with the splice acceptor, GFP and the neomycin/G418 selectable marker was excised with SalI.

The 3′ loxP site (in XhoI), the GFP cassette (in SalI) and the 3′ homologous region (in EcoRI) were put together in pBluescript and taken out with SpeI and PvuI. This fragment was inserted in the SalI site (3′ of the sixth and last exon) of the pSC3Z m*Gata1* gene with the 5′ loxP site (see Fig. 1). The construct was linearised with SpeI and used to electroporate E14 embryonic stem (ES) cells.

ES cells

E14 ES cells were electroporated with the targeting construct and grown in medium containing G418 to select for integration of the neomycin gene. Approximately 300 clones were picked and screened by Southern blotting for homologous recombination in the *Gata1* locus. The genomic DNA was digested with XbaI and the blot was hybridised with a probe (EcoRI–XbaI fragment) that is adjacent to the 3′ homology region (Fig. 1). Positive ES cell clones were transfected with a vector expressing Cre under the PGK promoter and a hygromycin resistance marker. Clones were screened for recombination by Southern blot analysis, using a probe (BamHI–NcoI fragment) just before the 5′ loxP site (Fig. 1).

DNA constructs and mice

We inserted the SV40 large T antigen nuclear localisation signal (NLS) at the N-terminus of Cre (30). The *Dhh-Cre* construct was prepared by introducing NLS-Cre in the first exon of the mouse *Dhh* gene, at the NcoI site coinciding with the initiation codon of the Dhh protein (28). A 20 kb NotI fragment was purified and used to generate transgenic mice according to standard procedures (Fig. 2A). The spatio-temporal expression pattern of Cre was assessed using the ROSA26 reporter strain *R26R* in which expression of the *lacZ* reporter gene is dependent on Cre-mediated recombination (31). Testes were isolated from *Dhh-Cre*/*R26R* compound males at day 18 of embryonic development, and day 15 after birth, and stained for β-galactosidase activity as described previously (32).

Chimeric mice were generated by injecting ES cells from the clones described above into C57BL/6 blastocysts. Chimeras were bred with wild-type C57BL/6 mice and screened for germline transmission. *Gata1*$^{floxed:floxed}$ female mice were mated to FVB males carrying the *Dhh-Cre* transgene to generate *Gata1*$^{floxed:y}$::*Dhh-Cre* compound transgenic males. To obtain sperm counts, seminal vesicles were isolated, flushed with phosphate-buffered saline (PBS), and a fraction of the cells was counted manually under the microscope.

RT–PCR and RQ–PCR

RNA was isolated with the tri reagent procedure according to the manufacturer's instructions (Sigma, St Louis, MO). A 5 µg aliquot of RNA was used for the reverse transcription reaction with oligo(dT) (18mer) and random hexamers. Real-time quantitative (RQ)–PCR was performed in triplicate reactions on a Bio Rad iCycler machine, using the Eurogentec (Seraing, Belgium) SYBR green kit. The oligonucleotides used for RT–PCR and RQ–PCR are listed in Table 1.

RESULTS

Introduction of loxP sites in the mouse *Gata1* locus

To create a 'floxed' allele of the mouse *Gata1* gene, we designed a targeting construct in which the coding exons of the *Gata1* gene are flanked by loxP sites (Fig. 1A). The 5′ loxP site was inserted in an EcoRI site in the first intron, between erythroid exon 1 and exon 2. The 3′ loxP was placed behind exon VI in the 3′-flanking region of the gene. The integrity and orientation of the loxP sites were determined by sequencing. A splice acceptor sequence, GFP cDNA and neomycin-selectable marker gene were introduced 3′ to the downstream loxP site, with the aim of activating GFP expression after Cre-mediated excision of the Gata1 coding sequences. E14 ES cells were electroporated with the targeting construct, and transformants were selected by growth in medium containing G418. Homologous recombination events were scored by Southern blot analysis. This revealed that of approximately 300 clones analysed, ~10% had recombined correctly at the 3′ end of the construct. Several clones were transfected with a Cre expression vector and screened for recombination. In one of these, we could demonstrate appropriate recombination (Fig. 1B). This clone was karyotyped and used for blastocyst injections. Chimeric mice were born and mated with C57BL/6 females. Offspring were checked for the conditional knockout ('floxed') *Gata1* locus and backcrossed to FVB mice. In further generations, hemizygous and homozygous floxed *Gata1* mice were born according to Mendelian distribution. These mice were healthy and fertile with no apparent phenotype, indicating that expression of Gata1 was not disturbed by the presence of two loxP sites and the GFP–neo cassette in the locus.

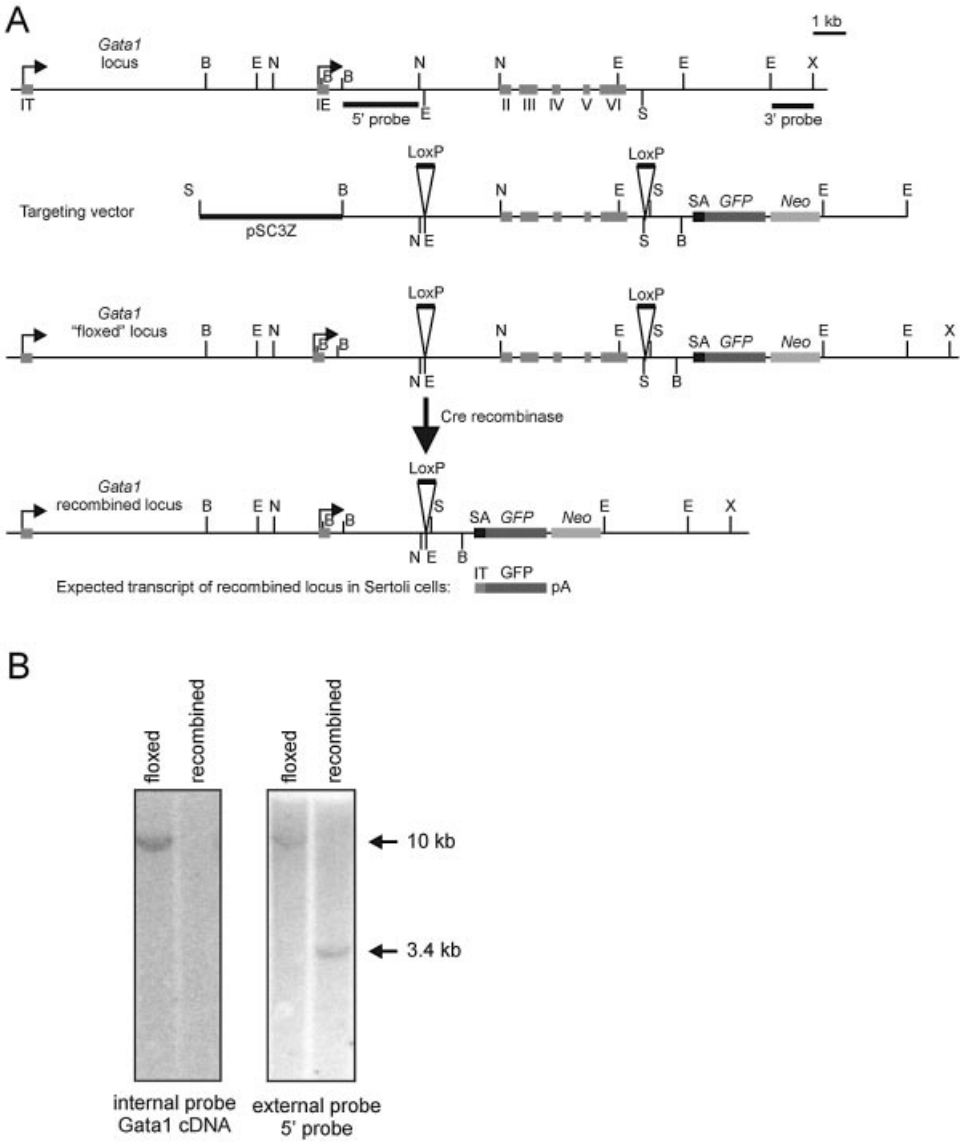

Figure 1. A conditional allele of the *Gata1* gene. (**A**) The mouse *Gata1* locus, with the testis-specific first exon (IT) ~8 kb upstream of the erythroid-specific first exon (IE). The coding exons (II–VI) are common to both mRNAs. After homologous recombination with the targeting vector, the *Gata1* locus has two loxP sites and the splice acceptor/GFP cassette inserted, and is referred to as the 'floxed' locus. After recombination of the floxed *Gata1* locus, the coding exons are deleted and the GFP transcript should be expressed. (**B**) Southern blot of BamHI-digested DNA from ES cells harbouring the floxed *Gata1* locus, before and after Cre transfection. Left panel: the blot was hybridised with a Gata1 cDNA probe. Because the coding sequences are removed upon recombination, no band is detected with the cDNA probe after recombination. Right panel: same blot, re-hybridised with the 5′ probe (A). The size of the 10 kb band is reduced to 3.4 kb after recombination.

Expression of Cre recombinase in Sertoli cells

To drive expression of Cre recombinase in the Sertoli cell compartment of the testis, we used an ~19 kb fragment of the *Dhh* gene. We introduced an NLS of the SV40 large T antigen at the N-terminus of Cre (30), and cloned this modified version of Cre in the initiation codon of the Desert Hedgehog protein (Fig. 2A). Vector sequences were removed by NotI digestion, and the ~20 kb insert was purified and used to generate transgenic mice. Two independent transgenic lines were obtained. To validate the expression pattern of Cre in these mice, they were crossed with a reporter strain in which expression of the bacterial gene for β-galactosidase (*lacZ*) is dependent upon Cre-mediated recombination [*R26R* (31)]. Since the reporter construct is knocked into the *ROSA26* locus, activation of lacZ expression can be monitored in every cell type at every stage of development (31). We isolated testes from male *Dhh-Cre/R26R* embryos at day 18 of gestation (E18), i.e. before Gata1 expression is activated, and at day 15 after birth (P15) when Gata1 expression peaks in Sertoli cells, just before the onset of spermatogenesis (5,6). As controls, we used testes from males carrying only the *Dhh-Cre* transgene or the *R26R* allele. We performed staining for lacZ activity on whole mounts of these testes. In the controls, no lacZ activity could be detected (data not shown). This is in contrast to the *Dhh-Cre/R26R* testes, which displayed strong blue staining,

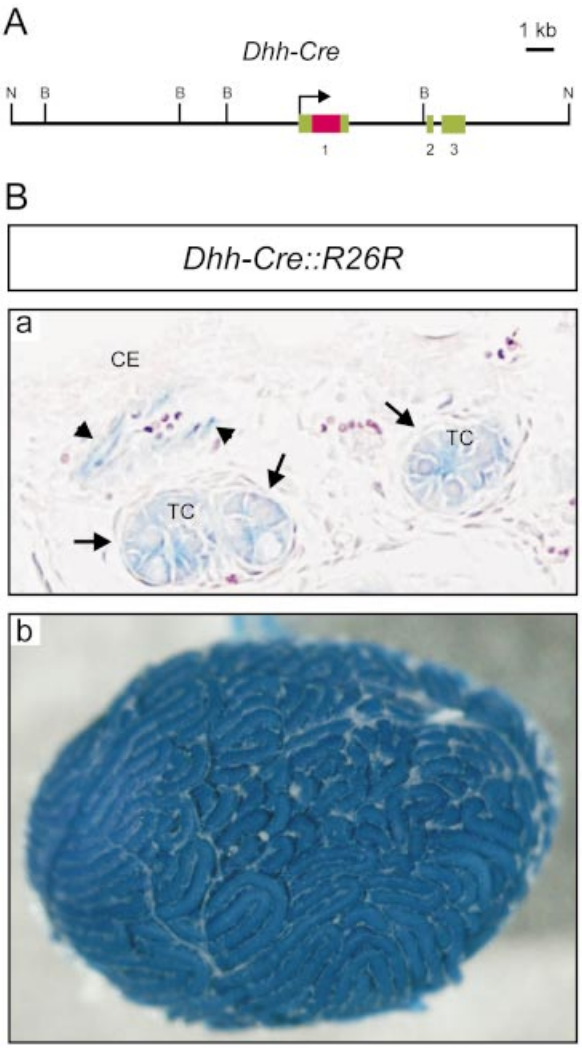

Figure 2. Expression of Cre in Sertoli cells driven by *Dhh* genomic sequences. (**A**) *Dhh-Cre* construct used to make transgenic mice. *Dhh* exons are in green; Cre-encoding sequences in red. B = BamHI; N = NotI. (**B**) (a) LacZ-stained section of E18 *Dhh-Cre/R26R* transgenic testis. CE = coelomic epithelium; TC = testis cords. Arrows point to pre-Sertoli cells; arrowheads indicate endothelial cells. Original magnification 25×. (b) LacZ-stained whole mount of P15 *Dhh-Cre/R26R* transgenic testis. All testis cords are stained intensely blue. Original magnification 2.5×.

indicating that the *lacZ* gene had been activated through Cre-mediated recombination (Fig. 2B). In cross-sections of E18 testes, we observed that staining was restricted to the pre-Sertoli cells in the testis cords, and endothelial cells of the walls of the blood vessels (Fig. 2B, a). This is in agreement with the expected expression pattern of the *Dhh* gene in the testis (28). Importantly, we observe blue staining of all the pre-Sertoli cells in consecutive sections across the testis. This demonstrates that the *Dhh-Cre* transgene is expressed pancellularly in pre-Sertoli cells, before Gata1 expression is activated. Furthermore, the same staining pattern is found at P15, just before the onset of spermatogenesis when Gata1 is expressed abundantly in the Sertoli cells. A whole mount of P15 testis is shown in Figure 2B, b. We conclude that the *Dhh-Cre* transgenics can be used to delete the floxed *Gata1* gene in the Sertoli cell compartment. Since the *Dhh-Cre* transgene is already active in pre-Sertoli cells, i.e. at a stage preceding the normal activation of Gata1 expression in this lineage, it can be used to assess the role of Gata1 in Sertoli cell function. Although the *Dhh-Cre* transgene is also expressed in other cell types, e.g. endothelial cells (Fig. 2B, b) and Schwann cells of the peripheral nervous system (M.Jaegle, M.Uyl and D.Meijer, in preparation), these cell types never express Gata1 and deletion of the *Gata1* gene would therefore be of no consequence to these cell types. Since we obtained essentially the same results with both *Dhh-Cre* transgenic lines, we used one of the lines (line a2) for the experiments described below.

Table 1. Oligonucleotides used for RT–PCR and RQ–PCR

Gene	Sense and antisense primer	Product size (bp)
Amh	5′-AGCTGGACACCATGCCTTTC-3′	
	5′-AGGGTCTCTAGGAAGGGGTC-3′	88
Cyclophilin A	5′-TCACCATTTCCGACTGTGGAC-3′	
	5′-ACAGGACATTGCGAGCAGATG-3′	120
Cyp 19	5′-TCGAGTACTTCCCTAAGCCC-3′	
	5′-GCCAAAAGGCTGAAAGTACC-3′	83
Fshr	5′-GCAAACTGGAGGCGGCAAAC-3′	
	5′-TCTGATCCCCAGGCTGAGTC-3′	97
Gata1	5′-TCCTCTGCATCAACAAGCCCA-3′	
	5′-GTTGAGCAGTGGATACACCTG-3′	297
Gata2	5′-AGAACCGGCCGCTCATCAAG-3′	
	5′-GGTGGTGGTTGTCGTCTGAC-3′	92
Gata4	5′-TCTCACTATGGGCACAGCAG-3′	
	5′-ACAGCACTGGATGGATGGAG-3′	82
Gata6	5′-ATCCCAGAACCCATTCATCC-3′	
	5′-AGACCAAATGGCTCCCAGTG-3′	82
Hprt	5′-CACAGGACTAGAACACCTGC-3′	
	5′-GCTGGTGAAAAGGACCTCTC-3′	247
Inhibin-α	5′-CTCCCAGGCTATCCTTTTCC-3′	
	5′-AGTGAAGAGGCCTTCCTCAG-3′	97
Inhibin-β-A	5′-AGCAGACCTCGGAGATCATC-3′	
	5′-CTGCCTTCCTTGGAAATCTC-3′	82
Inhibin-β-B	5′-AAACAATCCTTCGAGTGGCC-3′	
	5′-GGGTGTGTACGGACAGAAAAG-3′	98
Star	5-GGAAGTCCCTCCAAGACTAAAC-3′	
	5′-TGGTTGATGATTGTCTTCGG-3′	80

A Sertoli cell-specific knockout of the *Gata1* gene

To create a knockout of the *Gata1* gene in the Sertoli cell compartment, floxed *Gata1* mice were crossed with the *Dhh-Cre* transgenic mice. In male offspring, Cre-mediated recombination at the *Gata1* locus occurred only in mice harbouring the *Dhh-Cre* transgene and the floxed *Gata1* allele (*Gata1*$^{floxed:y}$::*Dhh-Cre*), as demonstrated by the complete absence of Gata1 mRNA in RT–PCR analysis of P14 testis RNA from compound mutant males (Fig. 3). Gata1 mRNA was also not detectable by RQ–PCR analysis of these samples. The threshold cycle for Gata1 expression in control testis was 30. With a maximum of 40 cycles performed and assuming an amplification efficiency of 1.8-fold per cycle, the Gata1 mRNA levels in the testes of compound mutant males are reduced at least 350-fold. We were unable to detect GFP expression by RT–PCR or by fluorescence microscopy. The *Dhh-Cre* transgene is also expressed in endothelial cells and the Schwann cells of the peripheral nervous system. However, Gata1 is not expressed in these cells and it is therefore not surprising that the compound mutant males did not show any obvious vascular abnormalities or neuropathological

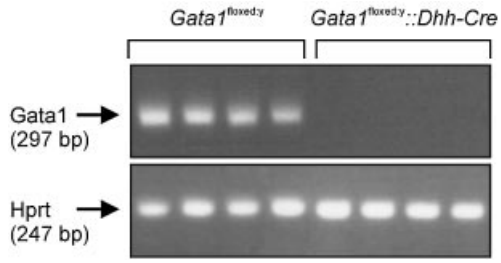

Figure 3. The *Dhh-Cre* transgene abolishes testis-specific Gata1 expression from the floxed *Gata1* allele. Testes were isolated from P14 *Gata1*$^{floxed:y}$ and *Gata1*$^{floxed:y}$::*Dhh-Cre* mice. RNA was extracted from total testis, and expression of Gata1 was assessed by RT–PCR analysis. The size of the expected product for Gata1 cDNA is 297 bp. RT–PCR analysis of Hprt expression was used as an internal control; expected product size of cDNA is 247 bp.

symptoms. Since the mice develop to maturity, it can be concluded that the *Dhh-Cre* transgene does not express the Cre protein in erythroid precursor cells, in agreement with the analysis of the *Dhh-Cre*/*R26R* mice described above. Because the *Dhh-Cre* transgene is active in fetal Sertoli cell precursors (Fig. 2B, a), long before Gata1 is normally expressed, we conclude that the *Gata1*$^{floxed:y}$::*Dhh-Cre* compound males, unlike control males, have never expressed the Gata1 protein in their Sertoli cell compartment.

Fertility of *Gata1*$^{floxed:y}$*::Dhh-Cre* males

Breeding performance of the compound mutant males was similar to that of control males. Litters produced by matings between compound mutant males and wild-type FVB females had an average size of eight pups, and the distribution between male and female pups was as expected (32 male pups versus 30 female pups, i.e. 52 versus 48%). Compound mutant males remained fertile until at least 18 months of age. To further analyse the testicular function of *Gata1*$^{floxed:y}$::*Dhh-Cre* males, testes, epididymides and seminal vesicles of *Gata1*$^{floxed:y}$ and *Gata1*$^{floxed:y}$::*Dhh-Cre* littermates of different ages were isolated. We compared the weights and gross morphology of these organs, but found no significant differences between the two groups. Sperm was isolated from epididymides and the sperm cell numbers were counted. Within the same age groups, no significant differences were observed between the mice analysed (Table 2). Collectively, we conclude that fertility and testicular function of *Gata1*$^{floxed:y}$::*Dhh-Cre* compound males are apparently normal.

Expression of potential Sertoli cell-specific Gata1 target genes

To determine whether the expression of putative Sertoli cell-specific Gata1 target genes was affected in *Gata1*$^{floxed:y}$::*Dhh-Cre* males, we isolated RNA from testes of 2-week-old *Gata1*$^{floxed:y}$::*Dhh-Cre* and *Gata1*$^{floxed:y}$ mice. At this age, the testis is composed for a large part of Sertoli cells, because the process of spermatogenesis is just starting. Furthermore, Gata1 expression normally peaks at this stage of testicular maturation (5). We performed an RQ–PCR analysis to compare the expression of putative Gata1 target genes Amh, Fshr, inhibin α-subunit and activin/inhibin β-A- and β-B-subunit. In addition, we determined the expression of Star (steroidogenic acute regulatory protein) and Cyp19 (PII aromatase) since these genes have essential Gata sites in their regulatory elements (33,34). We used the expression levels of Hprt and cyclophilin A as internal controls for the RQ–PCRs. We observed no significant differences in the expression levels of any of the genes analysed (Table 3). Thus, we conclude that Gata1 function is not essential for the appropriate regulation of the expression of these genes in Sertoli cells.

Expression of other Gata family members

In erythroid Gata1 null cells, the transcription of Gata2 is highly upregulated, implying some interchangeability between Gata factors (35). Furthermore, negative regulation of Gata2 transcription by Gata1 has been suggested (35). Finally, a transgene driving expression of Gata2 or Gata3 cDNA under *Gata1* regulatory sequences can rescue the embryonic lethal phenotype of Gata1 knockdown mice (36). To test whether there might be such a compensatory activity of other Gata factors in the testis of *Gata1*$^{floxed:y}$::*Dhh-Cre* males, we performed an RQ–PCR analysis to detect transcript levels of the different Gata family members that are expressed in the testis. We observed no significant differences in the expression levels of Gata2, Gata4 and Gata6 in testis RNA isolated from 2-week-old *Gata1*$^{floxed:y}$::*Dhh-Cre* and *Gata1*$^{floxed:y}$ mice (Table 3). It is therefore unlikely that the absence of phenotypic consequences in Sertoli cell function of *Gata1*$^{floxed:y}$::*Dhh-Cre* mice is due to compensatory upregulation of another Gata factor. However, it remains possible that Gata1 function is masked by the presence of these factors at levels similar to those observed in normal Sertoli cells.

Table 2. Comparison of testes, epididymides, seminal vesicles weights and sperm counts of *Gata1*$^{floxed:y}$ and *Gata1*$^{floxed:y}$::*Dhh-Cre* littermates at different ages

Age (days)	Genotype	*n*	Testis (mg)	SD	Epididymus (mg)	SD	Seminal vesicles (mg)	SD	Sperm count per epididymus ($\times 10^6$)	SD
24	floxed:y	1	35.5	–	10.0	–	5.7	–	–	–
	floxed:y::Dhh-Cre	3	42.1	3.9	10.5	2.1	6.4	1.3	–	–
52	floxed:y	1	67.5	–	23.2	–	52.3	–	18.0	–
	floxed:y::Dhh-Cre	1	90.5	–	29.3	–	60.5	–	19.0	–
74	floxed:y	3	101.0	15.9	40.4	9.8	65.5	5.6	31.7	6.6
	floxed:y::Dhh-Cre	4	102.0	11.5	38.0	1.8	61.6	9.6	28.5	6.6

Table 3. RQ–PCR expression analysis of putative Gata1 target genes and Gata transcription factors in testes from *Gata1*$^{floxed:y}$ and *Gata1*$^{floxed:y}$::*Dhh-Cre* mice at P14

	floxed:y Threshold cycle	SD	floxed:y::Dhh-Cre Threshold cycle	SD
Amh	26.6	1.2	25.5	0.7
Fshr	25.3	0.3	24.7	0.6
Inhibin α	23.5	0.4	24.1	0.6
Inhibin β-A	27.5	0.4	27.5	0.6
Inhibin β-B	22.8	0.1	23.1	0.1
Star	23.7	0.2	24.3	0.2
Cyp19	28.1	0.1	26.8	0.4
Gata2	27.6	0.4	26.4	0.6
Gata4	24.2	0.4	23.8	0.3
Gata6	24.7	0.2	24.6	0.3
Hprt	24.4	0.3	24.7	0.1
Cyclophilin A	20.2	0.1	19.7	0.0

DISCUSSION

A floxed allele of the *Gata1* gene

Here, we describe the generation of a mouse line in which the *Gata1* gene has been flanked by loxP sites. After Cre-mediated recombination, the coding sequences of the *Gata1* gene are deleted and a GFP reporter gene is brought under the control of the *Gata1* erythroid- and testis-specific promoters. However, we have been unable to detect expression of the GFP reporter from the recombined locus, in either Sertoli or erythroid cells (data not shown). Since expression of GFP was also negative by RT–PCR analysis, a likely explanation is that the part of the gene that is deleted by the recombination event contains important regulatory regions for the expression of the *Gata1* gene. The *Gata1* locus appears to contain multiple regulatory elements (13,37–40). It is now known that at least one of these elements resides in the area deleted in the recombined locus (37). Thus, our data support the notion that this element is important for appropriate erythroid expression of the *Gata1* gene in the context of the endogenous locus. Apart from the testis-specific promoter, testis-specific enhancer elements of Gata1 expression are not known. Our data suggest that such elements reside in the area deleted by Cre in the *Gata1* conditional knockout locus described here. Most importantly, insertion of the loxP sites and the GFP–neo cassette did not appear to affect the expression of Gata1, as the *Gata1* conditional knockout mice developed and reproduced normally.

The Sertoli cell-specific knockout of the *Gata1* gene

Previously, a 'knockdown' mutation of the testis promoter of the mouse *Gata1* gene has been generated. This mutation did not show a Sertoli cell-specific phenotype (41). However, in these experiments, it could not be excluded that residual Gata1 expression from the erythroid promoter masks the phenotype in Sertoli cells. In the present study, we have used the *Dhh-Cre* transgene to delete the *Gata1* gene in Sertoli cells. This promoter becomes active around E12.5 in pre-Sertoli cells, long before Gata1 expression is normally activated in this cell lineage at P7 (15,28). Thus, this is the first description of the consequences of a true Gata1 null mutation in Sertoli cells.

Our data demonstrate that a Sertoli cell-specific knockout of Gata1 does not result in an apparent phenotype based on the analysis of the expression of putative testis-specific Gata1 target genes, testis development, spermatogenesis and male fertility. This is surprising since specific modulation of the expression of Gata factors during testis development has been reported, and a number of potential target genes have been identified (5,14,15,21–23,42). These target genes have been found through *in vitro* promoter studies that utilise proximal promoter elements to stimulate the expression of reporter genes. These assays can only mimic the *in vivo* situation to a limited extent. For instance, important regulatory elements may be missing from the constructs used, the promoter tested is not in its normal chromatin environment, and the cells employed in these studies are usually transformed cells. Furthermore, these apparently conflicting data could be reconciled by the possibility that Gata factors have overlapping functions in Sertoli cells *in vivo*. In this scenario, expression of Gata target genes in Gata1 null Sertoli cells would be certified by the presence of another Gata factor. The 'endodermal' Gata4 and Gata6 are known to be expressed in Sertoli cells (16,43). Gata4 promotes the expression of several Sertoli cell-specific promoter constructs in tissue culture (4). The model implicating Gata1 in the downregulation of Amh expression predicts that in the absence of Gata1, Sertoli cells would sustain high-level Amh expression directed by Gata4. Aberrantly high Amh levels may result in a phenotype, since overexpression of Amh in male mice affects sexual development adversely (18). However, we do not find any changes in sexual development, or Amh expression at P14, in the Gata1 compound mutant males. Thus, we conclude that if Gata1 is involved in the physiological downregulation of Amh expression, it is not a pivotal regulator of this process.

Implications for the role of Gata factors in Sertoli cells

From our data, it would appear that the expression of Gata1 in Sertoli cells has no functional significance. We consider this unlikely, since Sertoli cell-specific expression of Gata1 is very specifically regulated, and is conserved between man and mouse (5). In this regard, it is intriguing that two human males bearing a GATA1 mutation (V205M) displayed cryptorchidism (17). Although our data suggest that loss of Gata function may not be responsible for the observed cryptorchidism, an alternative explanation is that the V205M mutation has a dominant-negative effect, resulting in a more drastic phenotype than complete loss of Gata1 activity in Sertoli cells. This suggests functional redundancy of Gata factors in Sertoli cell development. Opposing roles for endodermal (Gata4) versus haematopoietic (Gata1) factors have been proposed in Sertoli cells. Recently, it has been demonstrated that a Gata4 mutant has severe impact on gonadal development (44). Functional replacement of Gata1 by Gata2 has been established for erythroid cells (36). We therefore propose that the haematopoietic Gata2 factor may compensate for the loss of Gata1 function in Sertoli cells, since it is expressed in the testis (Table 3). The work reported here sets the stage to address this issue through the analysis of compound Sertoli cell-specific knockout mutants of Gata factors, using the *Dhh-Cre* mice as a tool to circumvent the embryonic lethality generally associated with Gata factor null mutations. Furthermore, the *Dhh* expression construct could be used to express a

dominant-negative Gata protein, such as a Gata–engrailed fusion protein (45–47) to prove that Gata factors have an essential role in Sertoli cells.

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