Sox9-dependent expression of *Gstm6* in Sertoli cells during testis development in mice

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Abstract

Glutathione *S*-transferases (GSTs) are an important family of multifunctional enzymes that play a role in the protection of tissues by the detoxification of hazardous and carcinogenic compounds. We found previously that *Gstm6* is upregulated in the somatic cells of male mouse fetal gonads relative to female gonads. In this study, we describe the spatial and temporal expression pattern of *Gstm6* during mouse development. We show that *Gstm6* is predominantly expressed in the reproductive system, at significantly higher levels in XY gonads compared with XX gonads from 11.5 dpc onwards, and remains expressed in the testes in adult mice. Its expression is associated with the Sertoli cell lineage, and is dependent on the expression of the male sex-determining gene *Sox9*. Our data suggest that *Gstm6* plays a male-specific role in gonad development or function, possibly by modulating the exposure of somatic tissue and/or germ cells to endogenous or exogenous toxicants.

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Introduction

Sex determination is achieved in three stages during development. First, genetic sex is established by inheritance of one of both sex chromosomes, X or Y, from the father. If the Y-chromosome is present, the male sex-determining gene *Sry* is activated in pre-Sertoli cells residing in the bipotential gonads, immediately followed by the activation of a second male-determining gene *Sox9*, resulting in the development of testes (Koopman *et al.* 1990, 1991, Kent *et al.* 1996). In the absence of *Sry*, such as in female embryos, or in the absence of *Sox9* function (Barrionuevo *et al.* 2006), the gonads develop into ovaries. Later in fetal life and during puberty, the testes and ovaries produce male and female sex hormones respectively, which are important for the development of the reproductive system and secondary sex characteristics.

Sex determination and gonad development are orchestrated by intricate genetic programming, the nature of which we are only beginning to understand. To obtain insight into these processes, diverse microarray screens have been carried out and have provided a large

number of candidate genes (Nef *et al.* 2005, Small *et al.* 2005, Beverdam & Koopman 2006, Bouma *et al.* 2007, Clement *et al.* 2007, Cory *et al.* 2007). Many of these genes are either unknown or have not been implicated in the genetic network underlying sex determination and gonad development. The major challenge of the next decade is to understand how these genes take part in the genetic networks controlling sex determination and gonad development.

Glutathione *S*-transferases (GSTs) are an important family of multifunctional isoenzymes. They catalyse the conjugation of reduced glutathione via the sulphhydryl group, to electrophilic centres on a wide variety of substrates (Douglas 1987). This activity is useful in the detoxification of endogenous compounds such as peroxidised lipids as well as the metabolism of xenobiotics (reviewed by Hayes & Pulford 1995, Hayes *et al.* 2005). In addition to their enzymatic activities, GSTs may also bind toxins and function as transport proteins.

Previously, we showed in a somatic gonad cell-type-specific microarray screen that glutathione *S*-transferase mu 6 (*Gstm6*) is highly upregulated (39-fold) in 11.5 days

post-coitum (dpc) pre-Sertoli cells when compared with 10.5 dpc pre-Sertoli cells and 11.5 dpc pre-granulosa cells, the female equivalent of Sertoli cells (Albrecht & Eicher 2001, Beverdam & Koopman 2006). Here, we describe in-depth the spatial and temporal expression pattern of *Gstm6* in the developing embryos and adult mouse tissues. We found that *Gstm6* is expressed at high levels in the gonads and at lower levels in other embryonic mouse tissues. *Gstm6* expression was initially restricted to testicular cords in XY gonads, but from around 14.5 dpc onwards *Gstm6* mRNA was also detected in the developing ovaries. In addition, we found that *Gstm6* expression is restricted to the Sertoli cell lineage, and analysis of *Sox9*-knockout gonads indicated that its expression was dependent on *Sox9*. These data suggest that *Gstm6* may have yet undiscovered roles in the earliest steps of male sex determination and/or gonad development.

Results

Gstm6 expression in mouse embryos and adult gonads

We first analysed the expression pattern of *Gstm6* in whole 13.5 dpc mouse embryos by section *in situ* hybridisation (SISH), and found robust expression of *Gstm6* in the developing testes, and low or background expression in other fetal tissues (Fig. 1A). These results were confirmed by quantitative, real-time RT-PCR (qRT-PCR) assays of a range of tissue samples from 13.5 dpc embryos (Fig. 1B). These analyses reveal that *Gstm6* is expressed at high levels in the developing testes compared with the developing ovaries and other fetal tissues. Furthermore, qRT-PCR of adult mouse gonadal tissue revealed high levels of expression in testes but not ovaries (Fig. 1C), indicating that testis-specific expression of *Gstm6* continued into adult life.

Temporal and cellular profile of *Gstm6* expression

We next analysed the temporal *Gstm6* expression profiles by qRT-PCR in embryonic XY and XX gonads at

stages just prior to, during and after the onset of male sex determination, which occurs around 11.5 dpc (Fig. 2A). We found that at 10.5 and 11.5 dpc, the expression levels of *Gstm6* were low both in XY and XX genital ridges. After 11.5 dpc, *Gstm6* expression levels increased sharply in XY gonads to 16.5 dpc, the last stage examined. Curiously, expression levels were lower at 13.5 dpc than at 12.5 or 14.5–16.5 dpc. This temporary decrease in expression levels was found consistently in several independent experiments and was also observed in *in situ* hybridisation experiments (see below). In XX gonads, expression levels remained low throughout the period tested, 10.5–16.5 dpc (Fig. 2A).

To analyse the spatial expression pattern of *Gstm6* in the developing gonads, we carried out *in situ* hybridisation assays on whole XY and XX gonads from 11.5 to 16.5 dpc (Fig. 2B). Using this technique, we detected *Gstm6* expression in XY but not in XX gonads at 11.5 dpc. From 12.5 dpc onwards, *Gstm6* staining was detected with increasing intensity in the developing testis cords, with lower levels of staining at 13.5 dpc, consistent with qRT-PCR data. In XX gonads, however, *Gstm6* mRNA signal was not visible until 14.5 dpc, from which stage it was observed at low levels throughout the developing ovary (Fig. 2B).

To determine the cell type(s) expressing *Gstm6* in XY gonads, we first carried out whole-mount *in situ* hybridisation (WISH) assays at 13.5 dpc on wild-type and *W^e/W^e* mutant testes that lack germ cells (Buehr *et al.* 1993). *Gstm6* expression was detected in testis cords in *W^e/W^e* samples at levels similar to wild-type XY gonads, suggesting that *Gstm6* is expressed in Sertoli cells and/or peritubular myoid cells, and is independent of the presence of germ cells (Fig. 3A).

We then performed SISH assays on sagittally sectioned XY wild-type embryos at 13.5 dpc, and we found that *Gstm6* was expressed within the testis cords in a pattern very similar to that of the Sertoli cell-specific marker anti-müllerian hormone (*Amh*). In both cases, staining was observed in the irregularly shaped cytoplasm of Sertoli cells, and was stronger towards the periphery of

Figure 1 *Gstm6* is specifically expressed in mouse embryonic and adult testes. (A) *In situ* hybridisation using *Gstm6* probe 1 on a sagittal section of a 13.5 dpc XY mouse embryo showing high levels of *Gstm6* expression in the male gonad, whereas other embryonic tissues show little or no *Gstm6* expression. Scale bar, 2 mm. (B) qRT-PCR assays showing high levels of *Gstm6* expression in 13.5 dpc testes and low levels in 13.5 dpc ovaries and other embryonic tissues. (C) qRT-PCR assays showing high levels of *Gstm6* expression in adult testes, and low expression levels in adult ovaries. The Y-axes display relative fold expression levels compared with *18S* rRNA with the maximum expression level set to 100%. Experiments were carried out three times and each experiment consisted of three replicates. The error bars represent the s.e.m. of three experiments.

Figure 2 *Gstm6* expression is upregulated in embryonic testes after sex determination. (A) qRT-PCR assays showing *Gstm6* mRNA levels in XX and XY genital ridges and gonads from 10.5 dpc to 16.5 dpc. *Gstm6* expression is low in both XY and XX genital ridges at 10.5 dpc, but from 11.5 dpc *Gstm6* expression is high in XY gonads, whereas it stays low in XX gonads. From 14.5 dpc, slightly higher levels of *Gstm6* expression are observed also in XX gonads. The Y-axis displays relative fold expression levels compared with *18S* rRNA with the maximum expression level of each experiment set to 100%. Experiments were carried out three times and each experiment consisted of three replicates. The error bars represent the S.E.M. of three experiments. (B) *In situ* hybridisation assays using *Gstm6* probe 2 on whole XY (upper panels) and XX (lower panels) genital ridges and gonads from 11.5 dpc to 14.5 dpc. *Gstm6* is first activated in 11.5 dpc XY embryos throughout the genital ridge, whereas no *Gstm6* mRNA was detected in 11.5 dpc XX genital ridges. From 12.5 dpc onwards, *Gstm6* expression is restricted to the testicular cords. At 14.5 dpc, *Gstm6* expression was detected throughout the developing ovaries.

the cords (Fig. 3B). This staining was distinct from that obtained with probes detecting the germ cell marker *Pou5f1*, which was localised in the smooth, annular cytoplasm surrounding the nuclei of germ cells, and was stronger towards the centre of the cords (Fig. 3B). In addition, *Gstm6* expression was also detected in a thin layer of cells surrounding the testis cords likely to be peritubular myoid cells (Fig. 3B, arrow).

qRT-PCR assays using RNA extracted from 13.5 dpc wild-type and W^e/W^e gonads corroborated our *in situ* hybridisation data (Fig. 3C–E). We analysed the expression levels of *Gstm6* in comparison with the Sertoli cell-specific gene *Sox9* and the germ cell marker *Ddx4* (*Mvh*, mouse vasa homologue; Toyooka *et al.* 2000). We found high levels of *Ddx4* expression in wild-type gonads, but not W^e/W^e gonads, confirming the absence of germ cells in the W^e/W^e gonads (Fig. 3C). *Sox9* expression levels were similar in wild-type and

W^e/W^e gonads, as expected from a Sertoli cell-specific gene (Fig. 3D). Similarly, *Gstm6* expression was detected in both wild-type and W^e/W^e gonads at similar levels (Fig. 3E).

Our previous microarray analyses suggested that *Gstm1* and *Gstm2* are also expressed in a sexually dimorphic manner in the somatic cells of the developing gonads, albeit at lower levels than *Gstm6* (Beverdam & Koopman 2006). To further characterise the expression pattern of these two genes in the developing gonads, we performed qRT-PCR on wild-type and W^e/W^e mutant 13.5 dpc testes and ovaries. We found that the expression levels of *Gstm1* and *Gstm2* were higher in 13.5 dpc XY WT gonads compared with 13.5 dpc XX WT gonads (Fig. 3F and G). In addition, *Gstm1* and 2 were expressed at even higher levels in the absence of germ cells (Fig. 3F and G), suggesting that they are exclusively expressed by somatic gonad cells and not by germ cells, corroborating our microarray data.

Gstm6 expression is dependent on *Sox9*

Sox9 is known to regulate testis-specific expression of a number of genes, including *Amh*, *Vanin1*, *Pgds2* and *Sf1* (Shen *et al.* 1994, De Santa Barbara *et al.* 1998, Wilson *et al.* 2005, Wilhelm *et al.* 2007). We therefore set out to determine whether *Gstm6* expression was also dependent on the expression of *Sox9*, using a conditional knockout mouse model, in which *Sox9* was inactivated in the developing gonads using the CRE/loxP recombination system, with CRE recombinase expressed under control of the cytokeratin 19 promoter (Barrionuevo *et al.* 2006). We determined the expression levels of *Sox9* and *Gstm6* by qRT-PCR in XY genital ridges and gonads from *Sox9* conditional homozygous mutant embryos at 11.5, 12.0 and 12.5 dpc, relative to wild-type XY and XX littermates. *Sox9* expression levels in wild-type XY genital ridges and gonads increased from 11.5 to 12.5 dpc and remained low in wild-type XX genital ridges and gonads, whereas *Sox9* levels were strongly reduced in mutant genital ridges and gonads as expected (Fig. 4A). *Gstm6* expression levels in wild-type XY genital ridges and gonads increased strongly between 11.5 and 12.5 dpc, but remained low in *Sox9* mutant genital ridges and gonads (Fig. 4B). In fact, *Gstm6* expression levels in *Sox9* mutant genital ridges and gonads were similar to those found in wild-type XX genital ridges and gonads. These results demonstrate that *Gstm6* expression in XY genital ridges and gonads depends, directly or indirectly, on the expression of *Sox9*. The lag between upregulation of *Sox9* and *Gstm6* in wild-type testes (Fig. 4), and the observation that both genes are expressed in Sertoli cells, further supports the hypothesis that *Gstm6* acts downstream of *Sox9* in the cascade of testis development.

Figure 3 *Gstm6* is expressed in Sertoli cells. (A) *In situ* hybridisation assays using *Gstm6* probe 2 showing *Gstm6* expression in 13.5 dpc wild-type (left panels) and *W^e/W^e* (right panels) gonads showing that *Gstm6* expression is maintained at similar levels in gonads lacking germ cells. (B) *In situ* hybridisation assays using *Gstm6* probe 1 showing expression of *Gstm6* (left panels), Sertoli cell marker *Amh* (middle panels) and germ cell marker *Pou5f1* (right panels) in 13.5 dpc XY gonads. *Gstm6* is expressed in a pattern similar to *Amh* but disparate to *Pou5f1*. Arrow points to presumptive peritubular myoid cells. Scale bars, 100 μ m. (C–E) qRT-PCR assays showing the absence of the germ cell marker *Ddx4* in *W^e/W^e* gonads (C). *Sox9* and *Gstm6* expression levels are similar in wild-type and *W^e/W^e* gonads (D and E). (F) qRT-PCR assays showing *Gstm1* expression levels in WT (dark bars) and *W^e/W^e* (light bars) XY and XX gonads. (G) qRT-PCR assays showing *Gstm2* expression levels in WT (dark bars) and *W^e/W^e* (light bars) XY (left) and XX (right) gonads. The Y-axis displays relative fold expression levels compared with 18S rRNA with the gene expression levels in WT gonads set to 100%. Experiments were carried out three times and each experiment consisted of three replicates. The error bars represent the S.E.M. of three experiments. WT, wild type; *W^e*, *W^e/W^e*.

Discussion

Here, we describe in detail the expression pattern during mouse gonadal development of *Gstm6*. We found *Gstm6* to be expressed at high levels in Sertoli cells of the testis from 12.5 dpc until at least 16.5 dpc, but barely or not at all in ovaries. *Gstm6* expression was dependent on the expression of *Sox9*, which controls male sex determination and testis development. Our data suggest that *Gstm6* plays a specific role in the development or function of testes. Because GSTs are implicated in the detoxification of endogenous compounds and xenobiotics, *Gstm6* expression may provide protection of germ cells against toxicants that may bring about DNA damage or mutation in the germ line. If so, our findings beg the question of why such a role appears to be dispensable in the developing ovaries.

Our experiments also suggested that *Gstm6* might be expressed in peritubular myoid cells. This expression may provide an additional level of protection of germ cells in addition to expression in Sertoli cells. Further investigation of this possibility awaits identification of a specific marker gene for peritubular myoid cells (Jeanes *et al.* 2005).

We observed a temporary downregulation in *Gstm6* expression levels in 13.5 dpc XY gonads in qRT-PCR and

WISH assays. This downregulation was observed consistently in sufficient independent trials to exclude error or artefact as an explanation. While the reason for this phenomenon is not understood, it may reflect a

Figure 4 *Gstm6* expression is dependent on *Sox9*. (A) *Sox9* expression levels are high in genital ridges and gonads of wild-type XY mice and increase over time, whereas they remain low in genital ridges and gonads of wild-type XX mice. In *Sox9*, conditional homozygous mutant XY gonads *Sox9* expression levels are strongly reduced when compared with wild-type XY gonads. (B) *Gstm6* expression levels are higher in XY genital ridges and gonads and they peak at 12.5 dpc, whereas those in genital ridges and gonads of wild-type XX littermates remain low. In *Sox9*, conditional homozygous mutant XY gonads *Gstm6* expression levels are strongly reduced when compared with wild-type XY gonads, but similar to expression levels found in wild-type XX gonads. The Y-axis displays relative fold expression levels compared with 18S rRNA with the maximum expression level of each experiment set to 100%. Each experiment consisted of three replicates. The error bars represent minimal and maximal ranges of gene expression in three replicates.

period of relatively little metabolic activity following the dramatic morphogenetic events of testicular cord formation. However, from 13.5 dpc onwards, a marked increase in *Gstm6* expression levels was observed, perhaps concomitant with the increase in the number of *Gstm6*-producing Sertoli cells in this period.

We found that that in addition to *Gstm6*, *Gstm1* and *Gstm2* also are expressed at higher levels in the somatic cells of XY gonads compared with XX gonads (present study and Beverdam & Koopman 2006), suggesting that *Gstm* genes may have redundant functions during gonadogenesis. Further experiments will be required to investigate whether *Gstm6* and possibly other *Gstm* members are indeed involved in detoxification during gonadogenesis or whether they have other, yet unidentified roles in the developing gonads.

Materials and Methods

Animals

Protocols and use of animals were approved by the Animal Welfare Unit of the University of Queensland and by the Animal Ethics Committee of Monash University, Australia. Embryos were collected from timed matings of the CD1 outbred strain, the *c-Kit*^{We+/−} inbred strain (Buehr *et al.* 1993) and a transgenic mouse line, maintained on a Swiss outbred background, constitutively expressing EGFP from the X-chromosome (gift of A Nagy; Hadjantonakis *et al.* 1998) and 0.5 dpc was defined as noon on the day of discovery of the copulation plug. Mouse embryos were staged according to limb bud morphology (Theiler 1989). Embryonic sex was established by assessing GFP fluorescence (for 10.5 and 11.5 dpc samples used for qRT-PCR analyses), by PCR on genomic DNA using *Zfy* forward primer: 5'-CCTATTGCATG-GACTGCAGCTTATG-3' and *Zfy* reverse primer: 5'-GACTAGACATGTCTTAACATCTGTCC-3' (for other 10.5 and 11.5 dpc samples) or by assessing gonad morphology (for samples 12.5 dpc and older). *Ck19:Cre/+; Sox9*^{fllox/fllox} embryos on a 129P2/OlaHsd × C57BL/6 mixed background were generated, sexed and genotyped as described previously (Daneau *et al.* 2002, Barrionuevo *et al.* 2006).

qRT-PCR assays

Of 10.5 and 11.5 dpc genital ridges and adult and 12.5 up to 16.5 dpc embryonic gonads with mesonephroi attached were pooled according to stage and sex. Total RNA was extracted from dissected embryonic and adult mouse tissues according to the manufacturer's instructions and including the optional genomic DNA degradation I step (Qiagen). Total RNA was reverse transcribed using random hexamer primers (Promega) and Superscript III (Invitrogen) using standard methodologies.

qRT-PCR primers were designed with Tm of close to 60 °C to generate 60–145 bp amplicons spanning introns. Target amplification efficiency of all primers was approximately equal. *Gstm6*-F: 5'-ATGGGCATGCTTTGCTACA-3', *Gstm6*-R:

5'-GGAAGTCCGAGTAGAGTTTCAGC-3', *Gstm1*-F: 5'-GCACCTCATCATGCTCTGTT-3', *Gstm1*-R: 5'-TTTTCTCAGGGATGCTCTCA-3', *Gstm2*-F: 5'-AGTTGGCCATGGTTTGCTAC-3', *Gstm2*-R: 5'-AGCTTCATCTTCTCAGGGAGAC-3', *Sox9*-F: 5'-AGTACCCGCATCTGCACAAC-3', *Sox9*-R: 5'-TACTTGTAATCGGGGTGGTCT-3', *Ddx4*-F: 5'-AAGCAGAGGGTTTCAAGC-3', *Ddx4*-R: 5'-GCCTGATGCTTCTGAATCG-3', *18S*-F: 5'-GATCCATTGGAGGGCAAGTCT-3' and *18S*-R: 5'-CCAAATGATCCAACTACGAGCTTTT-3'.

qRT-PCR assays were carried out in triplicate consisting of three replicates each using SYBR green PCR master mix, 3.5 μM primer pairs using an ABI Prism 7000 Sequence Detection System following the manufacturer's instructions. Target cDNA levels were analysed by the comparative cycle time (C_t) method of qRT-PCR and the values were normalised to *18S* rRNA expression levels.

In situ hybridisation

Embryos and embryonic gonads were dissected and fixed in 4% paraformaldehyde at 4 °C overnight and washed with PBS. For SISH, embryos were mounted in paraffin wax and sectioned at 7 μm using a microtome. SISH was carried out as described by Svingen *et al.* (2007) and WISH as described by Hargrave *et al.* (2006).

Gstm6 *in situ* hybridisation probes were generated by PCR using cDNA from 12.5 dpc mouse gonadal tissue. *Gstm6* probe 1 was generated using forward primer 5'-CATGCATG-GAGTGCCGGTGT-3' and reverse primer 5'-CACACAA-GACTGGGCTTCC-3'. *Gstm6* probe 2 was generated using forward primer 5'-ACAAGGTCACCCAGAGCAATGC-3' and reverse primer 5'-TGGCTTCCGTTTCTCAAAGTCAG-3'. PCR fragments were cloned into the pGEM-T Easy vector (Promega), according to the manufacturer's instructions and checked by sequencing. The specificity of each probe was checked by BLAST searching the probe sequence using the NCBI database. Templates for probes for *Amh* and *Pou5f1* were made as described previously (Munsterberg & Lovell-Badge 1991, Schepers *et al.* 2003). *Gstm6* probe 1 was used for SISH assays, but performed poorly in whole-gonad *in situ* hybridisation assays where *Gstm6* probe 2 was used.

Declaration of interest

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

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