RESEARCH REPORT

Female-to-male sex reversal in mice caused by transgenic overexpression of Dmrt1
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ABSTRACT

Genes related to Dmrt1, which encodes a DNA-binding DM domain transcription factor, act as triggers for primary sex determination in a broad range of metazoan species. However, this role is fulfilled in mammals by Sry, a newly evolved gene on the Y chromosome, such that Dmrt1 has become dispensable for primary sex determination and instead maintains Sertoli cell phenotype in postnatal testes. Here, we report that enforced expression of Dmrt1 in XX mouse fetal gonads using a Wt1-BAC transgene system is sufficient to drive testicular differentiation and male secondary sex development. XX transgenic fetal gonads showed typical testicular size and vasculature. Key ovarian markers, including Wnt4 and Foxl2, were repressed. Sertoli cells expressing the hallmark testis-determining gene Sox9 were formed, although they did not assemble into normal testis cords. Other bipotential lineages differentiated into testicular cell types, including steroidogenic fetal Leydig cells and non-miotic germ cells. As a consequence, male internal and external reproductive organs developed postnatally, with an absence of female reproductive tissues. These results reveal that Dmrt1 has retained its ability to act as the primary testis-determining trigger in mammals, even though this function is no longer normally required. Thus, Dmrt1 provides a common thread in the evolution of sex determination mechanisms in metazoans.

KEY WORDS: Sex determination, DM domain genes, Sox9, Evolution, Mouse

INTRODUCTION

DM domain genes encode transcription factors that contain a conserved DM-type DNA-binding domain that was originally identified in the sex regulators doublesex and male abnormal-3 in Caenorhabditis elegans (Raymond et al., 1998). In several non-mammalian vertebrate species, they act as triggers for primary sex determination. Examples include DMY in the medaka fish Oryzias latipes (Matsuda et al., 2002; Nanda et al., 2002; Otake et al., 2010), DM-W and DMRT1 in the amphibian Xenopus laevis (Yoshimoto et al., 2008, 2010) and DMRT1 in chicken (Smith et al., 2009; Lambeth et al., 2014). However, Dmrt1 has become dispensable for testis determination in mammals, as evidenced by the experimental observation that XY Dmrt1 null mutant mice are born as males with testes (Raymond et al., 2000). Nevertheless, Dmrt1 plays essential roles in maintaining Sertoli cell phenotype in postnatal mouse testes (Matson et al., 2011; Minkina et al., 2014).

Most eutherian mammals, including mice, use Sry, a newly evolved gene on the Y chromosome, as the trigger for primary male sex determination (Gubbay et al., 1990; Sinclair et al., 1990; Koopman et al., 1991). The expression of SRY in the pre-supporting cells in the developing fetal gonads (Albrecht and Eicher, 2001; Wilhelm et al., 2005) directs these cells to differentiate into Sertoli cells, which orchestrate the differentiation of other testis-specific cell lineages and their assembly into functional testes (Svingen and Koopman, 2013). Sry encodes a transcription factor that upregulates expression of the related HMG box transcription factor gene Sox9 (Sekido and Lovell-Badge, 2008). Unlike Sry, the involvement of Sox9 as a pivotal, early-acting effector of male sex determination has been documented in all vertebrates studied, leading to the prevailing view that the different switch mechanisms that have evolved in different vertebrate taxa all converge on Sox9 (Cutting et al., 2013).

In the current study, we investigated the ability of Dmrt1 to initiate male development in mammals using a transgenic mouse model. We overexpressed Dmrt1 in XX mouse fetal gonads using a Wt1-BAC transgene system (Polanco et al., 2010; Zhao et al., 2014a). Surprisingly, ectopic expression of Dmrt1 was sufficient to drive testicular differentiation at the fetal stage and male secondary sex development postnatally. Our results suggest that, despite the diversity and plasticity of sex determination mechanisms (Graves, 2008; Cutting et al., 2013), DM domain genes provide a common thread in the evolution of sex determination mechanisms in metazoans, with Sry likely having replaced Dmrt1 in this role in mammals.

RESULTS AND DISCUSSION

Transgenic overexpression of Dmrt1 causes female-to-male sex reversal of the fetal gonads

We set out to investigate whether overexpression of Dmrt1 might be sufficient to cause testis development in XX mice, and used a Wt1 enhancer in a piggyBac-based BAC vector to drive Dmrt1 expression in transgenic mice (Fig. 1A) (Zhao et al., 2014a). Using this enhancer, we have previously demonstrated that the transgene of interest is specifically expressed in XX and XY gonadal somatic pre-supporting cells, and not in the mesonephros, beginning at 10.5 days post coitum (dpc) (Polanco et al., 2010), before sex determination takes place.

We first confirmed that the Wt1::Dmrt1-IRES-EGFP transgene was expressed in developing fetal gonads. Strong EGFP fluorescence was observed in XX and XY transgenic gonads at 14.5 dpc (Fig. 1F-I). Furthermore, using immunofluorescence staining, we confirmed that DMRT1 and EGFP proteins were co-expressed in somatic cells of transgenic fetal gonads (both XX and XY; Fig. 1L,M), as expected, whereas in wild-type ovaries endogenous DMRT1 was only detectable in germ cells (Fig. 1J). Quantitative RT-PCR (qRT-PCR) analysis showed that Dmrt1 expression levels were increased 21-fold in XX and ninefold in XY transgenic fetal gonads, relative to wild type (Fig. 1R).
XX transgenic fetal gonads resembled testes in their gross morphology, with a volume larger than that of typical ovaries (Fig. 1B-E) and a prominent blood vessel on the coelomic surface, a hallmark of testis development (arrowheads, Fig. 1C-E,H,I). Double immunofluorescence analysis of XX transgenic gonads for the differentiation markers SOX9 and FOXL2 revealed the presence of Sertoli cells and absence of granulosa cells, indicating female-to-male gonadal sex reversal caused by transgenic overexpression of Dmrt1 (Fig. 1N-P). Quantitation of Sox9 and Foxl2 expression by qRT-PCR confirmed these findings (Fig. 1S,T).

As a sequence-specific transcription factor, DMRT1 is capable of binding to DNA and activating or repressing the expression of putative target genes (Murphy et al., 2010). Previously, it has been shown that DMRT1 binds near Sox9, Sox8, Wnt4 and Foxl2 and activates (Sox9, Sox8) or represses (Wnt4, Foxl2) their expression in postnatal testes (Matson et al., 2011). Wnt4 and Foxl2 are known to repress Sox9 expression (Ottolenghi et al., 2007). Therefore, activation of Sox9 in XX Dmrt1 transgenic fetal gonads might be...
a result of direct transactivation by DMRT1, or repression of Wnt4 (Fig. 2T; see below) and Foxl2 (Fig. 1T), or a combination of both. Unlike wild-type testes, which had well-defined cords at this stage (Fig. 1C,O), no cord structures were observed in XX or XY transgenic fetal testes (Fig. 1C-E,K-M,O-Q and Fig. 2A-F). The lack of distinct testis cords was confirmed by anti-laminin immunofluorescence (supplementary material Fig. S1). Failure of cord formation in XY transgenic testes suggests active repression of this process resulting from Dmrt1 overexpression. The expression of Pdgfra, an essential mediator of testis cord formation (Brennan et al., 2003), was unaltered in the transgenic fetal gonads (data not shown). Moreover, flattened, smooth muscle actin (SMA)-positive peritubular myoid cells were present in adult XX transgenic gonads (supplementary material Fig. S2F; see below), suggesting that a lack of myoid cells cannot account for the perturbed cord formation. These results uncouple the processes of Sertoli cell differentiation and testis cord formation, which were previously understood to be tightly linked.

**Differentiation of testicular cell lineages in XX Dmrt1 transgenic fetal gonads**

We examined in detail the differentiation of Sertoli, fetal Leydig and germ cells in XX transgenic fetal gonads at 14.5 dpc. We first

Fig. 3. Sex reversal in XX adult Dmrt1 transgenic mice. (A-C) External genitalia of XX wild-type (wt), XY wild-type and sex-reversed XX Dmrt1 transgenic (tg) mice at 4 weeks of age. Male development of an XX transgenic mouse is indicated by the development of a penis and increased ano-genital distance (bracket). (D-F) Male internal reproductive structures in an XX transgenic mouse. o, ovary; u, uterine horn; t, testis; e, epididymis; vd, vas deferens; sv, seminal vesicle. (G-L) Histological analysis of gonadal sections at 4 weeks using Hematoxylin and Eosin staining. XX transgenic testes did not show conspicuous seminiferous tubules (I,L), in contrast to wild-type testes (H,K). (M-O) Immunofluorescence analyses of gonadal sections at 4 weeks. (M-O) DMRT1 (magenta) and EGFP (green) were co-expressed in Sertoli cells in an XX transgenic testis (O). These double-positive Sertoli cells formed clusters but not intact seminiferous tubules, in contrast to wild-type testes (N). Germ cells (marked by MVH, grey) were absent from these Sertoli cell clusters. (P-R) SOX9-positive Sertoli cells (green) formed in XX transgenic testes, and no FOXL2-positive granulosa cells (magenta) were present (R; some non-specific staining of vasculature is seen using this antibody). (S-U) Adult Leydig cells in wild-type testes (T) and theca cells in the wild-type ovary (S) were positively stained for the steroidogenic marker HSD3β (magenta). The distribution pattern of HSD3β-positive cells in XX transgenic testes (U) resembled that of Leydig cells in wild-type testes (T). AMH (green) marks granulosa cells in wild-type ovaries (S). Nuclei were counterstained with DAPI (blue). (V-CC) qRT-PCR analyses of cell type marker genes. Compared with XX wild-type ovaries, genes expressed in Sertoli and/or Leydig cells (V-Y) were upregulated and those expressed in granulosa or theca cells (Z-CC) were downregulated in XX transgenic testes. Pink bars represent phenotypic females and blue bars phenotypic males. Means±s.e.m., n=3. Scale bars: 2 mm in D-F; 0.5 mm in G-I; 100 μm in J-L; 50 μm in M-U.
performed immunofluorescence for AMH, a marker of Sertoli cell differentiation and a direct transcriptional target of SOX9 (De Santa Barbara et al., 1998), and for HSD3β, a steroidogenic enzyme expressed by fetal Leydig cells. The presence of cells expressing AMH or HSD3β confirmed that both Sertoli and fetal Leydig cells had formed in the sex-reversed XX Dmrt1 transgenic testes, as in wild-type XY testes but not XX ovaries (Fig. 2A-D). These results were confirmed by qRT-PCR (Fig. 2I,O).

We next assayed the expression of additional Sertoli and fetal Leydig cell marker genes using qRT-PCR. Genes involved in the differentiation of Sertoli (Sox8, Sox10, Pigk and Dhh) and fetal Leydig (Star, Cyp11a1, Ins1 and Cyp17a1) cells were consistently upregulated in XX transgenic testes, as compared with wild-type ovaries (Fig. 2J-N,P; supplementary material Fig. S2A,B). By contrast, genes characteristic of ovarian development, such as Wnt4 and Fst, were markedly downregulated in XX transgenic testes, similar to the situation in wild-type testes (Fig. 2T,U). Similar results were obtained from samples at 13.5 dpc (data not shown).

Germ cells in fetal ovaries begin to enter meiosis around 13.5 dpc in response to retinoic acid (Bowles et al., 2006; Koubova et al., 2006). In fetal testes, CYP26B1 expressed by Sertoli and fetal Leydig cells (Bowles et al., 2006; Koubova et al., 2006) degrades retinoic acid so that male germ cells avoid meiosis and are instead mitotically arrested. Consistent with the presence of Sertoli and fetal Leydig cells, expression of Cyp26b1 was upregulated in XX transgenic testes to levels similar to wild-type XY testes, and well above those seen in wild-type ovaries (Fig. 2Q). Accordingly, SYCP3, a meiotic marker, labelled most germ cells in wild-type ovaries but none in XX transgenic testes (Fig. 2E-H). qRT-PCR analyses of Sycp3 and Stra8 (a target gene of retinoic acid signalling) further confirmed the absence of meiosis in XX transgenic testes (Fig. 2V,W). We also analysed the expression of Nos2 (Suzuki and Saga, 2008) and Dmnt3l (Bouc'his and Bestor, 2004), markers of male fetal germ cell fate, and found that both were upregulated in XX transgenic gonads relative to wild-type ovaries (Fig. 2R,S). Together, these results demonstrate that germ cells in XX Dmrt1 transgenic gonads had adopted a male fate.

Unexpectedly, the expression levels of several marker genes were lower in XY transgenic gonads than in XY wild-type testes (Fig. 2I,K,L,N-P,S; supplementary material Fig. S2). In addition, transgenic gonads were disorganised, without well-defined testis cords. By contrast, Sox9 was expressed at similar levels in XY wild-type and both XX and XY transgenic gonads (Fig. 1S). Based on these data, we hypothesize that high levels of Dmrt1 in XX and XY transgenic gonads may act as a double-edged sword, activating Sox9 expression (directly or indirectly) to induce fetal testis development but, at the same time, repressing the expression of other downstream genes important for proper testis morphogenesis.

**Female-to-male sex reversal in an adult XX mouse transgenic for Dmrt1**

In mammals, once the fetal gonads develop as testes, androgens produced by fetal and adult Leydig cells stimulate the differentiation of male external and internal reproductive organs. We next analysed transgenic gonads and the secondary sex phenotype at 4 weeks of age. Again, we observed female-to-male sex reversal, as characterised by male external genitalia similar to those of wild-type male littermates (penis and increased ano-genital distance; Fig. 3A-C) and the presence of a male reproductive tract (epididymides, vasa deferentia and seminal vesicles; Fig. 3D-F), albeit hypoplastic. Testes were small and did not descend (Fig. 3E,F).

No female internal genitalia developed. As with transgenic fetuses, no well-structured seminiferous tubules formed in XX transgenic testes (Fig. 3G-L; supplementary material Fig. S3A-F).

Persistent expression of the Dmrt1 transgene was confirmed by immunofluorescence and qRT-PCR (Fig. 3M-O,V; data not shown). Accordingly, several known DMRT1 targets, such as Espn, Csr9, Eid and Vsig1 (Murphy et al., 2010; Agbor et al., 2013), were massively upregulated in XX transgenic testes (supplementary material Fig. S3G-J). Genes and proteins expressed in Sertoli cells, such as SOX9 and Cyp26b1, were strongly expressed in XX transgenic testes (Fig. 3P-R,W,X), whereas markers of postnatal ovaries, including FOXL2, AMH, Wnt4, Cyp19a1 (aromatase) and Hsd17b1, were absent or greatly reduced (Fig. 3P-U,Z-CC; supplementary material Fig. S3L).

Adult Leydig cells expressing HSD3β, Cyp11A1 (SCC), Hsd17b3 and Ins13 were present in XX transgenic testes (Fig. 3S-U,Y; supplementary material Fig. S3D-F.K). However, androgen production might have been compromised, since expression of Hsd17b3 and Cyp17a1, which both encode steroidogenic enzymes, was suppressed in XX transgenic testes (Fig. 3Y; supplementary material Fig. S3M), perhaps accounting for the underdevelopment of male reproductive organs observed (Fig. 3F). As expected, germ cells were absent from XX transgenic testes (supplementary material Fig. S3A-C,N), similar to the situation in XX testes transgenic for Sry (Koopman et al., 1991), Sox9 (Vidal et al., 2001) or Sox10 (Polanco et al., 2010).
Conclusions and perspectives
DM domain genes act as the trigger for primary sex determination in several non-mammalian vertebrate species, acting upstream of Sox9, which appears to be the common point of convergence of the various switch mechanisms used among vertebrates (Cutting et al., 2013) (Fig. 4). The emergence of SRY in the common progenitor of marsupial and placental mammals has rendered Dmrt1 redundant, such that the regulatory elements required for sufficient expression in the supporting cell lineage (Lei et al., 2007; Agbor et al., 2013) were lost or degraded.

Our results here indicate that DMRT1 remains capable of driving testicular differentiation in mammals, provided that it is expressed at sufficient levels at the right place and time. Our marker analyses further suggest that it acts in this role upstream of Sox9, a conclusion that is supported by the recent observation that upregulation of DMRT1 precedes SOX9 expression during female-to-male sex reversal of polled intersex goats (Elziat et al., 2014). Thus, despite the existence of a variety of sex-determining mechanisms in the animal kingdom, our results support a model in which Dmrt1 provides a common genetic thread among metazoans (Fig. 4).

We suggest that a Dmrt1-related gene might have resided (or retained) the role of primary sex determinant in mammalian species that have lost Sry (Graves, 2002; Jiménez et al., 2013). Furthermore, the number of members of both the DM and Sox families of transcription factors to prime the male sex-determining pathway might have provided resilience to mutation, a mechanism for replacement of one sex-determining switch gene with another on an evolutionary time-scale, and a high degree of plasticity that has resulted in the variety of sex-determining mechanisms seen in metazoans today.

MATERIALS AND METHODS
Mouse transgenesis
A sequence containing the mouse Dmrt1 coding region followed by IRES-EGFP (Dmrt1-IRES-EGFP) was cloned into the PBWt1-Dest vector via Gateway LR recombination as described (Zhao et al., 2014a). Two transgenic founder mice (an infertile XX female and an XY male) were produced by pronuclear injection of PBWt1-IRES-EGFP vector DNA and hyperactive piggyBac transposase mRNA as described (Zhao et al., 2014a). The XY male founder transmitted the transgene through the germline and was mated to C57BL/6J females to generate embryos and pups for analyses. All animal procedures were approved by the University of Queensland Animal Ethics Committee. Genotyping protocols are described in the supplementary materials and methods.

mRNA and protein expression analyses
RNA extraction, cDNA synthesis, qRT-PCR and immunofluorescence were performed as described (Zhao et al., 2014b). Further details including PCR primers and antibodies are provided in the supplementary materials and methods and Tables S1 and S2. Bright-field and EGFP fluorescence images of freshly dissected organs were captured on a Leica M165 FC stereomicroscope. Confocal images were captured on a Zeiss LSM710 confocal microscope.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
P.K., L.Z. and T.S. designed experiments; L.Z. and E.T.N. performed the research; L.Z. and P.K. analysed data and wrote the paper.

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Supplementary material
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