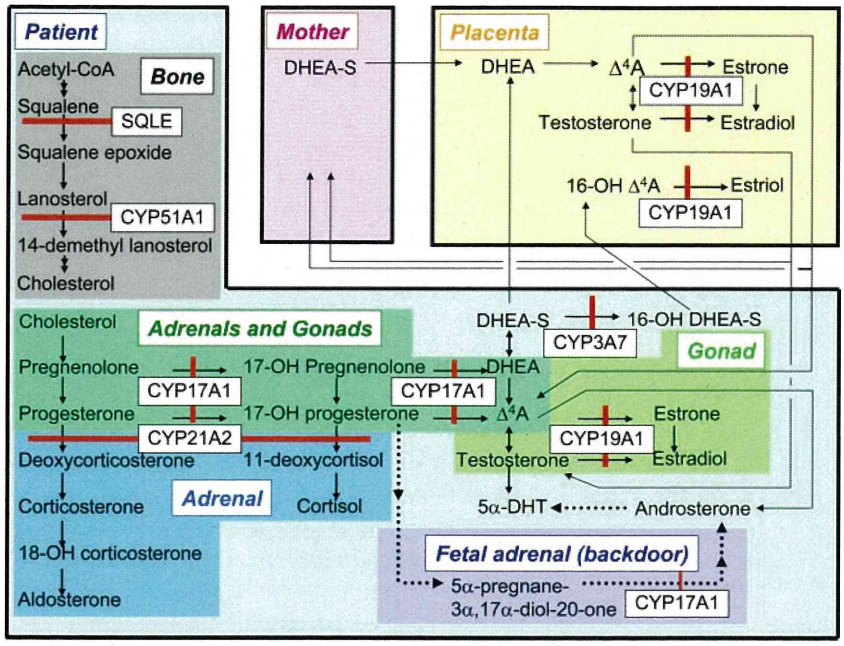

FIG. 4. Simplified schematic representation indicating impaired cholesterologenesis and steroidogenesis in PORD. DHEA, Dehydroepiandrosterone; DHEA-S, DHEA sulfate; Δ^4A, androstenedione; DHT, dihydrotestosterone. SQLE, CYP51A1, CYP17A1, CYP21A2, CYP19A1, and CYP3A7 are POR-dependent enzymes. The important Ms only are shown, and the reaction steps in which some Ms are omitted are indicated by two tandem *arrows*. Note that the amount of estriol synthesized in the placenta far exceeds the total amount of estrone and E_2 (~10 times) (34).

may primarily be due to the complexity of steroidogenesis in PORD (Fig. 4). For example, both production and degradation of 17-OHP are carried out by POR-dependent enzymes, and such enzymatic reactions would depend on the R457H dosage and the differential supporting activity of the R457H protein for target enzymes as well as the amount of substrates and products. Furthermore, the basal cortisol values imply that the baseline steroidogenic capacity can grossly be sustained, even in group B. Indeed, whereas basal blood 17-OHP values were significantly higher in group B than group A, some of them remained within the normal range, and several cases of both groups were not detected in neonatal mass screening. Nevertheless, the R457H dosage would have important clinical relevance, because the ACTH-stimulated blood cortisol was drastically reduced especially in group B, and adrenal crisis was observed only in group B. Furthermore, because 17,20 lyase activity alone was significantly different between groups A and B (Fig. 2B), this would provide further support for the previous finding that 17,20 lyase activity is the most sensitive index of defective POR activity (5, 15).

46,XY DSD was not so remarkable, whereas 46,XX DSD was invariably identified. This suggests a mildly reduced androgen production in genetic males and a definitely excessive androgen production in genetic females. In this context, there are three androgen sources during the fetal life in PORD, *i.e.* the fetal testis, backdoor pathway, and placenta (3, 4, 9, 25, 26) (Fig. 4). For fetal testicular T production specific to 46,XY cases, placental hCG-stimulated T production around the critical period for sex development would be more compromised in group B than group A because testicular T production is performed in a simple one-way manner, as in cholesterologenesis. Furthermore, because T responses to hCG stimulation were reduced, at least in the two examined cases of group B, this implies the compromised maximum T production capacity. By contrast, the backdoor- and placenta-derived androgen productions common to both 46,XY and 46,XX cases may be similar between groups A and B: 1) whereas 17-OHP as the source metabolite for the backdoor pathway is higher in group B than group A, the supporting activity for fetal adrenal CYP17A1 involved in the backdoor pathway would be lower in group B than group A; and 2) whereas fetal adrenal derived dehydroepiandrosterone as the source metabolite for placental androgens would be lower in group B than group A (4, 9, 25), the residual supporting activity for placental CYP19A1 would be lower in group B than group A. Thus, the total amount of androgens would be relatively well preserved in 46,XY cases with a mild difference in the fetal testis-derived T between groups A and B and invariably and similarly increased in 46,XX cases of both groups A and B. Furthermore, this notion explains why maternal virilization during pregnancy was similar between groups A and B because it is primarily due to androgens of the placental origin rather than the fetal gonadal or the backdoor origin (3, 4, 25).

Assessment of pubertal development was possible in a limited number of patients. However, pubertal development appeared to differ between groups A and B and between 46,XY and 46,XX cases. In this regard, T and E_2 biosynthesis during puberty is also performed in a simple one-way manner, and T production is mediated by CYP17A1 and E_2 production is mediated by both CYP17A1 and CYP19A1 (Fig. 4). Thus, gonadal steroid production would depend on the R457H dosage, with T production being less compromised than E_2 production. In addition, our observation suggests the frequent occurrence of PCO in infancy and puberty when gonadotropins are physiologically elevated (27) and the beneficial effect of estrogen replacement therapy in the amelioration of PCO.

Evaluation of growth pattern also remained fragmentary. However, two implications are possible. First, the intrinsic skeletal abnormalities may be relevant to the growth pattern. Indeed, relative tall stature in childhood may be compatible with the elongation of long bones as indicated by arachnodactyly and dolichostenomelia, and worsening of scoliosis during puberty in group B would also be consistent with the low POR activity (supplementary Fig. 1). Second, the spontaneous pubertal growth pattern of cases 2 and 3 without scoliosis is considered to represent a mild form of that of male patients with aromatase deficiency (28, 29). Such a qualitatively similar but quantitatively different pubertal growth pattern would be explained by assuming a drastically attenuated but not abolished *in vivo* supporting function of the R457H protein for aromatase.

Lastly, clinical features were similar between A503V-positive and -negative cases in group B. However, this would not argue

against a possible phenotypic effect of mildly hypomorphic A503V, because A503V of the four cases in group B was present on the alleles carrying apparently null mutations. Thus, it remains unknown whether A503V can modify phenotypic features in PORD, although the previous study argues against a modifying effect of A503V on clinical phenotypes in 21-hydroxylase deficiency (30). Furthermore, because A503V was absent from all of 47 alleles carrying R457H, this would provide further support for the previous notion that R457H is a founder mutation accompanied by a specific haplotype (6, 7). Thus, whereas A503V was identified in only eight of the 70 alleles (11.4%) in this study, this frequency is obviously biased by the high prevalence of R457H in Japanese patients. Rather, the frequency of A503V in R457H-negative alleles suggests that the prevalence of A503V is considerably high in the Japanese population, as reported in other populations (from 19.1% in African American to 36.7% in Chinese American) (15).

Remarks and conclusion

It should be pointed out that the results are totally based on the studies of Japanese patients. In this regard, A287P is common in Caucasian patients (4, 5), and clinical studies in 10 A287P-positive patients including three homozygotes (five with 46,XY and five with 46,XX) have suggested phenotypic similarities and differences between R457H-positive patients and A287P-positive patients: 1) skeletal phenotype is usually obvious and appears to be grossly dependent on the A287P dosage; 2) 46,XY DSD is variable and is apparently independent of the A287P dosage; 3) 46,XX DSD is also variable and absent in one A287P homozygote and one of four compound heterozygotes with A287P; and 4) maternal virilization during pregnancy is not described (1, 2, 5, 31, 32). Thus, skeletal phenotype would be explained by assuming that both R457H and A287P have drastically lost supporting activities for CYP51A1 and/or SQLE involved in cholesterologenesis, although functional studies have not been performed. Furthermore, clinical features relevant to steroidogenic dysfunction would be grossly consistent with the previous *in vitro* functional data. It has been reported that R457H yields only 1–3% supporting activities for 17α-hydroxylase and aromatase, and virtually no activity for 17,20 lyase, whereas A287P provides supporting activities of about 40% for 17α-hydroxylase, about 20% for 17,20 lyase, about 70% for 21-hydroxylase, and about 100% for aromatase (1, 5, 11, 33). Thus, the relative activities of frontdoor and backdoor pathways would be different largely between R457H-positive and A287P-positive patients, and placental T production would remain minor, if any, in A287P-positive patients. Collectively, the Japanese data would not apply simply to other populations.

In conclusion, the present study in Japanese patients argues against the heterozygote manifestation and suggests that the residual POR activity reflected by the R457H dosage constitutes the underlying factor for the clinical variability in some features but not other features, probably because of the simplicity and the complexity of the POR-dependent metabolic pathways relevant to each phenotype. Further studies including genotype-phenotype analyses in various ethnic groups will permit a better clarification of the molecular and clinical characteristics of PORD.

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OTX2 Loss of Function Mutation Causes Anophthalmia and Combined Pituitary Hormone Deficiency with a Small Anterior and Ectopic Posterior Pituitary

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Context: Orthodenticle homeobox 2 (OTX2) is a transcription factor necessary for ocular and forebrain development. In humans, heterozygous mutations of *OTX2* cause severe ocular malformations. However, whether mutations of *OTX2* cause pituitary structural abnormalities or combined pituitary hormone deficiency (CPHD) has not been clarified.

Objectives: We surveyed the functional consequences of a novel *OTX2* mutation that was detected in a patient with anophthalmia and CPHD.

Patient: We examined a Japanese patient with growth disturbance, anophthalamia, and severe developmental delay. He showed deficiencies in GH, TSH, LH, FSH, and ACTH. Brain magnetic resonance imaging revealed a small anterior pituitary gland, invisible stalk, ectopic posterior lobe, and Chiari malformation.

Results: Sequence analysis of *OTX2* demonstrated a heterozygous two bases insertion [S136fsX178 (c.576-577insCT)] in exon 3. The mutant Otx2 protein localized to the nucleus, but did not activate the promoter of the *HESX1* and *POU1F1* gene, indicating a loss of function mutation. No dominant negative effect in the presence of wild-type Otx2 was observed.

Conclusion: This case indicates that the *OTX2* mutation is a cause of CPHD. Further study of more patients with *OTX2* defects is necessary to clarify the clinical phenotypes and endocrine defects caused by *OTX2* mutations. **(*J Clin Endocrinol Metab* 94: 314–319, 2009)**

The proper development of the anterior lobe of the pituitary gland depends on several transcription factors. Defects in these transcription factors are associated with combined pituitary hormone deficiency (CPHD) (1, 2). In addition to CPHD, individuals with mutations in these transcription factor genes may present with other symptoms related to extrapituitary expression of the altered gene, such as in the nervous system and adrenal gland (1, 2).

Orthodenticle homeobox 2 (*OTX2*; MIM 600037), a bicoid-type homeodomain gene, is a vertebrate ortholog of *Drosophila* gene orthodenticle (*Otd*), which is required for anterior brain, eye, and antenna formation (3–7). Mouse *Otx1* and *Otx2* are expressed in developing neural and sensory structures, including the brain, ear, nose, and eye. Homozygous *Otx2* knockout mice die at midgestation with severe brain anomalies (3–6). Heterozygous knockout mice reveal variable phenotypes ranging from anencephaly, micrognathia, anophthalmia, and microphthalmia to normal, depending on the genetic background (3–6).

Consistent with these findings, heterozygous mutations of *OTX2* have been reported recently in patients with severe ocular

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Abbreviations: CPHD, Combined pituitary hormone deficiency; GFP, green fluorescent protein; MRI, magnetic resonance imaging; OTX2, orthodenticle homeobox 2; PRL, prolactin.

malformations and/or brain anomalies, seizures, and developmental delay (8). Functional analysis of these OTX2 mutations revealed that they are loss-of-function mutations (9). In humans, a deletion in the 14q22-23 region, including *OTX2*, causes anophthalmia and hypothalamic-pituitary anomalies (10, 11). Thus, a mutation in *OTX2* gene may be the cause of the ophthalmological anomaly observed in patients with CPHD.

Here, we report a patient with anophthalamia and CPHD, who had a novel mutation of *OTX2*.

Patient and Methods

Pituitary hormone assessment

GH provocative tests were performed using insulin (0.1 IU/kg), arginine (0.5 g/kg), and GHRH (1 μg/kg). Serum levels of LH and FSH were determined in response to GnRH (100 μg/m^2). Serum levels of TSH and PRL were determined in response to TRH (10 μg/kg).

DNA amplification and sequence analysis of *OTX2*

Informed consent to participate in the study was obtained from the parents. The ethical committee of our hospital approved this study. Genomic DNA was extracted from peripheral leukocytes, and each exon of the *OTX2* gene was amplified by PCR as described previously (8). After amplification, the PCR products were purified and sequenced directly using an ABI PRISM Dye Terminator Cycle Sequencing Kit and an ABI 373A automated fluorescent sequencer (Applied Biosystems, Foster City, CA).

Wild-type and mutant Otx2 cDNA construction and plasmid construction

Only eight amino acids (codon 33–40) in the N terminus of mouse Otx2 are different from the amino acid sequence of human OTX2 (96% identity). Thus, to examine the effects of this mutation on OTX2 activity, we created a corresponding mutation in the mouse Otx2 cDNA. Mouse Otx2 cDNA was inserted into pcDNA 3.1 (Wt-Otx2). The mutant cDNA was created by site-directed mutagenesis using an overlapping PCR strategy and was designated MT-Otx2. The mutation was verified by direct DNA sequencing.

Reporter plasmids

A DNA fragment containing −819 to +119 bp of the human *HESX1* upstream sequence was generated by PCR amplification using human genomic DNA as a template (12). The PCR product was cloned into the luciferase vector pGL3 by a standard technique. This *HESX1* upstream regulatory sequence construct was designated pGL3-HESX1. The 5′ upstream region of the human *POU1F1* sequence contains the putative DNA binding site to OTX2 (GGATTA, at position −134 to −129) (13, 14). The promoter of *POU1F1* was also generated by PCR amplification using human genomic DNA as a template according to a previous report (14). The PCR product was cloned into the luciferase vector pGL3 and was designated pGL3-POU1F1.

Cell culture

COS cells were obtained from American Type Cell Culture (Manassas, VA) and grown in DMEM supplemented with 10% fetal bovine serum.

Transient gene expression

To assay *HESX1* and *POU1F1* gene promoter activity, COS cells were plated in six-well plates, grown to 70% confluency, and transiently transfected by lipofectamine with: 1) empty expression vector (pCDNA3, 0.5 μg); 2) WT-Otx2 (0.5 μg); 3) MT-Otx2 (0.5 μg); or 4) WT-Otx2 (0.25 μg) plus MT-Otx2 (0.25 μg) together with each reporter vector (1.0 μg) (pGL3-HESX1 or pGL3-POU1F1).

Cell extracts were prepared 48 h after transfection, and luciferase assays were performed. Luciferase measurements were divided by the respective β-galactosidase activity to control for transfection efficiency. The mean of each triplicate reaction was expressed as a percentage of the empty vector control to allow comparison of data from different experiments.

Fluorescence analysis and microscopy

COS cells transfected with either WT-Otx2-green fluorescent protein (GFP) or MT-Otx2-GFP were placed onto glass coverslips and fixed in 4% (vol/vol) formaldehyde/PBS 24 h after transfection. Cells were permeabilized in 0.1% Triton/PBS and then examined on a Fuji fluorescence microscope (Tokyo, Japan).

Results

Case report

The patient is a Japanese boy, currently 6 yr old. He was born after 40 wk gestation by normal vaginal delivery and was the first child of nonconsanguineous parents. The patient had no siblings, and his parents were healthy. His birth weight was 3490 g and length was 49.5 cm. At birth he was noted to have bilateral anophthalmia. At 1 d after birth, he showed failure to thrive and hypoglycemia, but these symptoms improved by iv glucose supplementation. He also showed prolonged jaundice and was treated with UV light for 1 d. Further investigation was not performed until at 4 yr of age he was referred to our hospital because of short stature. On physical examination, his height was 81.8 cm (−5.3 SD for a normal Japanese boy), and his weight was 10.7 kg (−2.5 SD for a normal Japanese boy). His head circumference was 47.2 cm (−2.7 SD for a normal Japanese boy). He had a small penis and bilateral undescended testes. His psychomotor development was markedly delayed. Hormonal data showed central hypothyroidism, GH deficiency, gonadotropin deficiency, and cortisol deficiency (Table 1).

Brain magnetic resonance imaging (MRI) revealed a small anterior pituitary, invisible pituitary stalk, ectopic posterior lobe, bilateral anophthalamia, defect of the bilateral optic nerve, and Chiari malformation (Fig. 1). Thus, he was diagnosed as having CPHD, and replacement therapy with L-T_4 and hydrocortisone was started at that time. Treatment with GH replacement therapy was started 2 months later. At present, his body weight is 13 kg and height is 93.5 cm (−4.3 SD for a normal Japanese boy). The patient's development remains severely delayed.

Gene sequencing results

Sequence analysis of *OTX2* demonstrated a heterozygous two bases insertion in exon 3 [S136fsX178 (c.576-577insCT)] (Fig. 2A). This insertion mutation caused amino acid changes and a premature stop codon 184 bases downstream (codon 178). Thus, this mutant lacks the C-terminal region of OTX2 (Fig. 2B). Neither the patient's parents nor 50 normal Japanese subjects showed these base changes.

TABLE 1. Endocrinological findings in the patient

	Basal	Max	Normal range Basal	Normal range Max
Free T_3 (pg/ml)	2.41		3.23–5.11	
Free T_4 (ng/dl)	0.75		1.09–2.55	
IGF-I (ng/ml)	12.0		29–173	
GH (ng/ml)				
GHRH test	0.4	9.1		>15
Insulin tolerance test[a]	0.4	0.6		>6.0
GnRH test				
LH (mIU/ml)	0.1	0.3	0.2–1.2	1.3–10.0
FSH (mIU/ml)	0.9	1.0	1.4–3.0	9.0–26.0
TRH test				
TSH (mU/ml)	2.64	21.57 (after 120 min)	0.3–3.50	10–35
PRL (ng/ml)	55.2	69.4 (after 60 min), 63.2 (after 120 min)	1.7–15.4	29.4–35.8
Insulin tolerance test[a]				
ACTH (pg/ml)	18	21	9.8–27.3	97–360
Cortisol (mg/dl)	10.2	12.2	15–20	>20

Conversion factors to SI units: free T_3, 1.54 (pmol/liter); free T_4, 12.87 (pmol/liter); IGF-I, 0.131 (nmol/liter); GH, 1.0 (μg/liter); LH, 1.0 (IU/liter); FSH, 1.0 (IU/liter); TSH, 1.0 (mIU/liter); PRL, 1.0 (mg/liter); cortisol, 27.59 (nmol/liter); and ACTH, 0.2202 (pmol/liter).

[a] By insulin stimulation, blood glucose decreased from 86 to 39 mg/dl in 15 min.

Functional analysis and subcellular localization

Although we have studied this mutation in the context of the mouse Otx2 cDNA, the findings are likely to apply to human OTX2 because the amino acids sequence of the relevant regions of the human OTX2 and mouse Otx2 are identical.

WT-Otx2 activated the *HESX1* promoter activity, whereas MT-Otx2 did not (Fig. 2C). Using the *POU1F1* promoter, MT-Otx2 partially activated promoter activity compared with empty vector; however, activation was less than 50% of that by WT-Otx2 (Fig. 2C). Cotransfection of MT-Otx2 and equivalent amounts of WT-Otx2 did not affect WT-Otx2, which excluded dominant negative effects (Fig. 2C).

We analyzed the subcellular localization of WT-Otx2 and MT-Otx2. As shown in Fig. 2D, both WT-Otx2 and MT-Otx2 proteins localized to the nucleus. These results are in accordance with the results reported by Chatelain *et al.* (9).

FIG. 1. A sagittal image showing a small anterior pituitary (*arrow*) and an ectopic posterior gland (*arrowhead*).

Discussion

We identified a novel *OTX2* mutation in a patient with anophthalmia and CPHD. By the nature and the location of the mutation, impaired function is expected, and our *in vitro* experimental evidence confirms this.

We summarized the clinical phenotypes, MRI findings, mutations, and functional consequences of *OTX2* reported thus far (8, 9) (Table 2). To date, six heterozygous mutations of *OTX2* in seven patients have been reported. The locations of these mutations are shown in Fig. 2B. Three mutations cause a frame shift (p.P28fs, p.G40fsX86, and p.L156fsX178), two are nonsense mutations (Q99X and Y179X), and one is a missense mutation (R89G). Chatelain *et al.* (9) demonstrated that p.P28fs and p.G40fsX86 do not have DNA binding activity, whereas Q99X, p.L156fsX178, and Y179X retain the DNA binding activity but do not activate the target gene promoter. These findings indicate that the homeodomain of Otx2 is sufficient for DNA binding, whereas the C-terminal region is required for the target gene activation. Therefore, although we did not perform a DNA binding study, the functional alteration of our mutant (S136fsX178) is likely due to the lack of C-terminal region and not the lack of DNA binding activity, similar to the p.L156fsX178 and Y179X mutants.

Clinical phenotypes and MRI findings of patients with mutations of *OTX2* are variable, even in the same family, as shown in Table 2. For instance, ophthalmological symptoms ranged from bilateral anophthalmia to microphthalmia. In addition, four patients showed developmental delay. In MRI findings, the development of the optic nerve and chiasm varies from normal to absent. Thus, bilateral anophthalmia, developmental delay, and absence of optic nerve in our patient are consistent with a mutation of *OTX2*.

In regard to endocrine function, our case demonstrated that CPHD was caused by pituitary and hypothalamic disturbance,

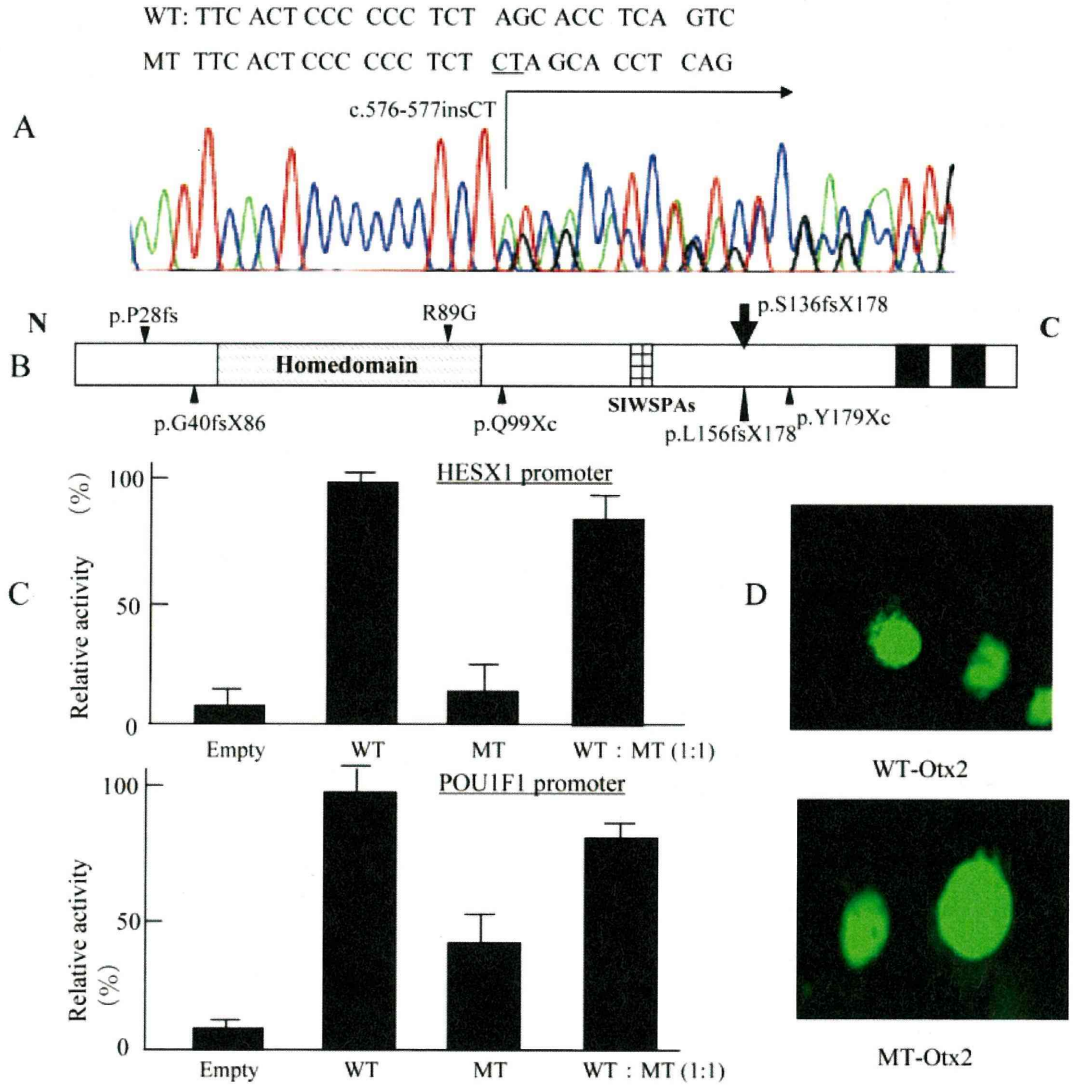

FIG. 2. A, Sequence analysis demonstrated an insertion of CT nucleotides. Note the *double bands* present after the mutation site [c.576-577insCT (S136fsX178)]. B, Schematic representation of the *OTX2* gene. The *hatched box* represents the homeodomain. The SIWSPA motif is conserved between OTX families. The two *black boxes* in the C terminus represent the tandem repeated conserved tail motif. The mutation reported in this study introduces a premature stop codon at 178, denoted by an *arrow*. Six previously reported mutations are shown by *arrowheads*. C, Transactivation functions of WT-Otx2 and MT-Otx2. Cotransfection of WT-Otx2 with either *HESX1* or *POU1F1* promoter stimulated the luciferase reporter gene relative to the empty vector. In the *HESX1* promoter, MT-Otx2 abolished the activation function; however, MT-Otx2 was hypomorphic using the *POU1F1* promoter. Cotransfection of MT-Otx2 and WT-Otx2 did not impair the transactivation capacity of WT-Otx2, suggesting no dominant negative effect of the mutant protein. D, Subcellular localization of WT-Otx2 and MT-Otx2. COS cells expressing WT-Otx2-GFP or MT-Otx2-GFP were visualized directly by GFP fluorescence. Both WT-Otx2 and Mt-Otx2 are localized to the nucleus.

consistent with the findings of MRI. In a previous report, endocrine investigations of patients with *OTX2* mutation were not described (8). Three patients showed short stature; however, two had normal pituitary glands as demonstrated by MRI (Table 2). These findings suggest that they did not have severe CPHD.

To explain the hypothalamic-pituitary abnormality and the endocrinological findings in our patient, we investigated potential targets for this transcriptional factor. One possible target is the *HESX1* gene, because three *OTX2* binding sites are found in the *HESX1* promoter region, and these sites are required for gene activation (12). In addition, murine *Hesx1* failed to be transcribed in the anterior neural plate in *Otx2* knockout mice (15). Furthermore, mutations of *HESX1* cause CPHD and pituitary abnormalities in humans (1, 2, 16). In this context, our mutation was unable to activate the *HESX1* gene promoter adequately, perhaps resulting in the observed hypothalamic-pituitary abnormality and CPHD observed in our patient.

Another potential target gene is the *POU1F1* gene, which also contains an OTX2 binding site. Using the *POU1F1* promoter, mutant Otx2 showed poor activity compared with that of WT-Otx2. It is known that mutations of *POU1F1* cause GH, TSH, and prolactin (PRL) deficiency in humans (1, 2, 17–19). Therefore, additive effects of the impairment of *HESX1* and *POU1F1* gene activation may be involved in the development of CPHD in this patient. Further studies are required to determine whether OTX2 interacts with other genes, leading to the hypothalamic-pituitary abnormality and CPHD.

TABLE 2. Clinical, MRI, and molecular findings in seven patients and our patient with *OTX2* mutations

Case	Gender	Age at evaluation (yr)	Ocular symptoms	Clinical findings	MRI	Mutations of the *OTX2* cDNA[a] protein	Degree of loss of function[b]
1	M	4	Right anophthalmia, left microphthalmia	Developmental delay, no short stature	Normal pituitary, thin optic chiasm, hypoplastic optic nerve, partial absence of corpus callosum, hippocampal malrotation	c.464ins GC, p.L156fsX178	Severe
2	M	6.3	Bilateral microphthalmia	Normal development, no short stature	Normal pituitary, absence of optic nerve and chiasm	c265C>G, p.R89G	Partial
3	F	12	Bilateral microphthalmia	Normal development	Reduced optic nerve on CT	c.81delC, p.P28fs	Severe
4[c]	F	33	Bilateral microphthalmia	Short stature (<3rd percentile), severe developmental delay, seizure	Not available	c.537T>A, p.Y179Xc	Severe
4[d]	M	25	Bilateral microphthalmia	Short stature (<3rd percentile), mild developmental delay	Normal pituitary, normal optic nerve and chiasm	c.537T>A, p.Y179Xc	Severe
5	M	24	Bilateral anophthalmia	Short stature (<3rd percentile), normal development	Normal pituitary, hypoplastic optic nerve and chiasm	c.117delCC, p.G40fsX86	Severe
6	M	11	Bilateral anophthalmia	Severe developmental delay, seizure	Absence of optic nerve and chiasm, hippocampal malformation	c.259C>T, p.Q99Xc	Severe
Our case	M	4	Bilateral anophthalmia	Severe short stature, severe developmental delay	Hypoplastic anterior pituitary, ectopic posterior, absence of optic nerve and chiasm, Chiari malformation	c.576-577insCT, p.S136fsX178	Severe

These patients were reported by Ragge *et al.* (8). Results of *in vitro* functional study were described in Chatelain *et al.* (9). Pituitary findings of patients 3, 4[c], and 6 were not reported in the literature (8). M, Male; F, female.

[a] The A of the initiation codon (ATG) is considered as position 1. Therefore, the nucleotide number of *OTX2* mutations is different from that of Ragge *et al.* (8).

[b] All these mutants did not show dominant negative effect.

[c,d] These subjects are siblings.

In conclusion, we report a patient with CPHD, anophthalmia, and developmental delay caused by an *OTX2* mutation. To understand clinical phenotypes and endocrinological findings caused by *OTX2* mutations, further study of more patients with *OTX2* defects is necessary.

Acknowledgments

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Disclosure Statement: The authors have nothing to declare.

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Sry: the master switch in mammalian sex determination

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Summary

SRY, the mammalian Y-chromosomal testis-determining gene, induces male sex determination. Recent studies in mice reveal that the major role of SRY is to achieve sufficient expression of the related gene *Sox9*, in order to induce Sertoli cell differentiation, which in turn drives testis formation. Here, we discuss the cascade of events triggered by SRY and the mechanisms that reinforce the differentiation of the testes in males while actively inhibiting ovarian development.

Key words: *Sry*, Sex determination, *Sox9*, Testis, Sertoli cell

Introduction

The development of two sexes is observed in most animals and is essential for their survival and evolution. Disorders of sex development (DSDs; see Glossary, Box 1) are among the most common genetic diseases in humans and are often associated with genital ambiguity (Kronenberg and Williams, 2007). Because of its clinical and biological importance, identifying the mechanism of sex determination – the developmental decision to generate either testes or ovaries – continues to attract the attention of a broad range of researchers, including developmental biologists, biomedical scientists, evolutionary biologists and ecologists.

In mammals, two major breakthroughs have shaped our current understanding of sex determination. First, in 1959, two human DSDs, Turner syndrome (XO females) and Klinefelter syndrome (XXY males) were identified and reported (Ford et al., 1959; Jacobs and Strong, 1959), and these studies established that the Y chromosome carries a gene that determines maleness. It would take another 30 years before the second breakthrough was made: the discovery of *SRY* (sex-determining region on the chromosome Y, denoted *Sry* in species other than humans). The human *SRY* gene was identified by searching for conserved sequences among translocated Y chromosomal DNA from four XX male patients (Sinclair et al., 1990). The presence of a similar gene, *Sry*, on the mouse Y chromosome was consistent with this gene having a sex-determining function (Gubbay et al., 1990). The role of *Sry* as the switch gene for mammalian sex determination was confirmed in experiments in which XX mice were converted to males by the introduction of *Sry* (Koopman et al., 1991).

Sry and the molecular mechanisms of sex determination have continued to be studied intensely over the past 20 years. Unlike other developmental systems that are well conserved through evolution, sex determination is highly variable in the animal kingdom, and the genetic mechanisms involved in common laboratory model organisms, such as flies, nematode worms, chickens and frogs, bear little, if any, resemblance to those used in mammals. Indeed, *Sry* is found only in mammals, though not in all mammalian orders – monotremes (see Glossary, Box 1), for example, lack *Sry*. Most of our current understanding of *Sry* and

Box 1. Glossary

Cell-autonomous. Occurring within a cell, not involving signalling between cells.

Chromatin immunoprecipitation (ChIP). A method used to identify the transcriptional targets of a given transaction factor by precipitating the transcription factor while it is bound to DNA, then characterizing the bound DNA.

Coelomic epithelium. Layer of cells lining the body cavity of an embryo.

Disorder of sex development (DSD). Any one of a spectrum of conditions where the development of internal or external sexual organs differs from 'typical' male or female, or is not as expected given the sex chromosomes present.

Eutherian mammals. A subclass of mammals that have a placenta.

Genital ridges. Pair of thickened rows of coelomic epithelial cells either side of the midline in the trunk of an embryo that are the precursors of the gonads.

Granulosa cells. The 'nurse' cells in ovarian follicles that nurture germ cells.

High-mobility group. A specific family of transcription factors that have structurally related DNA-binding domains ~80 amino acids long.

Leydig cells. Cells in the interstitium of testes that produce androgens.

Mesonephros. Embryonic structure attached to each genital ridge, from which the male or female internal sexual ducts arise.

Monotremes. A subclass of mammals, represented by platypus, that lay eggs instead of giving birth to live young.

Nuclear localization signal. A short sequence of amino acids that allows proteins such as transcription factors to move from the cell cytoplasm into the cell nucleus.

Ovotestis. A gonad containing both ovarian and testicular tissue.

Paracrine signalling. Short-range chemical communication between cells.

Pre-Sertoli cells. Cells in an XY genital ridge that have activated *Sry* and *Sox9* expression, but have not yet assembled into testis cord structures.

Sertoli cells. Testicular cells that form testis cords and interact with and nurture germ cells.

Testis cord. The precursors of the adult spermatogenic tubules, composed of germ cells enclosed by a layer of Sertoli cells.

Theca cells. Cells in the outermost layer of the ovarian follicle that produce androgen as a source for neighbouring granulosa cells to convert to estrogens.

WT1(+KTS)/WT1(–KTS). Isoforms of the Wilms tumour suppressor protein (WT1) that have three amino acids (lysine, threonine, serine: KTS) inserted or excluded, respectively.

ZZ/ZW sex determining system. A system in which males have two identical sex chromosomes and females have two different sex chromosomes, in contrast to the situation with an XX/XY sex-determining system.

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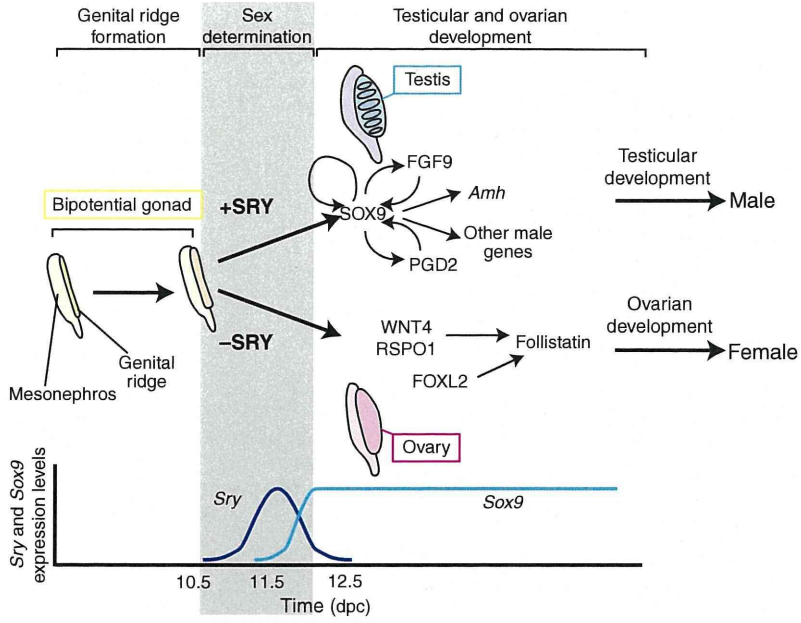

Fig. 1. Overview of sex determination in mice. Chronological flow of early mouse sex differentiation; the grey area indicates the period of sex determination. During mouse embryogenesis, bi-potential gonads (yellow) arise from the genital ridges by 10.5 dpc. In somatic cells of XY genital ridges, *Sry* expression (shown in dark blue beneath the schematic) starts at 10.5 dpc, reaches a peak at 11.5 dpc and then wanes by 12.5 dpc. A few hours later, *Sox9* expression (shown in light blue beneath the schematic) is upregulated to induce differentiation of Sertoli cells. *Sox9* expression peaks at 11.5-12.5 dpc, continues to be expressed postnatally and is supported by several positive-feedback loops (including FGF9, prostaglandin D2 and SOX9 itself), and SOX9 subsequently activates many male-specific genes, including *Amh*. At 12.5 dpc, testis cords have formed, and morphological differences between testis (blue) and ovary (pink) are evident. In the absence of SRY, genes such as *Wnt4, Rspo1* and *Foxl2* are expressed in a female-specific manner and induce ovarian development, as characterized by the expression of follistatin and many other ovary-specific genes. Abbreviations: *Amh*, anti-Müllerian hormone; dpc, days post coitum; FGF9, fibroblast growth factor 9; FOXL2, forkhead box L2; PGD2, prostaglandin D2; RSPO1, R-spondin 1; SOX9, SRY box containing gene 9; SRY, sex-determining region on the chromosome Y; WNT4, wingless-type MMTV integration site family, member 4.

mammalian sex determination has come from studies in mice. In this primer, we summarize the results of these studies and discuss the insights they have provided into the molecular and cellular biology of mammalian testis development.

An overview of the mammalian sex determination pathway

The most detailed studies of mammalian sexual determination have been carried out using mice as a model (Fig. 1). In mice, the gonadal primordia, which are called the genital ridges (see Glossary, Box 1) arise at 10.0 days post coitum (dpc). At this stage, there are no morphological or functional differences between male and female genital ridges, and both structures contain precursor cells that have the ability to differentiate into Sertoli cells (which support germ cells in the testis, see Glossary, Box 1) or granulosa cells (which have a cognate role in the ovary, see Glossary, Box 1).

Sex-specific gonadal development is triggered by *Sry* expression in somatic gonadal cells of XY genital ridges at 10.0-10.5 dpc (Fig. 2A). SRY activity upregulates *Sox9* (SRY box containing gene 9) transcription in Sertoli cell precursors, which in turn upregulates other genes involved in the differentiation of Sertoli cells. Logically, SRY activity must also directly or indirectly suppress the female sex-determining pathway, which would otherwise continue to be active, as it is in XX genital ridges. Differentiating Sertoli cells then assemble into testis cords (tubular structures that contain the germ cells; see Glossary, Box 1) (Fig. 2). The Sertoli cells then stimulate the sex-specific development of germ cells, androgen-producing Leydig cells (see Glossary, Box 1), testis vascular cells and other interstitial (i.e. non-cord) cell types. The formation of testes is the hallmark of male sex determination.

By contrast, in the absence of *Sry* in XX gonads, genes such as *Wnt4* (wingless-type MMTV integration site family, member 4) and *Foxl2* (forkhead box L2) start to be expressed in a female-specific manner at 11.5-12.5 dpc, and upregulate other downstream female genes, such as follistatin. The female-specific programme of gene expression leads to the differentiation of granulosa cells and theca cells (see Glossary, Box 1), the production of oocytes, and the formation of ovarian follicles (Fig. 1). These events also occur in XY gonads that lack SRY function, supporting the key role for SRY in both activating the testis-determining pathway and suppressing the ovarian-determining pathway.

SRY structure and function

SRY is the founding member of the SOX (SRY-related HMG box) family of transcription factors. The SOX family is found throughout the animal kingdom, and comprises 20 members in mice and humans. SOX proteins have diverse roles in embryogenesis and in the development of many organs, typically acting as cell differentiation switches (reviewed by Bowles et al., 2000). SRY, like other SOX transcription factors, is characterized by a high mobility group (HMG; see Glossary, Box 1) DNA-binding domain (Fig. 3A). This domain binds to the sequence (A/T)ACAA(T/A) in the minor groove of DNA, inducing a 60-85° bend (Harley and Goodfellow, 1994) (Fig. 3B). Biochemical analysis of mutant human SRY protein from XY females has revealed that both DNA-binding and

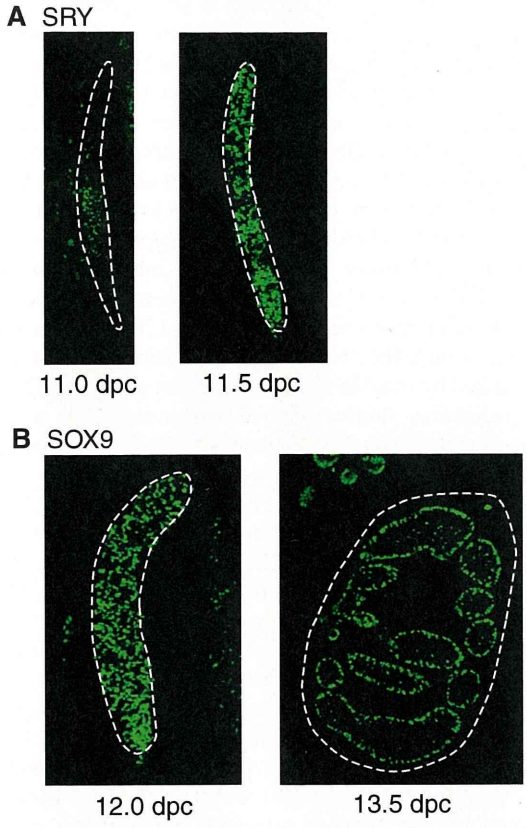

Fig. 2. Chronological sequence of SRY and SOX9 expression during sex determination and early testis development in mice. (**A**) At 11.0 dpc, SRY (sex-determining region on the chromosome Y) protein is expressed initially in a group of somatic cells (green) in the centre of the genital ridge (indicated by a broken line). The domain of SRY-expressing cells then expands to occupy the entire length of the genital ridge; these cells are referred to as supporting cell precursors. (**B**) By 12.0 dpc, SOX9 (SRY box containing gene 9) expression is activated in these same cells, now referred to as pre-Sertoli cells. The SOX9-expressing cells assemble into testis cords by 12.5 dpc and are referred to Sertoli cells. SOX9 continues to be expressed in Sertoli cells at 13.5 dpc and beyond. Proteins were visualized by immunofluorescence using antibodies to SRY and SOX9. Images are courtesy of Dr Dagmar Wilhelm (The University of Queensland, Brisbane, Australia).

-bending are essential for SRY function. Furthermore, most human XY females have mutations in the HMG domain, reflecting the importance of this domain for the function of SRY (reviewed by Harley and Goodfellow, 1994).

In contrast to the HMG domain, the remaining parts of SRY are poorly conserved between species, and no additional conserved functional domains have been identified (Fig. 3A). For example, in mice, only two amino acids make up the N-terminal region that usually comprises 30-60 amino acids in other species (Gubbay et al., 1990). In the C-terminal region of mouse SRY, a long glutamine (Q)-rich domain is present that is not found in other mammalian species but that might act as a transcriptional activation domain (Dubin and Ostrer, 1994). *Sry* transgene constructs that lack this domain fail to cause male development (Bowles et al., 1999), although it is not known whether this failure reflects a reduced stability of the truncated protein. XX transgenic mice that express human or goat *SRY* develop as males (Lovell-Badge et al., 2002; Pannetier et al., 2006), suggesting that the non-HMG-domain regions of SRY either are conserved in function but not in sequence, or have no function. In support of the former possibility, the human SRY protein has to be of full length to show normal DNA-binding ability in vitro (Sanchez-Moreno et al., 2008).

SRY protein also carries two nuclear localization signals (NLSs; see Glossary, Box 1) and target sites for acetylation and phosphorylation (Fig. 3A). The NLSs lie at each end of the HMG domain and are conserved between mouce and human (Sudbeck and Scherer, 1997); these bind calmodulin (a calcium-binding protein) and importin β (a nuclear import protein), respectively (Harley et al., 1996; Forwood et al., 2001; Sim et al., 2005) (Fig. 3A). Mutations in either NLS can cause human XY sex reversal, indicating that they are not functionally redundant (Battiloro et al., 1997; Veitia et al., 1997; Harley et al., 2003; Sim et al., 2005). Human SRY is acetylated at a single lysine residue that is well-conserved between species (Fig. 3A); acetylation enhances the nuclear localization of SRY by facilitating its interaction with importin β (Thevenet et al., 2004). Furthermore, human SRY is phosphorylated by cAMP-dependent protein kinase (PKA) on serine residues (S31-S33) located in the N-terminal part of the protein. This PKA-dependent phosphorylation of SRY increases its DNA-binding ability and its subsequent transcriptional activity, and is conserved across primates (Desclozeaux et al., 1998). Other possible phosphorylation residues (serine or threonine) are conserved in the N-terminal domain of SRY in all eutherian mammals (see Glossary, Box 1), except rodents, but the precise function of these residues has not been elucidated.

A number of proteins, including KRABO (kruppel-associated box domain only) (Oh et al., 2005; Peng et al., 2009), WT1 (Wilms tumour 1) (Matsuzawa-Watanabe et al., 2003), SIP1 (SRY-interacting protein 1) (Poulat et al., 1997) and PARP1 [Poly (ADP-ribose) polymerase] (Li et al., 2006) have been shown to interact with mouse and human SRY (Fig. 3A). However, most studies of these protein-protein interactions to date have been based on in vitro systems, and so their physiological significance is as yet unclear.

In summary, our understanding of the molecular mode of action of SRY remains rudimentary. Available evidence points to it having a role as a transcription factor that enters the nucleus, binds to DNA and then upregulates the expression of *Sox9* (see below), but detailed structure-function relationships and how SRY functions in different mammalian species, despite its high sequence divergence, remain to be determined.

Sry expression and its regulation

Most data on of the regulation of *Sry* have been obtained from studies in mice, a species in which the expression of *Sry* in gonads is tightly regulated in both space and time. In the mouse embryo, *Sry* expression starts at 10.5 dpc in somatic cells of XY genital ridges, reaches a peak at 11.5 dpc and wanes by 12.5 dpc (Koopman et al., 1990; Hacker et al., 1995; Jeske et al., 1995; Bullejos and Koopman, 2001; Wilhelm et al., 2005) (Fig. 1, Fig. 2A). Its expression is associated with the differentiation of Sertoli cells in the testis but, clearly, continued *Sry* expression in mice is not needed to maintain the Sertoli cell phenotype.

In all other species studied, *Sry* expression in the gonads is maintained rather than transient, for reasons that are not clear. Furthermore, in some species, such as humans and wallabies, *Sry* expression is not limited to the gonads, and is instead expressed in many tissues during foetal development (Clepet et al., 1993; Harry et al., 1995). In these species, the function of SRY beyond its role in testis development has not been determined.

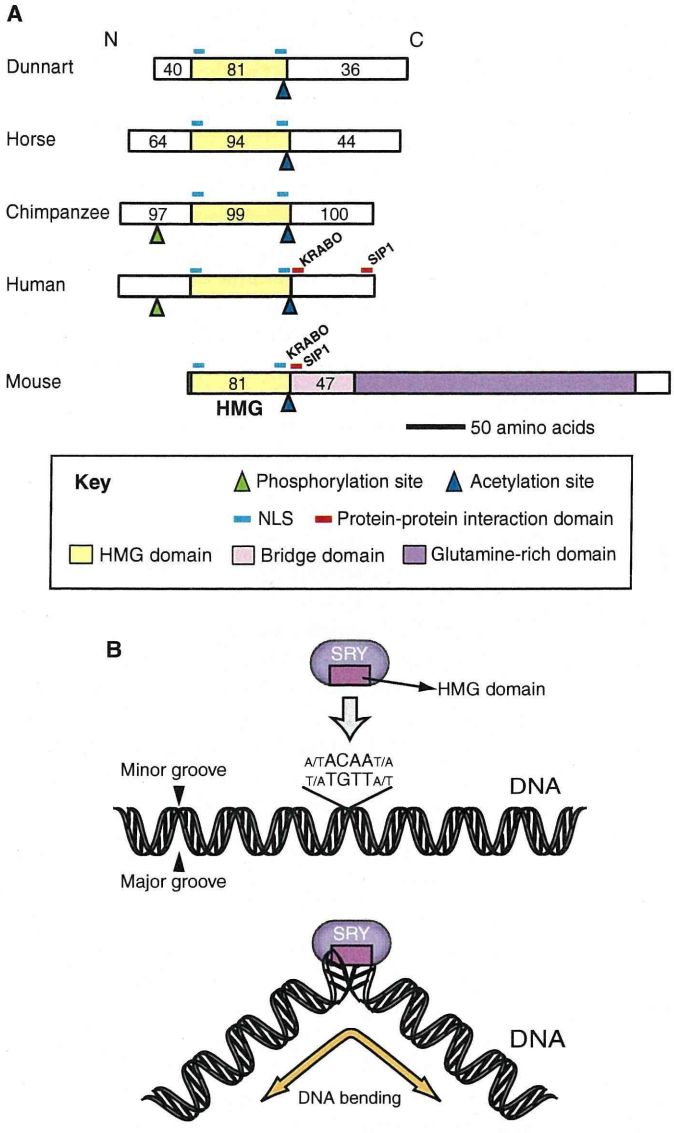

Fig. 3. SRY protein structure and function. (**A**) A comparison of SRY (sex-determining region on the chromosome Y) protein structure among five mammalian species: dunnart (*Sminthopsis macroura,* a marsupial), horse (*Equus caballus*), chimpanzee (*Pan trglodytes*), human (*Homo sapiens*) and mouse (*Mus musculus*). The HMG (high-mobility group) domain is shown in yellow. Numbers indicate the percentage of amino acids identical to human SRY. The locations of post-translational modification sites [nuclear localization signals (NLSs), phosphorylation sites, acetylation sites] and protein-protein interaction domains are indicated. Mouse SRY has a unique glutamine-rich domain, shown in purple. Between the HMG and glutamine-rich domains, mouse SRY has a 'bridge' domain of unknown function, indicated in pink. Kruppel-associated box domain only and SRY-interacting protein 1 interacting sites are indicated (KRABO and Slp1). (**B**) SRY protein binding to DNA. The double-helix structure of DNA contains two different grooves, major and minor. The HMG domain of SRY binds to the sequence motif (A/T)ACAA(T/A) in the minor groove of DNA, bending double-stranded DNA at 60-85°.

One of the unique characteristics of mouse *Sry* expression is its peculiar spatio-temporal wave-like pattern: *Sry* expression is initiated in the centre of the genital ridge before extending to the whole length of the gonad over a period of several hours (Fig. 2A) (Bullejos and Koopman, 2001; Wilhelm et al., 2005). Thus, not all parts of the genital ridge are exposed to *Sry* transcripts, or protein, at the same time. The transient and dynamic expression of *Sry* in mice has highlighted the concept that *Sry* functions within a critical window of time in individual somatic cells of the developing gonad. This concept was suggested after observations of the phenomenon known as B6-YDOM sex reversal, which arises when specific variants of the Y chromosome from the mouse species *Mus domesticus* (YDOM) are crossed onto the genetic background of the C57BL/6J (B6) inbred mouse strain. Repeated crossing to B6 mice leads to a variety of phenotypes, ranging from delayed testicular development to the development of ovotestes (see Glossary, Box 1) or ovaries (Eicher et al., 1982). Curiously, when ovotestes develop, they tend to have a testicular structure in the centre, flanked by ovarian regions (Eicher et al., 1982). Detailed in situ hybridization studies have demonstrated that a delay in initiating the expression of *Sry* underlies B6-YDOM sex reversal: the peak level of *Sry* expression in XY B6 Ypos gonads is delayed by up to 10 hours (Bullejos and Koopman, 2005). Thus, a critical window of time during which *Sry* can direct Sertoli cell development appears to close while the *Sry* expression wave is still confined to the central region of these XY gonads (Fig. 4A).

The existence of a narrow and crucial time window for the expression and function of *Sry* has been confirmed in transgenic mice that carry a *SRY* gene driven by the heat shock protein 70.3 (*Hsp70.3*) promoter, which allows for the experimental induction of *Sry* at various time points. In this system, a 6-hour delay of *Sry* induction results in a failure to initiate the testis development pathway (Hiramatsu et al., 2009). The limits of this window may be dictated by the need to pre-empt the pathway of ovarian granulosa cell development that occurs in the absence of *Sry*, or by the availability of partner proteins required for SRY protein to activate *Sox9*.

In contrast to the timing of *Sry* expression, the initiation of which is particularly important, the duration of *Sry* expression appears to be immaterial, as it varies between species. For example, in humans and goats, *Sry* gene expression persists well beyond sex determination and is observed even after birth (Hanley et al., 2000; Pannetier et al., 2006). Taken together, the crucial factor that determines the ability of SRY to induce Sertoli cell differentiation appears to be whether or not it is able to exceed a required threshold level of expression in any precursor cell within a given window of opportunity (Fig. 4A).

In addition to the threshold of *Sry* expression being required for Sertoli cell differentiation, there is another threshold to consider: the number of Sertoli cells required for proper testicular development. In experiments in which chimaeras were generated by combining XX and XY early mouse embryos, gonads containing various proportions of XY cells were produced. When 35-40% of somatic cells were XY, testes were formed, but when fewer than 10% were XY, ovaries were formed (Burgoyne et al., 1988) (Fig. 4B). Intermediate proportions of XY cells resulted in ovotestes.

A further observation to arise from these studies was that most, but not all, Sertoli cells in XX↔XY chimaeras were XY (Palmer and Burgoyne, 1991). These findings suggested that SRY expression normally drives Sertoli cell differentiation; however, it was also clear that SRY expression might not be an absolute requirement, and that XX cells could be recruited to the Sertoli cell population, perhaps by paracrine signalling (see Glossary, Box 1). More recently, studies of normal mouse testicular development using SOX9 and SRY antibodies have revealed the existence of pre-Sertoli cells (see Glossary, Box 1) that express SOX9 without

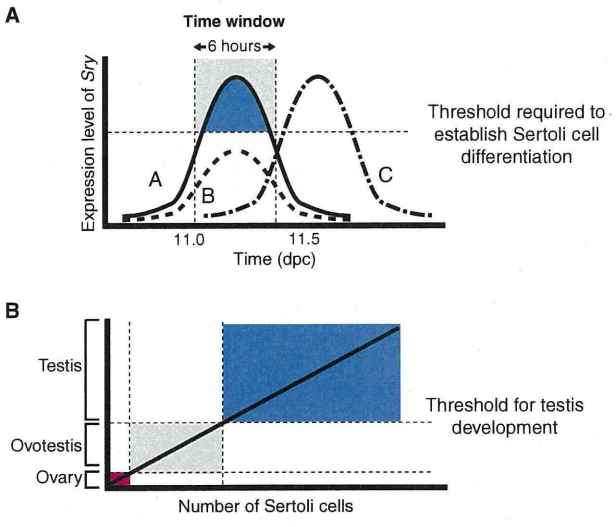

Fig. 4. Two thresholds important for mouse sex determination. (**A**) *Sry* expression levels must exceed a certain threshold (blue) within a narrow time window (grey) in individual somatic gonadal cells of developing mouse gonads in order to induce Sertoli cell differentiation (curve A). If the level of *Sry* expression does not reach the threshold, as in WT1(+KTS)-null mice (curve B), or is delayed and misses the critical window, as in YDOM sex reversal (curve C), somatic gonadal cells fail to differentiate into Sertoli cells. (**B**) The number of supporting cell precursor cells that differentiate as pre-Sertoli cells must also reach a certain threshold level in order to induce testis formation (blue). In XX-XY mouse chimaera experiments (Burgoyne et al., 1988; Palmer and Burgoyne, 1991; Patek et al., 1991), in which the proportions of XY and XX cells making up an embryo varies, the gonad fails to form a testis if the number of Sertoli cells is below a threshold, resulting in the development of an ovotestis (grey) or an ovary (red). Abbreviation: *Sry*, sex-determining region on the chromosome Y.

having first expressed SRY (Wilhelm et al., 2005). Moreover, in in vitro cultures that contain a mix of XX genital ridge cells that constitutively express a fluorescent marker and XY wild-type genital ridge cells, it has been shown that when the XY cells are in contact with the XX cells, the XX cells are induced to express *Sox9*, confirming the existence of a paracrine recruitment mechanism (Wilhelm et al., 2005). This phenomenon can be artificially induced by prostaglandin D2 (PGD2), and inhibited by a chemical blocker of the PGD2 receptor, implicating PGD2 in this paracrine recruitment mechanism (Wilhelm et al., 2005). Accordingly, *Pgds* (prostaglandin D synthase)-knockout mice show decreased *Sox9* expression in male gonads (Moniot et al., 2009). This mechanism is likely to provide a backup system to ensure and to reinforce male pathway activation by SRY.

In spite of the importance of its correct temporal and spatial expression, the regulation of *Sry* is still poorly understood. Regions that flank *Sry* are remarkably poorly conserved between mammalian species, hampering efforts to find potentially important regulatory elements. Although sequence analysis has revealed four intervals of relatively high DNA sequence conservation upstream of *SRY* among human, bovine, pig and goat genomes (Ross et al., 2008), the physiological significance of these sequences has not been determined. Transgenic mouse assays (in which critical regulatory regions are pinpointed by sequential deletion analysis) have so far not been useful for identifying a gonadal enhancer of *Sry*.

Despite these complications, several proteins have been implicated in regulating *Sry* expression (Table 1). Analyses of gene knockout mouse models have shown that the absence of these proteins leads to reduced levels of *Sry* expression, and results in XY sex reversal. In most cases, it is not clear whether the loss of these factors reduces the level of *Sry* expressed per cell or the overall number of cells that express *Sry*. How loss of their function leads to reduced *Sry* is also unclear for most of these proteins. For example, FOG2 (friend of GATA2; now known as ZFPM1 – Mouse Genome Informatics) is known to be involved in the repression rather than in the activation of several GATA-dependent target genes, such as anti-Müllerian hormone (*Amh*) and inhibin α (Robert et al., 2002). CBX2 (chromobox homologue 2), MAP3K4 (mitogen-activated protein kinase kinase kinase 4) and insulin receptors are not transcription factors, and so how they might influence *Sry* regulation remains to be determined. A recent bioinformatics study identified a region of mouse chromosome 1 between 33 and 49 cM that controls the expression of *Sry*, but none of the above genes is located within this region (Munger et al., 2009).

Among the molecules described above, much attention has focused on WT1 as a potential regulator of *Sry*. When WT1(+KTS) (see Glossary, Box 1) is reduced in humans, XY sex reversal occurs, accompanying a condition known as Frasier syndrome (Barbaux et al., 1997). Studies in mice show that

Table 1. Genes implicated in regulating *Sry* expression

Gene	Type of protein	Loss-of-function phenotype		References
		Human	Mice	
Wt1 (+KTS)	Transcription factor/ RNA-binding protein	Frasier syndrome (XY sex reversal, glomerulo-nephropathy)	XY sex reversal, dysplastic kidney	Barbaux et al., 1997 Hammes et al., 2001
Cbx2	Transcriptional co-factor	XY sex reversal	XY sex reversal, skeletal defects	Katoh-Fukui et al., 1998 Biason-Lauber et al., 2009
Gata4/Fog2	Transcription factor/co-factor (co-repressor)	*	XY sex reversal, foetal lethal	Tevosian et al., 2002
Map3k4	Kinase (enzyme)	*	XY sex reversal, spina bifida	Bogani et al., 2009
Ir, Irr, Igf1r†	Membrane receptors	*	XY sex reversal, neonatal death	Nef et al., 2003

*No mutations in human sexual reversal patients identified.
†Loss-of-function phenotype observed in triple knockout mice.
Cbx2, chromobox homolog 2 (also known as *m33*); *Fog2*, friend of GATA2; *Gata4*; Gata binding protein 4; *Igf1r*, insulin-like growth factor 1 receptor; *Ir*, insulin receptor; *Irr*, insulin receptor-related receptor; *Map3k4*; mitogen-activated protein kinase kinase kinase 4; *Wt1*(+KTS), Wilms tumour 1 gene, encoding an isoform that includes a three-amino acid motif (lysine-threonine-serine).

WT1(+KTS) plays a cell-autonomous (see Glossary, Box 1) role in regulating *Sry* expression in individual cells of the Sertoli cell lineage in vivo (Bradford et al., 2009b), but it is not clear whether *Sry* is a direct or indirect transcriptional target of WT1. In in vitro systems, WT1(+KTS) activates the promoter of mouse but not of human *Sry* (Shimamura et al., 1997; Hossain and Saunders, 2001; Miyamoto et al., 2008); moreover, WT1(+KTS) preferentially binds to unspliced pre-mRNAs, promoting gene expression by mRNA processing (Morrison et al., 2008). Thus, the mechanism of *Sry* regulation by WT1(+KTS) is unclear and requires further study.

In summary, the precise regulation of both the levels and onset of *Sry* expression is important for Sertoli cell differentiation and hence for testis development. The mechanistic details of how this regulation is achieved, however, remain unknown.

Mechanism of SRY action

After the discovery of *Sry*, attention turned to the issue of whether it might directly upregulate the male sex-determining pathway, or repress a repressor of that pathway (see Box 2). Much of this work focused on the relationship between *Sry* and *Sox9*, another important and early-acting gene that is expressed in Sertoli cells. In particular, studies have aimed to address whether SRY might regulate *Sox9* expression directly or indirectly, and whether *Sox9* might be just one of many targets of SRY.

Sox9: a major SRY target

Sox9, which encodes another member of the SOX transcription factor family, is expressed in several developing vertebrate organ systems, including the skeleton, heart, kidneys and brain (Wright et al., 1995). In XY mouse gonads, *Sox9* expression is upregulated in pre-Sertoli cells immediately after the onset of *Sry* gene expression, and mimics the wave-like pattern of *Sry* expression (Kent et al., 1996; Morais da Silva et al., 1996; Sekido et al., 2004; Bullejos and Koopman, 2005; Wilhelm et al., 2005). However, unlike *Sry* in mice, *Sox9* expression persists in the gonad beyond 12.5 dpc (Fig. 2B) (Kent et al., 1996; Morais da Silva et al., 1996), suggesting that it is able to sustain its own expression once initiated (Fig. 1), and that its continued expression might be linked to the maintenance of the Sertoli cell phenotype (DiNapoli and Capel, 2008).

The importance of *SOX9* for sex determination was revealed when heterozygous human *SOX9* mutations were found to be associated with a skeletal deformity syndrome: campomelic dysplasia (CD) (Foster et al., 1994; Wagner et al., 1994). In 75% of XY individuals with CD, partial or complete male-to-female sex reversal occurs, indicating that *SOX9* is necessary for testis determination in humans (Mansour et al., 1995). In addition, a duplication of *SOX9* had previously been identified in a mosaic XX sex-reversed individual (Huang et al., 1999). These observations indicated that *SOX9* is sufficient and necessary for testis determination, a conclusion confirmed by the sex reversal phenotype of XY *Sox9*-null (Chaboissier et al., 2004; Barrionuevo et al., 2006) and XX *Sox9*-overexpressing mice (Bishop et al., 2000; Vidal et al., 2001). Consistent with the role of SOX9 as a testis-determining factor, several genes that have a crucial role in testicular development, such as *Amh* and *Pgds*, have been identified as direct targets of SOX9 (De Santa Barbara et al., 1998; Wilhelm et al., 2007).

Unlike *Sry*, *Sox9* is conserved among non-mammalian vertebrate species. In addition, *Sox9* is linked with sexual dimorphism and with gonadal development in species such as the chicken, which has a ZZ/ZW sex-determining system (see Glossary, Box 1) (Schmid et al., 1989), and red-eared slider turtles and alligators, which have a temperature-dependent sex-determining system (Spotila et al., 1998; Western et al., 1999). Therefore, the role of SOX9 in sex determination is considered to be ancestral and pivotal among vertebrates. Furthermore, because the phenotype of *Sox9* overexpression in mouse XX gonads recapitulates that of *Sry* overexpression, *Sox9* is thought to be the only gene that is required downstream of *Sry* to activate the remainder of the testis-determining programme.

How does SRY upregulate *Sox9* expression? A recent analysis using transgenic reporter assays and in vivo chromatin immunoprecipitation (ChIP, see Glossary, Box 1) assays identified a gonad-specific enhancer of mouse *Sox9*, called TESCO (for testis-specific enhancer of *Sox9* core) (Sekido and Lovell-Badge, 2008). TESCO is a 1.4 kb sequence that is located 11-13 kb upstream of the *Sox9* transcription start site and is highly conserved in rat, dog and human genomes. SRY and SF1 (steroidogenic factor 1), an orphan nuclear receptor that regulates many genes involved in the differentiation of gonadal and adrenal cells, were found to cooperatively upregulate mouse *Sox9* by directly binding to TESCO (Sekido and Lovell-Badge, 2008) (Fig. 5), providing the first evidence that SRY acts as a transcriptional activator in vivo.

The discovery of TESCO also clarified another significant mechanism of *Sox9* regulation: an auto-regulatory positive-feedback loop (Fig. 5). SOX9 itself binds to TESCO with SF1 to stimulate its continued expression (Sekido and Lovell-Badge, 2008). This positive feedback loop is important not only to maintain *Sox9* expression after *Sry* subsides in mice, but also to ensure that the SRY signal is captured and amplified in each developing Sertoli cell. A positive-feedback loop is also observed between fibroblast growth factor 9 (FGF9) and SOX9 (Kim et al., 2006). *Fgf9* has not been proven to be a target of SOX9, but is known to be necessary for the maintenance of *Sox9* expression.

Box 2. The quest to find the molecular function of SRY

Despite being long suspected, it took over a decade to prove that SRY (sex-determining region on the chromosome Y) regulates *Sox9* (SRY box containing gene 9) directly. Why did it take so long? *Sry* has some peculiar features that make it particularly difficult to work with. The non-HMG domain sequence of mouse *Sry* gene does not have obvious functional domains and is poorly conserved between species. As a result, it was not known whether SRY acts as a transcriptional activator or a repressor, or even as an architectural factor that acts by changing DNA structure. The fact that SRY binds to a short (seven-base) target sequence that occurs frequently in the genome made it difficult to identify potential target genes bioinformatically. Furthermore, a lack of suitable cell lines and antibodies hampered molecular approaches. Useful and specific antibodies to SRY now exist, although cell lines remain a problem, as cells typically lose SRY expression after a few passages in culture.

Nor has it been easy to work backwards from *Sox9* to find out what regulates its expression, or whether SRY is involved. Translocations and deletions affecting *Sox9* function indicate that regulatory elements exist 1 Mb or more upstream of this gene in mice and humans (Bishop et al., 2000; Pfeifer et al., 1999). Painstaking deletion analysis in transgenic *Sox9* reporter mice finally revealed the gonadal-specific enhancer of *Sox9*, called testis-specific enhancer of *Sox9* core (TESCO), on which SRY acts directly (Sekido and Lovell-Badge, 2008). Even now it is not clear whether TESCO is the only element important for *Sox9* regulation during sex determination.

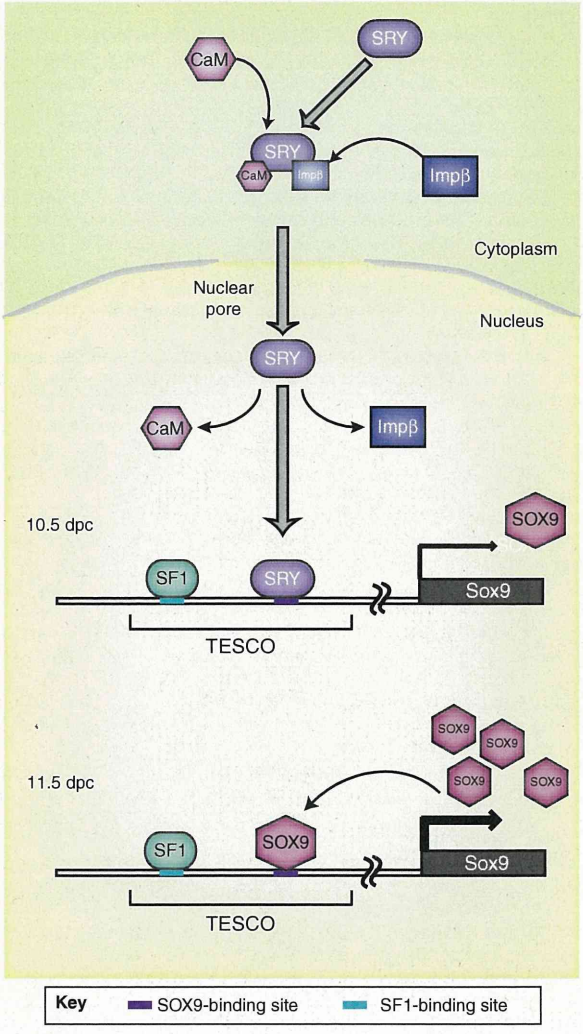

Fig. 5. Cellular mechanism of SRY function. A schematic of the cellular mechanisms of SRY action. In the cytoplasm, SRY is bound by calmodulin (CaM) and importin β (Impβ), which recognize the N- and C-terminal nuclear localization signals (NLSs) on SRY, respectively, and recruit it to enter the nucleus. At 10.5 dpc, SRY and steroidogenic factor 1 (SF1) bind directly to specific sites ('TESCO', testis-specific enhancer of *Sox9* core) that lie within the gonadal specific enhancer of *Sox9* (indicated by the coloured regions on the DNA) and upregulate *Sox9* expression cooperatively. At 11.5 dpc, after initiation of *Sox9* expression, an auto-regulation system operates in which SOX9 also binds directly to TESCO with SF1 to prolong and amplify *Sox9* expression. Abbreviations: SOX9, SRY box containing gene 9; SRY, sex-determining region on the chromosome Y.

Fgf9-null mice or mice lacking FGFR2, the main receptor for FGF9, show XY sex reversal (Colvin et al., 2001; Kim et al., 2007), attesting to the importance of FGF9 in the testis-determining pathway. Furthermore, a centre-to-pole diffusion of FGF9 appears to be essential for proper testicular development in the polar extremities of male mouse gonads, because polar fragments of mouse foetal XY genital ridges cultured separately from central fragments did not maintain *Sox9* expression or form testis cords (Hiramatsu et al., 2010).

Thus, at least three mechanisms are used to ensure the continued expression of *Sox9* and to recruit gonadal somatic cells to the *Sox9*-positive Sertoli cell population: cell-autonomous *Sox9* autoregulation, PGD2-mediated signalling and FGF9-mediated signalling from Sertoli cells. These regulatory loops induce the activation of other downstream male pathway events, such as *Amh* expression, cell proliferation in the coelomic epithelium (see Glossary, Box 1), cell migration from the mesonephros (see Glossary, Box 1) and testis-specific glycogenesis (Martineau et al., 1997; Capel et al., 1999; Schmahl et al., 2000; Schepers et al., 2003; Matoba et al., 2005; Matoba et al., 2008).

Box 3. *Sry* and sex-specific brain function

In mice, *Sry* (sex-determining region on the chromosome Y) is not only expressed in developing gonads, but also in the brain. It is tempting to hypothesise that SRY has some direct role in brain sex determination. So far, a few genes encoding proteins such as monoamine oxidase A (MAO-A) and tyrosine hydroxylase (TH), have been identified as potential targets of SRY in the brain (Milsted et al., 2004; Dewing et al., 2006; Wu et al., 2009). However, the physiological significance of these pathways and the regulatory mechanisms of *Sry* in the brain have yet to be elucidated. Because so little is known about sex-specific brain differentiation, and because the topic itself is fraught with controversy, it is a challenging task to prove that SRY has a direct role.

Additional SRY targets in gonads

Other than *Sox9*, few SRY target molecules in gonads have been reported. A recent in vivo ChIP study showed that the cerebellin 4 precursor gene (*Cbln4*) is a direct target of mouse SRY (Bradford et al., 2009a). *Cbln4* encodes a transmembrane protein and is expressed in a male-specific manner. However, the function of *Cbln4* product in testicular development is not known.

Human and mouse SRY have also been found to interact with β-catenin and to repress β-catenin-mediated TCF-dependent gene activation (Bernard et al., 2008; Tamashiro et al., 2008; Lau and Li, 2009). β-Catenin is the downstream effector molecule of WNT4/R-sponsin 1 (RSPO1) signalling and appears to be essential for initiating the development of the female gonad (Maatouk et al., 2008). It is attractive to speculate that, in order to activate the male pathway efficiently, SRY suppresses the function of the key female molecule β-catenin. However, evidence that SRY affects β-catenin function is based solely on in vitro data and its physiological significance in vivo has not yet been demonstrated.

In summary, the only clear direct target of SRY during testis determination is *Sox9*, and so understanding the role of SRY in engaging the testis-determining pathway becomes an issue of studying the molecular roles of SOX9. Interestingly, in mice, *Sry* is also expressed in the brain, where other potential targets of its protein have been identified (see Box 3); these targets are brain specific and so are not relevant to gonadal development.

Conclusion

Despite its dramatic biological role, *Sry* is a fragile and partly debilitated gene. The structure and regulatory sequences of *Sry* may have been degraded because of its location on the rapidly degrading Y chromosome (see Box 4). To respond to the weak *Sry* expression signal and to establish the male pathway efficiently, *Sox9*, apparently the only meaningful target of SRY, has acquired support mechanisms for its own regulation, in the form of cell-autonomous and intercellular signalling-based positive-feedback loops. Thus, SRY provides the trigger for male sex determination,

Box 4. Y-chromosome evolution and *Sry*

Sry (sex-determining region on the chromosome Y) determines the sexual fate of the organism, but also the fate of the Y chromosome itself. Gene-mapping analysis suggests that the X and Y chromosomes evolved from a pair of identical chromosomes (Graves, 2006) that began to differentiate when one copy of *Sox3* (SRY box containing gene 3) acquired a new function in male sex determination. The chromosome carrying this new gene, *Sry*, became the Y chromosome. The ancestral *Sox3*, the role of which is largely restricted to brain development, resides on what has become the X chromosome.

Having established a new Y chromosome, subsequent selection favoured restricted recombination with the new X, so that Y genes would stay on the Y chromosome. Because recombination is part of the mechanism used by cells to proofread and repair genes, genes in the non-recombining region of the Y chromosome tend to become lost and/or degraded. As a result, the human Y chromosome has shrunk to ~60 Mb and contains only 50 functional genes, whereas the human X chromosome is ~165 Mb and carries ~1000 genes (Graves, 2002; Graves, 2006; Wallis et al., 2008). Erosion of the Y chromosome is reflected in the accumulation of *Sry* mutations, explaining the high level of sequence divergence between mammalian species and perhaps accounting for its relatively low expression levels in mice. Not surprisingly, the use of *Sry* as a testis-determining trigger seems to have been lost altogether in some species, such as the mole voles *Ellobius lutescens* and *E. tancrei*, and the Japanese spinous country rats *Tokudaia osimensis osimensis* and *T. osimensis* (Just et al., 1995; Sutou et al., 2001). The sex chromosomes in these species are XO or XX; no Y chromosome or any trace of *Sry* are present, suggesting the existence of a completely new sex-determining system.

but it is actually SOX9, and not SRY, that is the key element that orchestrates and stabilizes Sertoli cell differentiation to lock in the testis-determining program. At the same time, SOX9 provides a means of blocking the pathway of gene activity that leads to the differentiation of ovarian cells, but the mechanism by which it does so remains unclear.

Intensive studies spanning more than 20 years since the discovery of *Sry* have identified several genes important for sex determination and gonadal development, and yet 30-40% of human XY DSD cases still remain undiagnosed. Given the importance of correct *Sry* regulation, as demonstrated in experimental studies, it may be that regulatory mutations in the unidentified enhancer sequences of *SRY* may be an important contributing factor to human DSDs. Further study is required to test this possibility. Other fundamental questions about this gene and its protein still remain unanswered: what are precise molecular mechanisms of *Sry* regulation; what are the functions of the non HMG-domain regions of the protein; and what is the physiological significance of its protein interactions? Recent technical advances, including bioinformatic analysis and the generation of genetically modified mice, will help to answer the remaining questions surrounding the process of mammalian sex determination, and may provide valuable information for the diagnosis and management of human DSDs.

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Patient Report

Two preterm infants with late onset circulatory collapse induced by levothyroxine sodium

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Key words adrenal insufficiency, circulatory collapse, hypothyroidism, levothyroxine sodium (L-T4), preterm infant.

Systemic hypotension in the postnatal period is associated with increasing mortality of preterm infants. Although several clinical aspects of developmental cardiovascular physiology explain some mechanisms of this phenomenon, the precise mechanism has not yet been elucidated.[1] Adrenal insufficiency is believed to be one of the causes of vasopressor-resistant hypotension occurring in the immediate postnatal period.[2,3] Two current randomized studies indicate that prophylactic therapy with glucocorticoids is effective for systemic hypotension in the early postnatal period[4,5] and this adrenal insufficiency is thought to be physiologically transient with recovery in the first week of life.[3]

In spite of these reports, some very-low-birthweight infants (VLBWI) manifest systemic hypotension two to four weeks after birth without any obvious causes such as infection, patent ductus arteriosus (PDA) or hypovolemia.[6,7] In such late-onset circulatory collapse, the mechanisms are not documented precisely yet. Empirical treatment with glucocorticoids is often performed[6] and recently it was reported that adrenal insufficiency might also have some role in the development of late-onset circulatory collapse.[7]

Here, we report two patients with late onset circulatory collapse immediately after administration of levothyroxine sodium (L-T4) for hypothyroidism. Although the hypotension was resistant to volume expansion therapy, inotropic agents and vasopressors, the glucocorticoid therapy improved blood pressure efficiently.

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Abbreviations: L-T4, levothyroxine sodium; TSH, thyroid stimulating hormone; VLBWI, very-low-birthweight infants; PDA, patent ductus arteriosus; HDC, hydrocortisone.

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Case Report

Case 1

Case 1 was a female infant born after 28 weeks 0 days of gestation, with a birthweight of 1076 g. Apgar scores were 7 and 9 at 1 and 5 min after birth, respectively. During pregnancy, no risk factors for congenital hypothyroidism were identified. Immediately after birth, the infant was treated in our neonatal intensive care unit (NICU). She was treated with the usual treatment for a VLBW infant including oxygen therapy without mechanical ventilation, infusion therapy, inotropic agent administration (5 μg/kg/min of dopamine), and antibiotics. Tube feeding of breast milk was started three days after birth. Except for continuous oxygen therapy (inspiratory oxygen fraction 0.25–0.3), all other interventions were ceased. We initiated erythromycin administration 13 days after birth, considering the possibility of chronic lung disease.

At the age of 11 days, her thyroid stimulating hormone (TSH) level was within the normal range on the neonatal mass-screening. However at the age of 26 days, she presented hypothyroidism (TSH: 83.41 μU/mL, free thyroxine [fT4]: 0.59 ng/dL) (Table 1) on the routine screening of our NICU. We then started L-T4 administration (6 μg/kg/day) by tube-feeding. After twelve hours of L-T4 treatment, she experienced apnea-bradycardia attacks, and sudden onset of hypotension, oliguria and hyponatremia (Na: 118 mEq/L; K: 5.82 mEq/L) (Table 2).

We immediately started intensive therapy with oxygen supplied by nasal-continuous positive airway pressure, infusion therapy, inotropic agents and diuretics. In spite of these therapies, she did not show sufficient improvement. Any underlying causes such as infections, PDA or hypovolemia were not documented. Considering the clinical course and the results of the examination, we suspected adrenal insufficiency and started hydrocortisone administration (HDC, 5 mg/kg/dose) via intravenous injection (Fig. 1a). Immediately after the HDC treatment, the patient's blood pressure and respiratory condition improved and the electrolytes imbalance was corrected.