Table 1 Endocrinological data of the two patients on neonatal mass screening. In both cases, TSH increased compared to the first result and the levels of fT4 were below the normal range

		Case 1		Case 2	
		Day 11	Day 26	Day 17	Day 19
TSH	(μU/mL)	10.29	83.41	75.2	311.9
fT4	(ng/dL)		0.59		0.04
17-OHP	(ng/ml)	0.36		24.01	

fT4, free thyroxine; 17-OHP, 17-hydroxyprogesterone; TSH, thyroid stimulating hormone.

Thereafter, her clinical course was satisfactory without any abnormal findings on brain magnetic resonance imaging, including the pituitary gland. At present, she is one and a half years old, and thus far, no abnormality has been observed in her development or growth. The thyroid function is within the normal range (TSH: 2.09 μU/mL, fT4: 1.62 ng/dL, fT3: 4.60 pg/mL) by administration of L-T4 (25 μg/day).

Case 2

Case 2 was a female infant born after 24 weeks and 5 days of gestation. Her birthweight was 774 g and Apgar scores were 6 and 8 at 1 and 5 min after birth, respectively. During pregnancy, no risk factors for congenital hypothyroidism were identified. Immediately after her birth, she was admitted to our NICU. We treated her with our routine intensive therapy regimen including mechanical ventilation with surfactant therapy, infusion therapy, antibiotics and glucose insulin therapy. Indomethacin was also given for her PDA due to prematurity. The clinical course was satisfactory and she was allowed to have breast milk 5 days after birth. In spite of her stable clinical condition, oxygen administration was necessary (inspiratory oxygen fraction 0.4–0.5 on mechanical ventilation). We suspected chronic lung disease, and started erythromycin administration at 10 days after birth. Except for these treatments, we were able to cease other therapies.

At the age of 17 days her TSH level was elevated (TSH: 75.2 μU/mL) on the neonatal mass-screening (Table 1). We re-examined her thyroid function 19 days after birth. The level of TSH was remarkably elevated (TSH: 311.9 μU/mL, fT4: 0.04 ng/dL) (Table 1) and L-T4 administration (8 μg/kg/day) was started.

Twenty-four hours after the initiation of L-T4, she developed hypotension, oliguria, hyponatremia and hyperkalemia (Na: 117 mEq/L; K: 7.58 mEq/L) (Table 2). We treated her with oxygen (inspiratory fraction 0.7–1.0 supported mechanical ventilation), infusion therapy, cardiotropic agents (dopamine and dobutamine: =<13 μg/kg/min) and diuretics. In spite of these therapies, her clinical condition did not show any remarkable improvement. We could not detect any underlying causes of the circulatory collapse. As in case 1, we suspected adrenal insufficiency, and initiated hydrocortisone administration (5 mg/kg/dose). Her clinical condition remarkably improved and all signs and symptoms disappeared (Fig. 1b). At present, she is six months old and has normal development and growth. The thyroid function is within the normal range (TSH: 3.20 μU/mL, fT4: 1.25 ng/dL, fT3: 3.56 pg/mL) by administration of L-T4 (6 μg/kg/day).

In both cases, any signs and symptoms that indicate hypothyroidism were not observed before the L-T4 treatment. Glucocorticoids were not given to the mothers during pregnancy or the patients before the onset of the circulatory collapse. There were no exposures to excess iodine such as repeated topical treatment of povidone-iodine antiseptic solutions.

Discussion

We should consider some clinical points from our experience. First, late onset circulatory collapse due to adrenal insufficiency should be considered even in patients who have not shown any signs or symptoms of adrenal insufficiency in their first two weeks of life. Second, thyroid hormone replacement therapy precipitates adrenal insufficiency and when initiating the treatment, we should take great care to monitor closely for the development of adrenal insufficiency.

Hypothyroidism often masks adrenal insufficiency, and L-T4 supplementation precipitates the adrenal insufficiency.[8] It is believed that thyroxin increases the clearance of cortisol,[9] and in

Table 2 Clinical data before and after the onset of circulatory collapse. In addition to hypotension and hyponatremia, metabolic acidosis and hyperkalemia were also observed

		Case 1		Case 2	
		Day 28	Day 29	Day 19	Day 21
CRP	(mg/dL)	<0.3	<0.3	<0.3	<0.3
WBC	(/μL)	–	–	22 810	23 400
pH		7.375	7.299	7.300	7.204
pCO_2	(Torr)	45.6	39.2	44.6	53.2
HCO_3^-	(mmol/L)	26.1	18.8	21.5	20.5
BE	(mmol/L)	0.4	–7.0	–4.9	–7.9
Na	(mEq/L)	133	118	127	117
K	(mEq/L)	5.68	5.82	4.88	7.58
Urine Volume	(mL/kg/hr)	3.0	0.08	2.0	0.00
FiO_2		0.24	0.26	0.60	1.0

BE, base excess; CRP, C-reactive protein; FiO_2, fraction of inspired oxygen; HCO_3^-, bicarbonate; pCO_2, carbon dioxide partial pressure; WBC, white blood cells.

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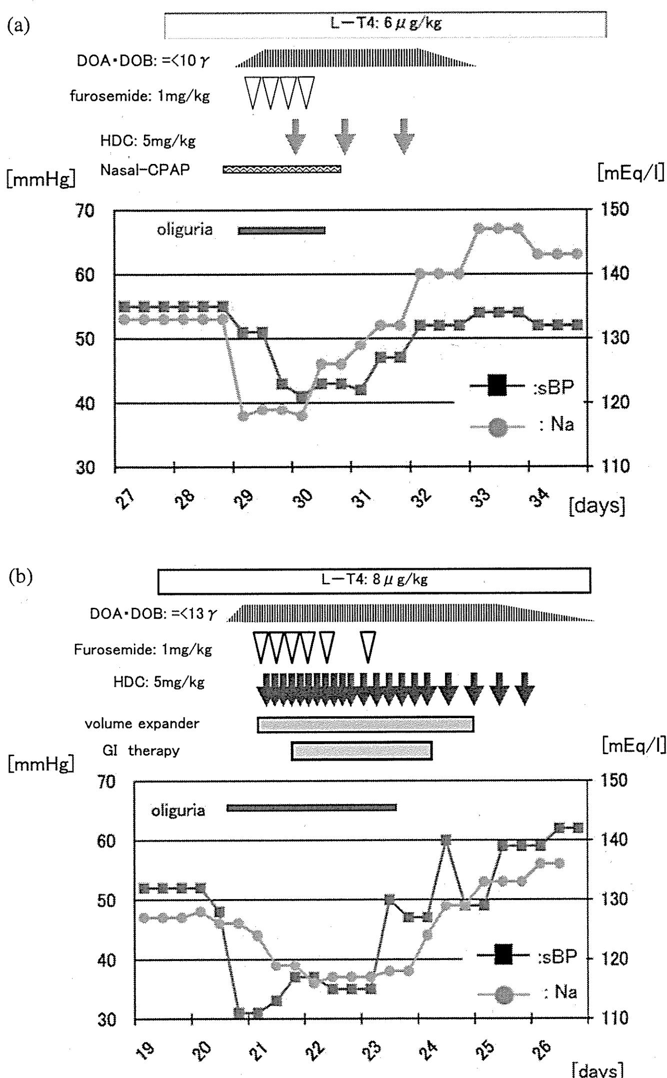

Fig. 1 The clinical courses of (a) Case 1 and (b) Case 2. Immediately after the initiation of levothyroxine sodium (L-T4), hypotension and hyponatremia appeared. These clinical symptoms and signs were promptly corrected with hydrocortisone (HDC) treatment. CPAP, continuous positive airway pressure; DOA, dopamine; DOB, dobutamine; GI, glucose insulin infusion; sBP, systolic blood pressure.

order to avoid adrenal crisis, glucocorticoid administration prior to thyroxin administration is recommended in patients suspected to have both hypothyroidism and adrenal insufficiency. Although we could not obtain precise endocrinological data of our patients during circulatory collapse due to severe illness, the clinical courses strongly suggest that the sudden onset of systemic hypotension in both patients was caused by adrenal insufficiency precipitated by administration of L-T4.

In preterm infants, physiological transient adrenal insufficiency is documented in the immediate postnatal period.[3,10] This adrenal insufficiency may recover during in the second week of their life[3] and is thought to be one of the major factors responsible for hypotension in the early postnatal period. Adrenal shock is vasopressor-resistant and two recent randomized control studies recommend glucocorticoid therapy for the treatment of refractory or pressor-resistant hypotension in their first week of life.[4,5] In general, transient adrenal insufficiency is thought to be caused by immature function of the hypothalamus-pituitary-adrenal axis.[2,3]

In comparison with early-onset hypotension, less attention has been paid to late-onset systemic hypotension. A recent Japanese nationwide surveillance reported that about 4% of VLBWI were treated with glucocorticoid because of late-onset systemic hypotension.[6] Although there are no apparent endocrinological data that indicate adrenal insufficiency,[7] some authors have suggested that transient adrenal insufficiency could be prolonged and might occasionally result in late-onset vasopressor-resistant systemic hypotension.[3,10] These data suggest that late-onset circulatory systemic hypotension induced by adrenal insufficiency is more common than previously believed. From our experience, we should consider adrenal insufficiency as the cause of systemic hypotension in preterm infants at any period, especially those who do not show any signs or symptoms of adrenal insufficiency during the first two weeks of life.

It is a difficult problem how to predict and prevent this kind of circulatory collapse precipitated by L-T4 administration. Prophylactic treatment with glucocorticoid could be one option to prevent adrenal insufficiency prompted by L-T4 administration. However, in the prophylactic treatment, the side-effects of glucocorticoid should be considered. Besides the usual side-effects of glucocorticoid, we should consider the particulars of preterm infants, e.g. the effect on neurological development, growth and other serious complications such as intestinal perforation. Facile treatment with glucocorticoids is still cautioned by the recent reports.[11,12] In contrast to the immediate postnatal period, adrenal insufficiency is estimated to be relatively uncommon in the late postnatal period.[7] Therefore, prophylactic treatment during the late postnatal period will likely result in the treatment of patients with normal adrenal function.

Here we have reported two preterm infants with late onset of circulatory collapse induced by L-T4 administration for the treatment of hypothyroidism. When initiating L-T4, we should consider the possibility of subclinical adrenal insufficiency. Adrenal insufficiency has a sudden onset and does not have any specific signs or symptoms. Therefore careful observation is necessary. The mechanisms and the effective treatment are not yet determined and further research including prospective clinical trials are necessary in order to determine the best method for preventing and treating adrenal insufficiency in the late postnatal period.

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The Effect of Intramuscular Testosterone Enanthate Treatment on Stretched Penile Length in Prepubertal Boys With Hypospadias

Tomohiro Ishii, Mie Hayashi, Ayuko Suwanai, Naoko Amano, and Tomonobu Hasegawa

OBJECTIVES To define the responses of stretched penile length (PL) to intramuscular testosterone enanthate (TE) treatment in prepubertal boys with hypospadias.

METHODS We examined 17 Japanese boys with hypospadias at 1.4 ± 1.3 (mean ± SD) years of age. Their PLs were 2.79 ± 0.37 cm and −1.16 ± 0.88 SD of the mean. The etiology of hypospadias included sex chromosome disorders of sex development in 5, mastermind-like domain-containing 1 deficiency in 1, and unknown cause in the others. No mutation was identified in the *SRD5A2* or *AR* gene. All the boys received as many as three intramuscular injections of TE 25 mg every 4 weeks (one injection in 3 boys, two in 5, and three in 9).

RESULTS The TE treatment significantly increased PL by 1.01 ± 0.50 cm and 2.27 ± 0.99 SD (cm, *P* = .0002; SD, *P* = .0002). Age, body surface area (BSA), and PL before the treatment did not significantly correlate with the effect of TE treatment on PL. The effect of TE treatment on PL at the first injection in Japanese boys with hypospadias (0.35 ± 0.20 cm and 0.91 ± 0.62 SD) was significantly less than that in micropenis at 2.6 ± 3.1 years of age (0.64 ± 0.26 cm and 1.37 ± 0.68 SD) (cm, *P* = .0008; SD, *P* = .02).

CONCLUSIONS These data indicate that (1) the intramuscular TE treatment significantly increases PL for hypospadias in prepubertal boys, with no demonstrable *SRD5A2* or *AR* mutation; (2) age, BSA, and PL before the treatment are not significantly contributing factors to the effect of TE treatment; and (3) the effect of TE treatment for hypospadias is significantly less than that for micropenis. UROLOGY 76: 97–100, 2010. © 2010 Elsevier Inc.

Testosterone treatment has been advocated to increase penile length.[1] Testosterone enanthate (TE) is usually given intramuscularly in doses of 25 mg during childhood and can be repeated three times every 4 weeks until sufficient penile length has been achieved.[2-5] We previously reported that administration of TE 25 mg is effective for micropenis, which was defined as significantly small penis without hypospadias, in prepubertal boys with no *SRD5A2* or *AR* mutation, with variable but significant increase in stretched penile length (PL).[6] Thus, the TE treatment is advocated for boys with micropenis, efficiently leading to the enlargement of penis and the potential establishment of gender identity for them.

The TE is also administered to patients with hypospadias that is often accompanied by significantly small penis with compromised formation of corpora spongiosum of the penis. Gearhart et al.[7] and Luo et al.[8] administered intramuscular TE to patients with hypospadias before reconstructive surgery for hypospadias and demonstrated that the preoperative TE treatment resulted in a significant increase in penile length without apparent side effects, ensuring the sufficient penile size to permit easier surgical repair of hypospadias. Both studies, however, did not comprehensively assess the effect of TE treatment on penile size compared with age-matched reference values and did not analyze the *AR* and *SRD5A2* genes, which have a crucial role in the PL response to exogenous androgen. In this study, we systemically studied the responses of PL to TE treatment and the relevance of the effect of TE to age, body size, and PL before the treatment among patients with no demonstrable *AR* or *SRD5A2* gene mutation.

MATERIAL AND METHODS

Subjects With Hypospadias

The present study consisted of 17 Japanese boys with hypospadias at 0-5 years of age (mean ± SD 1.4 ± 1.3) who were seen at the outpatient clinic, Department of Pediatrics, Keio University Hospital, Tokyo, Japan, from 1998 through 2008. Their PLs ranged 2.0-3.5 cm (2.79 ± 0.37) and −3.3-0.5 SD of the mean as compared with the age-matched Japanese standards (−1.16 ± 0.88).[9] The severity of hypospadias varied among patients whose hypospadias were penile in 1 boy, penoscrotal in

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13, and perineal in 3 based on the location of urethral meatus. Cryptorchidism was present in 3 patients for bilateral testes and in 3 for unilateral. All boys underwent repair surgery for hypospadias at 1 or 2 years of age, but 2 after 3 years. One underwent the surgery at 6 years of age after his parents decided to change legal sex from female to male, and the other at 3 years of age until seizures became tractable by anticonvulsant. Five of 17 boys had sex chromosome disorders of sex development (DSD), 1 had mastermind-like domain-containing 1 (MAMLD1) deficiency,[10] and the remaining 11 had unknown cause for hypospadias. The 11 patients with unknown cause for hypospadias did not have any detectable mutations in the *AR* and *SRD5A2* genes by polymerase chain reaction and direct sequence (data not shown).

Six boys had cryptorchidism, unilateral in 3 and bilateral in 3, that required either orchidopexy or orchiectomy, depending on the position of the testes and the severity of testicular hypoplasia. Basal and peak levels of serum luteinizing hormone and follicle-stimulating hormone after GnRH provocation (100 $\mu g/m^2$ intravenously; blood sampling at 0, 30, 60, 90, and 120 min) were elevated in 4 and 8 boys, respectively, and serum testosterone level after hCG provocation (3000 IU/m^2/dose intramuscularly for 3 consecutive days; blood sampling on days 1 and 4) was low in 1 boy, compared with the age- and pubertal tempo–matched Japanese reference data.[11,12]

Subject With Micropenis

To assess the effect of intramuscular TE injection in boys with hypospadias, we used data from boys with micropenis who were involved in our previous study and younger than 6 years old as a reference group. The ages in the micropenis group varied 0-5 years (2.6 ± 1.8), and the PLs in that group ranged 1.5-2.5 cm (2.09 ± 0.34) and −4.7-−2.2 SD (−3.01 ± 0.75).

Measurement of PL

The PLs were determined by the same physician before and after each TE injection. PL was obtained with a ruler placed against the dorsum of stretched penis by measuring the distance between the tip of the glans and the pubic symphysis, while depressing the suprapubic fat pad as completely as possible.[4]

Testosterone Treatment

All boys with hypospadias in this study received intramuscular injections of TE with a dosage of 25 mg. The injection was repeated every 4 weeks up to 3 times until PL was definitely above the mean of age-matched reference (1 injection in 3 boys, 2 in 5, and 3 in 9).

Statistical Analysis

The statistical significance of the difference was examined by Student's *t*-test and paired *t*-test for the means between 2 independent and dependent groups, respectively. The statistical significance of the regression was analyzed by the multiple regression analysis. A *P* value of less than 0.05 was considered significant.

RESULTS

The change in actual PL and PL SD score for each patient was shown in Fig. 1. The TE treatment significantly increased PL by 0.5-2.5 cm (1.01 ± 0.50) and 0.9-5.1 SD (2.27 ± 0.99) (cm, *P* = .0002; SD, *P* = .0002). The increment of PL at the first injection was 0.0-0.8 cm (0.35 ± 0.20) and 0.0-2.0 SD (0.91 ± 0.62) and was not significantly correlated with age, body surface area (BSA), actual PL before the treatment, or PL SD score before the treatment (Fig. 2). The increment of PL at the first injection in boys with hypospadias was significantly less than that in micropenis (n = 19, 0.2-1.1 cm [0.64 ± 0.26] and 0.3-3.3 SD (1.37 ± 0.68]) (cm, *P* = .0008; SD, *P* = .02) (Fig. 3). When boys with cryptorchidism were excluded, the increment of PL at the first injection was still significantly decreased in boys with hypospadias (n = 10, 0.0-0.5 cm [0.32 ± 0.19] and 0.0-1.3 SD [0.77 ± 0.52]) than in boys with micropenis (n = 18, 0.2-1.1 cm [0.66 ± 0.25] and 0.3-3.3 SD (1.40 ± 0.69]) (cm, *P* = .001; SD, *P* = .02). We did not find any adverse events such as gynecomastia, growth spurt, or appearance of pubic hair.

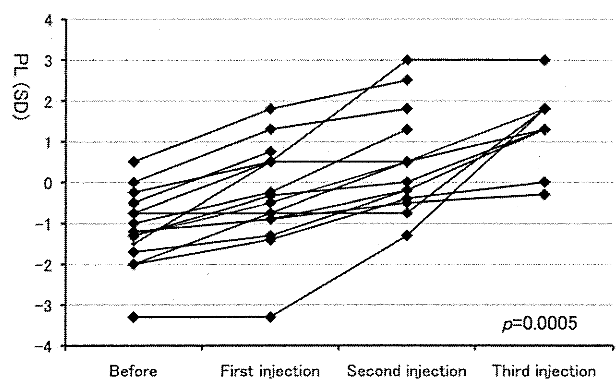

Figure 1. Stretched penile length (PL) before and after testosterone enanthate (TE) treatment.

COMMENT

The present study indicates that the intramuscular injection of TE 25 mg is an effective therapeutic method for increase in PL for hypospadias boys with preserved AR and SRD5A2 functions. Such beneficial effects of intramuscular TE treatment has been described in the literature reported by Luo et al.,[8] although they did not analyze the *AR* and *SRD5A2* genes. The mean increment of PL in this study (1.0 cm, n = 17, age 0.2-5.0 years) surpasses that reported by Luo et al. (0.4 cm, n = 25, age 0.8-1.0 years). We assume that the defect of androgen production or action might be less severe in our study population than in that of Luo et al., aside from the ethnic difference in androgen sensitivity. In fact, PL before TE treatment in this study (2.8 ± 0.4 cm) is longer than that in Luo et al. (2.0 ± 0.2 cm). Thus, our results provide additional credence for the application of intramuscular TE treatment for hypospadias in infancy to childhood.

Age, BSA, and PL before the treatment are not significant contributing factors to the effect of TE treatment on PL. We also previously reported that these factors did not significantly affect the effect of TE treatment for micropenis.[6] It still remains unknown what affects the

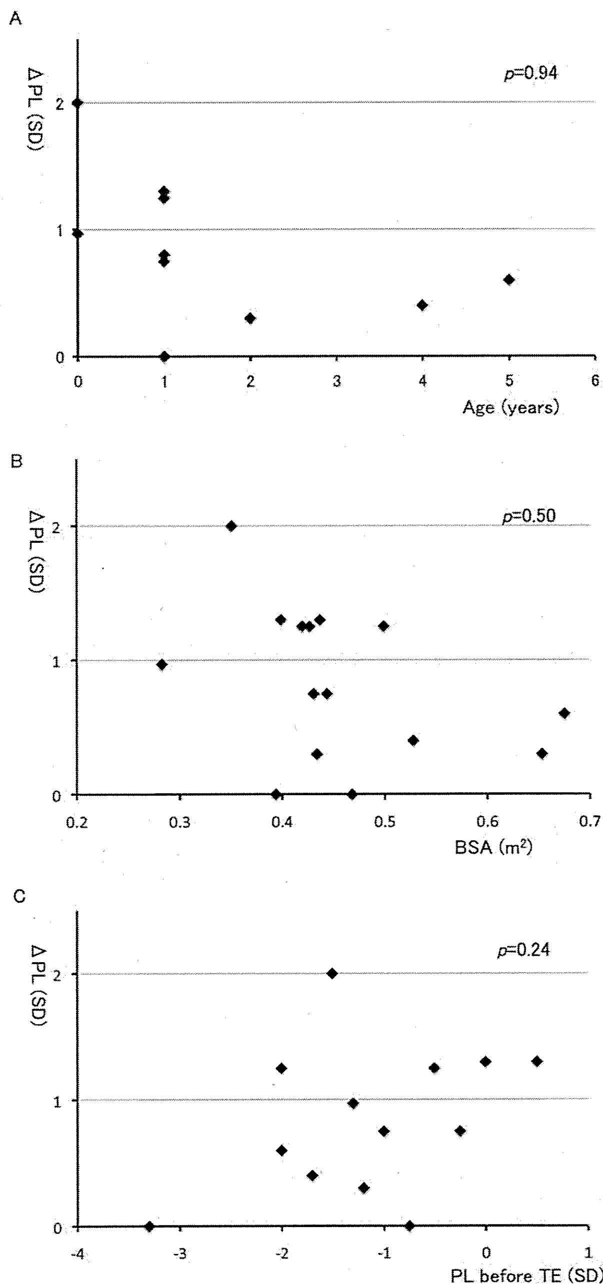

Figure 2. Relationship of the increment of PL at the first injection of TE to age **(A)**, BSA **(B)**, or stretched PL before the treatment **(C)**.

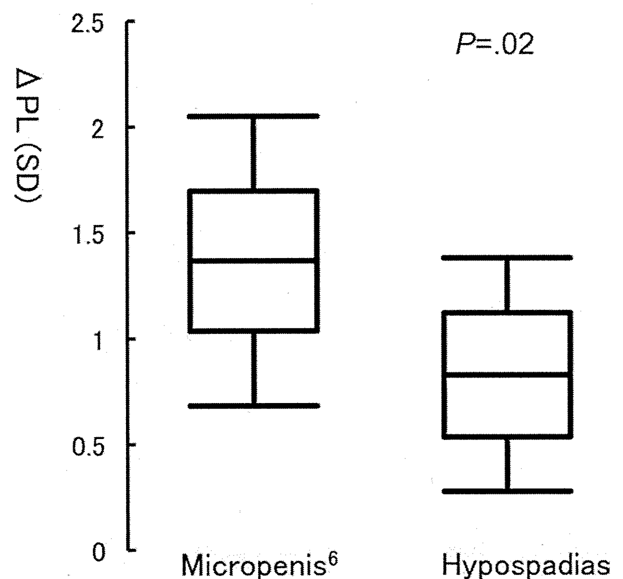

Figure 3. Comparison of the increment of stretched PL at the first injection of TE between boys with hypospadias and those with micropenis. The horizontal line within each box represents the mean, the limits of each box 95% confidence interval of the mean, and the whiskers mean ± SD.

variation in the effect of TE treatment for hypospadias and micropenis. We administered the same dosage of TE 25 mg for all boys, with no dosage adjustment to age or BSA. This simple and convenient method appears to still be clinically acceptable until future studies with a large number of subjects may reveal the contributing factors to the effect of TE treatment for hypospadias or micropenis.

Although the TE treatment significantly increased PL in all the boys with hypospadias in this study, the increments of PL were variable, with those at the first injection ranging from 0.0-0.8 cm (0.0-2.0 SD). Two boys showed no response to TE at the first injection, and one also exhibited no response at the second injection. However, thereafter they exhibited much better response to TE with 0.8 cm (2.0 SD) and 1.2 cm (3.1 SD) at the second and third injections, respectively, in one, and 1.0 cm (2.6 SD) at the third injection in the other. The variation in the increment of PL among boys with hypospadias would primarily be caused by the heterogeneity of the underlying factors for etiology of hypospadias in this study. The variation in the increment of PL in the same individual at different injections remains elusive.

The increment of PL for hypospadias at the first injection of TE is significantly less than that for micropenis when the possibility of androgen insensitivity syndrome or 5 alpha-reductase deficiency is excluded. The presence of cryptorchidism does not affect the significance of difference in PL response between those groups. In consistent with our data, Velasquez-Urzola et al.[13] compared the effects of intramuscular testosterone heptylate (TH) treatment between 2 groups having equivalent degree of small penis with and without hypospadias and showed that the increase in penile length after TH treatment was less in the children with hypospadias than in those without hypospadias, although they did not analyze *AR* and *SRD5A2* genes among their subjects (Table 1). Thus, it is inferred that a certain number of boys with hypospadias might have a defect in the anlage formation or in the downstream signal transduction of AR during the development of genital tubercle. Otherwise, the hypoplasia of corpora spongiosum accompanied with hypospadias itself would result in the poor response of PL to parenteral testosterone therapy.

The construction of a symmetric conically-shaped glans is one of objectives of the repair surgery for hypospadias. The surgery might be more difficult in boys with

Table 1. The effect of intramuscular injection of testosterone compound on the increment of penile length (ΔPL) in different conditions: micropenis v.s. hypospadias

	Condition	n	Age (years)	PL Before Injection (SD)*	Testosterone Compound	ΔPL (SD)*
Velasquez-Urzola A et al.[3]	Micropenis	13	0-2/12	−4.6 ± 1.0	Heptylate (100 mg/m^2)	2.8 ± 0.7
			2/12-2	−2.5 ± 1.0		1.5 ± 0.2
			2-10	−3.0 ± 1.0		2.3 ± 1.0
	Hypospadias	27	0-2/12	−4.0 ± 2.0		1.9 ± 1.2
			2/12-2	−2.5 ± 0.0		0.8 ± 0.8
			2-10	−3.0 ± 0.0		0.5 ± 0.2
This study	Micropenis	19	0-5	−3.0 ± 0.8	Enanthate (25 mg)	1.4 ± 0.7
	Hypospadias	16	0-5	0.8 ± 0.6		0.8 ± 0.6

* Mean ± SD.

a small penis than in those with a penis of normal size. We could not evaluate the relevance of the increase in penile size by the TE treatment and the outcome of reconstruction surgery for hypospadias because of lack of untreated subjects as a control. In this context, Gearhart et al.[7] and Luo et al.[8] noted that successful surgery was attributable to the achievement of adequate penis size. Both groups also mentioned the safety of TE treatment for hypospadias as shown in our study. At this point, it is reasonable to consider the potential contribution of the preoperative TE treatment to the successful surgery in patients with hypospadias.

This study involves some limitations. First, our institution has a special care unit for children with DSD, leading to a potential selection bias in this study. Our patients have markedly severe hypospadias, suggesting this conclusion may not apply to mild hypospadias such as the subcoronal or glanular type. Second, we did not set clear criteria to decide whether the injection of TE should be continued because of the nature of a retrospective study. Thus, we could evaluate the effect of TE on PL only at the first injection of TE. Third, a small number of patients did not allow us to generalize the conclusion from this study and to compare the effect of TE between the groups of hypospadias with and without significantly small penis.

CONCLUSIONS

The present study indicates that the intramuscular injection of TE significantly increases PL for hypospadias in prepubertal boys with no demonstrable *SRD5A2* or *AR* mutation. The effect of TE treatment on PL does not significantly correlate with age, BSA, and PL before the treatment, suggesting that the TE treatment can be applied at the same dosage of 25 mg irrespective of age, BSA, and PL. Finally, the results show that the TE treatment is significantly less effective in boys with hypospadias than in those with micropenis, implying the difference in the pathophysiological aspect between the development of hypospadias and micropenis.

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Antagonistic regulation of *Cyp26b1* by transcription factors SOX9/SF1 and FOXL2 during gonadal development in mice

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ABSTRACT **Sex determination in fetal germ cells depends on a balance between exposure to retinoic acid (RA) and the degradation of RA achieved by the testis-specific expression of the catabolic cytochrome P450 enzyme, CYP26B1. Therefore, identification of factors regulating the expression of the *Cyp26b1* gene is an important goal in reproductive biology. We used *in situ* hybridization to demonstrate that *Cyp26b1* and transcription factor genes steroidogenic factor-1 (*Sf1*) and *Sry*-related HMG box 9 (*Sox9*) are coexpressed in Sertoli cells, whereas *Cyp26b1* and *Sf1* are coexpressed in Leydig cells in mouse fetal testes. In the mouse gonadal somatic cell line TM3, transfection of constructs expressing SOX9 and SF1 activated *Cyp26b1* expression, independently of the positive regulator RA. In embryonic gonads deficient in SOX9 or SF1, *Cyp26b1* expression was decreased relative to wild-type (WT) controls, as measured by quantitative RT-PCR (qRT-PCR). Furthermore, qRT-PCR showed that *Cyp26b1* up-regulation by SOX9/SF1 was attenuated by the ovarian transcription factor Forkhead box L2 (FOXL2) in TM3 cells, whereas in *Foxl2*-null mice, *Cyp26b1* expression in XX gonads was increased ~20-fold relative to WT controls. These data support the hypothesis that SOX9 and SF1 ensure the male fate of germ cells by up-regulating *Cyp26b1* and that FOXL2 acts to antagonize *Cyp26b1* expression in ovaries.—Kashimada, K., Svingen, T., Feng, C.-W., Pelosi, E., Bagheri-Fam, S., Harley, V. R., Schlessinger, D., Bowles, J., Koopman, P. Antagonistic regulation of *Cyp26b1* by transcription factors SOX9/SF1 and FOXL2 during gonadal development in mice. *FASEB J.* 25, 000–000 (2011). www.fasebj.org**

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SEXUAL DIFFERENTIATION IN mammals is a unique process in that two completely different organs, testes and ovaries, arise from a common precursor, the bipotential genital ridge. The Y-linked gene *Sry* is the master switch of mammalian male determination (1), and its major role is to up-regulate an autosomal but related gene, *Sry*-related HMG box 9 (*Sox9*; ref. 2). SOX9 directly up-regulates a number of male-specific genes, such as *Amh*, *Pgds*, *Vnn1*, and *Sox9* itself, in conjunction with the transcription factor steroidogenic factor-1 (SF1; also known as NR5a1 and Ad4BP), during Sertoli cell differentiation (2–5). Sertoli cells orchestrate testis development, including the differentiation of androgen-producing Leydig cells, testis vascular cells, and other interstitial cells. In the absence of *Sry*, female genes, such as *Wnt4* and Forkhead box L2 (*Foxl2*), are up-regulated by default, leading to ovarian development (6, 7).

As the progenitors of the gametes, germ cells also undergo sexual differentiation in mammalian gonads. The earliest known sex-specific event in germ cell development is entry into meiosis in ovaries and not in testes. Approximately 13.5 days *post coitum* (dpc), germ cells stop proliferating and enter the prophase of meiotic division in mouse fetal ovaries, whereas, in testes, germ cells arrest in G_0 or G_1 of the mitotic cycle, resuming mitosis after birth (8, 9). Studies using XX↔XY chimeric mice showed that both XX and XY germ cells enter meiosis in developing ovaries (10), suggesting that sexual differentiation of bipotential germ cells is not cell-autonomous but instead that the tissue environment determines the initial sex differentiation of germ cells (11, 12).

Recent studies identified retinoic acid (RA) as an environmental factor that controls germ cell meiosis (13, 14). RA is a metabolite of vitamin A that mediates many physiological functions, including embryogenesis and organogenesis. RA binds to nuclear receptors, particularly retinoic acid receptors (RARs), which form heterodimers with retinoid X receptors (RXRs). The

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RAR/RXR complex binds to retinoic acid response elements in the regulatory regions of target genes (15, 16). It is believed that fetal gonads are exposed to RA that is synthesized in the adjacent mesonephros in a non-sex-specific manner. In the gonad, the concentration of RA is regulated by a P450 cytochrome enzyme, CYP26B1, that catabolizes all-*trans*-RA into inactive oxidized metabolites (13, 17, 18). *Cyp26b1* is initially expressed in fetal testes and ovaries and then becomes male-specifically expressed at 11.5–12.5 dpc (13). In developing testes, germ cells are prevented from entering meiosis by the presence of the RA-degrading enzyme, CYP26B1. Hence, CYP26B1 plays a key role in determining whether or not germ cells enter meiosis by controlling local distribution of RA (13, 14).

Despite its important role in the control of germ cell sexual differentiation, the mechanisms of *Cyp26b1* gene regulation are not known. Because *Cyp26b1* is up-regulated in developing testes as early as 11.5 dpc and is expressed by both Sertoli and interstitial cells (13), we hypothesized that *Cyp26b1* is regulated by the transcription factors SF1 and/or SOX9, both being expressed during the early stages of gonadal sex differentiation.

We report here that the spatial and temporal expression patterns of *Cyp26b1* and *Sf1* or *Sox9* overlap during testicular development *in vivo*. Further, we find that in TM3 cells, a model of fetal testicular somatic cells, exogenous *Sox9* or *Sf1* significantly activated *Cyp26b1* expression. *Cyp26b1* regulation by SOX9 and SF1 did not require RA, another positive regulator of *Cyp26b1*. In AMH-Cre:*Sox9$^{flox/flox}$* mice (19), which lack *Sox9* expression, or in *Cited2*-null mice, a mouse model with impaired *Sf1* expression (20–22), *Cyp26b1* expression was significantly decreased at 13.5 and 12.25 dpc, respectively. Furthermore, we find that one of the female sex-determining factors, FOXL2, attenuates *Cyp26b1* up-regulation by SOX9 and SF1 in TM3 cells and that *Cyp26b1* expression was significantly increased in XX gonads of *Foxl2*-null mice. Our results indicate that the testicular transcription factors SOX9 and SF1 and the ovarian transcription factor FOXL2 have mutually antagonistic actions that ensure correct sexual differentiation of fetal germ cells through regulation of *Cyp26b1* expression.

MATERIALS AND METHODS

Animals

Protocols and use of animals were approved by the Animal Welfare Unit of the University of Queensland. Mouse embryos were collected from timed matings of the Swiss Quackenbush and CD1 outbred strain, with noon of the day on which the mating plug was observed designated as 0.5 dpc. Embryos were sexed at 10.5–11.5 dpc using an X-linked GFP marker (23) and at 12.5–13.5 dpc by gonadal morphology. AMH-Cre:*Sox9$^{flox/flox}$* mice were obtained as described previously (19). In brief, *Sox9$^{flox/flox}$* mice produced by homologous recombination in embryonic stem cells were bred to AMH-Cre transgenic mice on a C57BL/6 background, and the resulting AMH-Cre:*Sox9$^{flox/+}$* offspring were backcrossed to *Sox9$^{flox/flox}$* mice to obtain AMH-Cre:*Sox9$^{flox/flox}$* embryos. The *Cited2*-knockout mice on a C57BL/6 background have been described previously (24), and embryo sex was determined by UBEX1 PCR analysis (25). The generation of *Foxl2*-null mice was reported previously (26), and these mice were maintained on a mixed C57B6/J/129/SVJ genetic background.

Transfection into TM3 cells

The murine testicular somatic cell line TM3 was obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM (Invitrogen, Carlsbad, CA, USA) with 10% FBS (AusGenex, Loganholme, QLD, Australia) at 37°C in 5% CO_2. Cells were plated at 2.5×10^5/well in 6-well plates 12 h before transfection. Cells were transfected with 0.5, 1, or 2 μg of expression vector (pSG.*Sox9*, ref. 27; pcDNA.*Sf1*, ref. 28; pcDNA.HA-*Sry*, ref. 29; or pcDNA.*Foxl2*, ref. 30) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The total amount of transfected plasmid was standardized using the empty expression vector as control. All-*trans*-RA was purchased from Sigma-Aldrich (R2625; Sigma-Aldrich Corp., St. Louis, MO, USA). RAR antagonist AGN193109 was provided by Vitae Pharmaceuticals (Fort Washington, PA, USA).

Real-time quantitative RT-PCR (qRT-PCR)

qRT-PCR analysis of TM3 cells and mouse gonads (except *Foxl2*-null mice) was conducted as follows. Total RNA was collected at 48 h post-transfection, except for experiments depicted in **Fig. 2A**. For tissue analysis, embryonic gonads without mesonephroi were dissected in ice-cold PBS at the appropriate stages. Total RNA from cells and tissue was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) or Micro Kit (Qiagen) including DNase treatment. Total RNA (500 ng for TM3 or 300 ng for gonads) was used as a template for synthesis of cDNA using SuperScript III (Invitrogen) and random primers (Invitrogen), according to the manufacturer's instructions. cDNA samples were diluted 1:4, and 1 μl was used in each 25 μl of qRT-PCR reaction, containing SYBR Green PCR Master Mix (Applied Biosystems, Foster City, Ca, USA). Transcript levels were analyzed on an ABI Prism 7500 Sequence Detector System (Applied Biosystems) over 40 cycles of 95°C for 15 s and 60°C for 1 min in a 2-step thermal cycle, preceded by an initial 10-min step at 95°C. Ribosomal protein S29 (*Rps29*) served as the normalizing gene to standardize qRT-PCR data (31). For analysis of *Foxl2*-null mice, we collected gonadal samples at 13.5 dpc, and total RNA was obtained from dissected gonads by enzymatic extraction (MELT system; Ambion, Austin, TX, USA) followed by linear RNA amplification using Ovation Pico (NuGEN, San Carlos, CA, USA). qRT-PCR (TaqMan; Applied Biosystems) was performed using an ABI 7900HT system (Applied Biosystems). *Sdha* was used as the housekeeping gene to standardize the data as reported previously (32), and the relative expression levels to XX wild-type (WT) mice were calculated. All qRT-PCR primers are listed in **Table 1**. The means ± SE of 3 biological replicates measured in triplicate were calculated. We used an unpaired Student's *t* test to demonstrate statistical significance of differences between the control and the given sample.

In situ hybridization and immunohistochemistry

In situ hybridization was performed as described previously (33). In brief, sequential sections of paraformaldehyde-fixed, paraffin-embedded embryos were dewaxed, rehydrated, incubated in proteinase K, refixed with 4% paraformaldehyde, acetylated, and prehybridized. Hybridization was performed overnight at

TABLE 1. *Primers used for qRT-PCR analysis*

Gene	Description	Forward primer	Reverse primer
SYBR qRT-PCR			
Rn18s	18S rRNA	GATCCATTGGAGGGCAAGTCT	CCAAGATCCAACTACGAGCTTTTT
Rps29	Ribosomal protein S29	TGAAGGCAAGATGGGTCAC	GCACATGTTCAGCCCGTATT
Cyp26b1	Cytochrome P450 26B1	TGGACTGTGTCATCAAGGAGGT	GTCGTGAGTGTCTCGGATGCTA
Sox9	Sry-related HMG box 9	AGTACCCGCATCTGCACAAC	TACTTGTAATCGGGGTGGTCT
Sf1	Steroidogenic factor 1	TCCAGTACGGCAAGGAAGA	CCACTGTGCTCAAGCTCCAC
Foxl2	Forkhead box L2	GCTACCCCGAGCCCGAAGAC	GTGTTGTCCCGCCTCCCTTG
TaqMan qRT-PCR			
Sdha	Succinate dehydrogenase complex, subunit A, flavoprotein	Mm01352366_m1	
Cyp26b1	Cytochrome P450 26b1	Mm00558507_m1	
Sox9	Sry-related HMG box 9	Mm00448840_m1	
Sf1 (Nr5a1)	Steroidogenic factor 1	Mm00496060_m1	

60°C. After 2 h of blocking, anti-digoxigenin antibody (Roche Diagnostics, Indianapolis, IN, USA) at 1:2000 in blocking solution was added, and sections were incubated overnight at 4°C. After washing, sections were equilibrated in NTM buffer and incubated in color solution.

Immunofluorescence of a 7-μm paraffin section was performed as described previously (33) using 12.5 dpc mouse fetus samples fixed in 4% paraformaldehyde. The primary antibody used for this study was rabbit anti-SF1 (1:1000, kindly provided from Dr. Ken-Ichirou Morohashi, Kyushu University, Fukuoka, Japan). The secondary antibody (goat anti-rabbit Alexa Fluor 488 or goat anti-rabbit Alexa Fluor 594) was obtained from Molecular Probes (Invitrogen) and used at 1:200 dilution.

RESULTS

Cyp26b1, Sf1, and *Sox9* have overlapping expression profiles in fetal testes

First, to clarify the relationship between *Cyp26b1* and *Sox9/Sf1*, we performed a time-course expression analysis from 10.5 to 13.5 dpc using qRT-PCR analysis. *Sox9, Sf1,* and *Cyp26b1* were expressed in a testis-specific manner (**Fig. 1*A***). In particular, *Cyp26b1* and *Sox9* had very similar temporal expression profiles: *Cyp26b1* started to be expressed in male genital ridges at 11.5 dpc and reached a peak level at 12.5–13.5 dpc, as did *Sox9* (Fig. 1*A*). *Sf1* was expressed more strongly in testes than ovaries by 11.5 dpc, and expression continued to increase until 13.5 dpc in testes (Fig. 1*A*).

SOX9 and SF1 are transcription factors and, if both are involved in *Cyp26b1* regulation, spatial expression patterns of *Sox9/Sf1* and *Cyp26b1* should overlap. Therefore, we analyzed the expression of each gene in sequential sections using *in situ* hybridization. *Sox9, Sf1,* and *Cyp26b1* are known to be expressed in Sertoli cells (13, 34–36). Thus, expression of all three genes was detected in irregular-shaped cells within the testis cords, consistent with the features of Sertoli cells and distinct from the large, round germ cells also found within testis cords (Fig. 1*B*). In

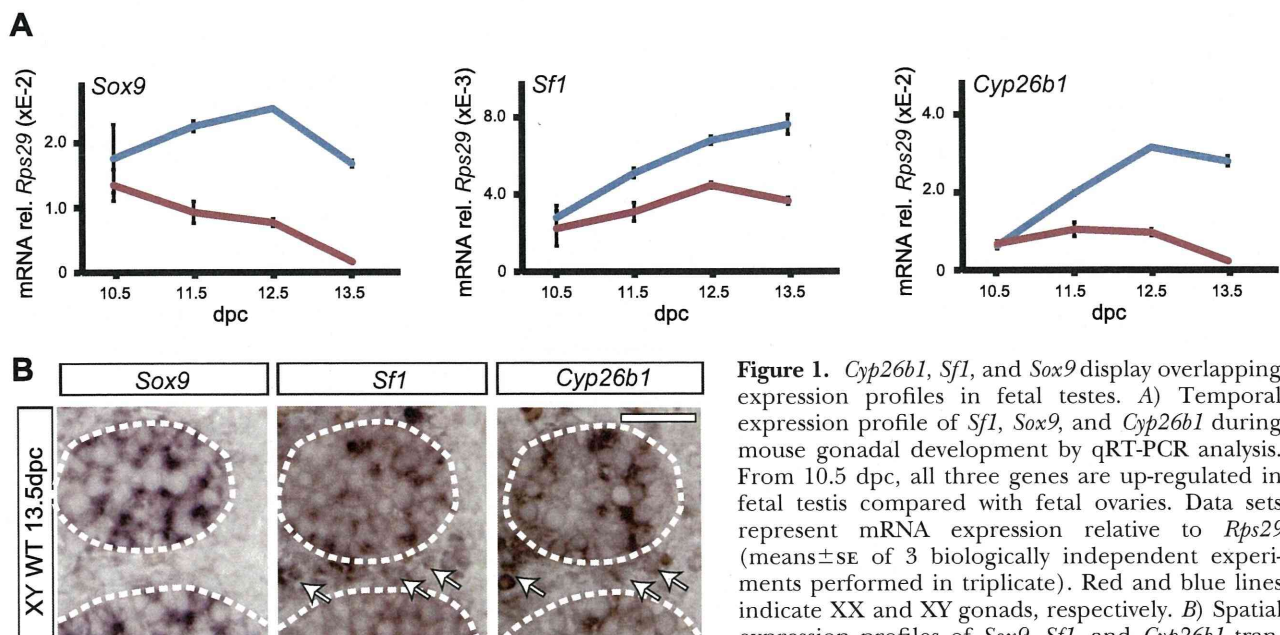


Figure 1. *Cyp26b1, Sf1,* and *Sox9* display overlapping expression profiles in fetal testes. *A*) Temporal expression profile of *Sf1, Sox9,* and *Cyp26b1* during mouse gonadal development by qRT-PCR analysis. From 10.5 dpc, all three genes are up-regulated in fetal testis compared with fetal ovaries. Data sets represent mRNA expression relative to *Rps29* (means±SE of 3 biologically independent experiments performed in triplicate). Red and blue lines indicate XX and XY gonads, respectively. *B*) Spatial expression profiles of *Sox9, Sf1,* and *Cyp26b1* transcripts in fetal testes by *in situ* hybridization. Serial sagittal sections of 13.5 dpc testes show that *Cyp26b1* expression overlaps with that of the Sertoli cell marker *Sox9* and with the Sertoli/Leydig cell marker *Sf1*. Arrows indicate interstitial cells expressing both *Sf1* and *Cyp26b1*. Scale bar = 50 μm.

addition, *Sf1* is known to be expressed in Leydig cells residing in the interstitial space; in our analyses, both *Sf1* and *Cyp26b1* were expressed in cells with a similar number and distribution in the interstitium, consistent with coexpression in Leydig cells (Fig. 1*B*, arrows). These findings support the hypothesis that SOX9 and SF1 are together involved in *Cyp26b1* regulation in Sertoli cells and that SF1 might regulate *Cyp26b1* in Leydig cells, during testicular development.

SOX9 and SF1 up-regulate endogenous *Cyp26b1* expression in TM3 cells

To investigate potential functional involvement of SOX9 and SF1 in *Cyp26b1* regulation, we performed *in vitro* experiments using the mouse gonadal cell line TM3 (29, 37). These cells express several genes characteristic of Sertoli cells, as assayed nonquantitatively by RT-PCR (29, 37). However, SOX9 protein expression was reported as undetectable by immunofluorescence in these cells (29), and using qRT-PCR we found that the expression levels of endogenous *Sox9*, *Sf1*, and *Cyp26b1* were extremely low compared with those in fetal testes (Supplemental Fig. S1). Therefore, any effects on *Cyp26b1* regulation in this cell culture system must reflect the effects of transfected rather than endogenous SOX9/SF1.

First, we investigated the time course of *Cyp26b1* expression after transfection with *Sox9* or *Sf1* expression constructs. *Cyp26b1* expression started to increase 12 h after *Sox9* or *Sf1* transfection and continued to increase until at least 48 h post-transfection (**Fig. 2*A***). On the basis of these data, we used 48 h after transfection as our standard time point for further analyses.

We next introduced varying amounts of *Sox9* or *Sf1* expression vector into TM3 cells. Exogenous *Sox9* and *Sf1* expression induced *Cyp26b1* expression in a dose-dependent manner (Fig. 2*B*, *C*). In contrast, *Sry* did not up-regulate *Cyp26b1* expression, suggesting that *Sry* is not directly involved in *Cyp26b1* regulation (Fig. 2*B*). It has been reported that SOX9 and SF1 target genes, such as *Sox9* itself, *Vnn1*, and *Amh*, are synergistically up-regulated by SOX9 and SF1 (2, 4). To investigate a potential cooperative action of SOX9 and SF1 in *Cyp26b1* regulation, we introduced both *Sox9* and *Sf1* expression vector into TM3 cells. Individual introduction of 1 μg of *Sox9* or *Sf1* expression vector increased *Cyp26b1* expression by ~6 and ~10-fold, respectively (Fig. 2*D*). Simultaneous introduction of both *Sox9* and *Sf1* increased *Cyp26b1* expression by ~18-fold, indicating that SOX9 and SF1 regulate *Cyp26b1* expression additively in TM3 cells (Fig. 2*D*), consistent with the similar temporal profile of *Cyp26b1* transcriptional response to either SOX9 or SF1 (Fig. 2*A*).

Up-regulation of *Cyp26b1* by SOX9 or SF1 does not require RA action

It has been reported that cytosolic class-1 aldehyde dehydrogenase (Aldh1a1) is expressed in a male-specific manner in fetal gonads and that *Aldh1a1* is genetically downstream of *Sox9* (38). Previous studies involving mRNA injection into *Xenopus* embryos have established that ALDH1A1 can catalyze RA synthesis (39) and RA has been shown to positively regulate *Cyp26* genes, including *Cyp26b1* (40, 41). In agreement with published results, we found that exogenous RA strongly up-regulated *Cyp26b1* expression in TM3 cells (**Fig. 3*A***). This up-regulation was abolished by the synthetic antagonist AGN193109, which suppresses RA action by binding to RARs but not RXRs (42), confirming that *Cyp26b1* up-regulation by RA is mediated by RARs (Fig. 3*A*).

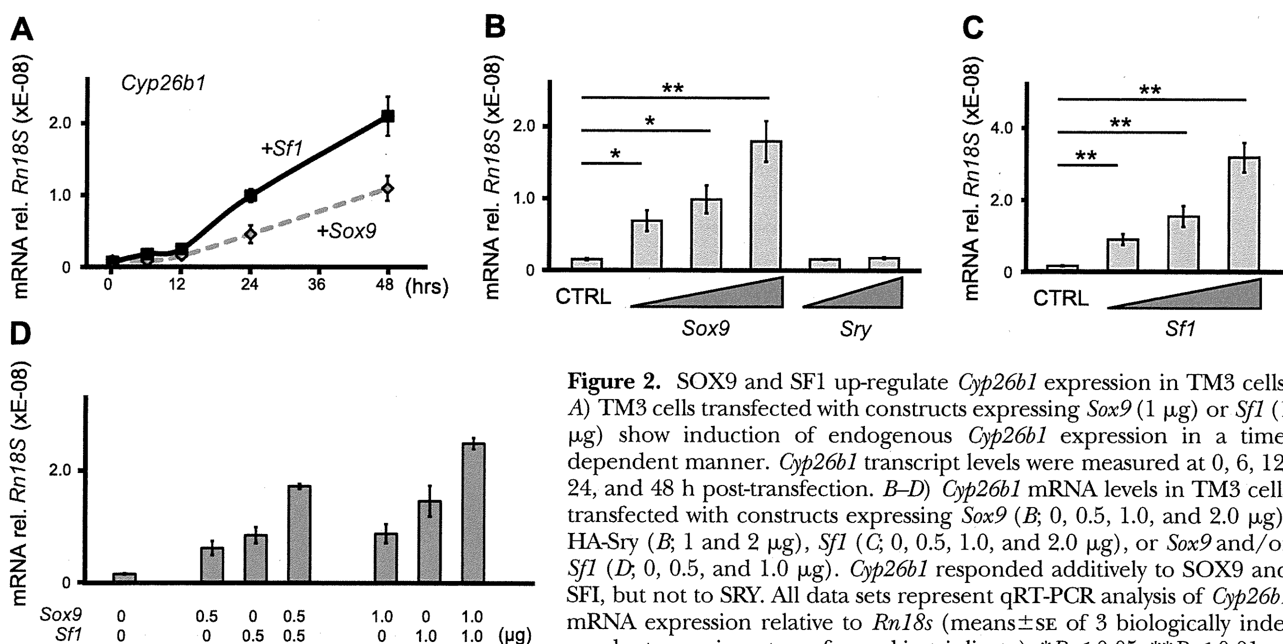
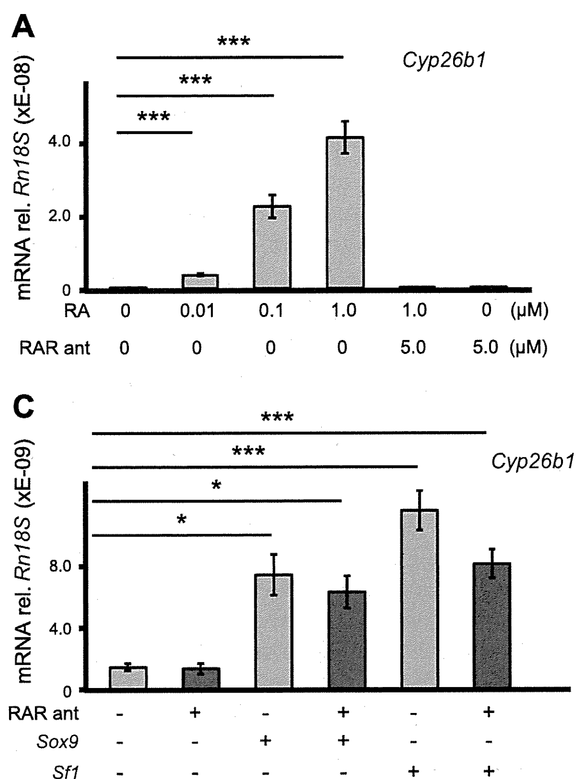


Figure 2. SOX9 and SF1 up-regulate *Cyp26b1* expression in TM3 cells. *A*) TM3 cells transfected with constructs expressing *Sox9* (1 μg) or *Sf1* (1 μg) show induction of endogenous *Cyp26b1* expression in a time-dependent manner. *Cyp26b1* transcript levels were measured at 0, 6, 12, 24, and 48 h post-transfection. *B–D*) *Cyp26b1* mRNA levels in TM3 cells transfected with constructs expressing *Sox9* (*B*; 0, 0.5, 1.0, and 2.0 μg), HA-Sry (*B*; 1 and 2 μg), *Sf1* (*C*; 0, 0.5, 1.0, and 2.0 μg), or *Sox9* and/or *Sf1* (*D*; 0, 0.5, and 1.0 μg). *Cyp26b1* responded additively to SOX9 and SFI, but not to SRY. All data sets represent qRT-PCR analysis of *Cyp26b1* mRNA expression relative to *Rn18s* (means±SE of 3 biologically independent experiments performed in triplicate). $^*P < 0.05$, $^{**}P < 0.01$ *vs.* control (CTRL); unpaired Student's *t* test.



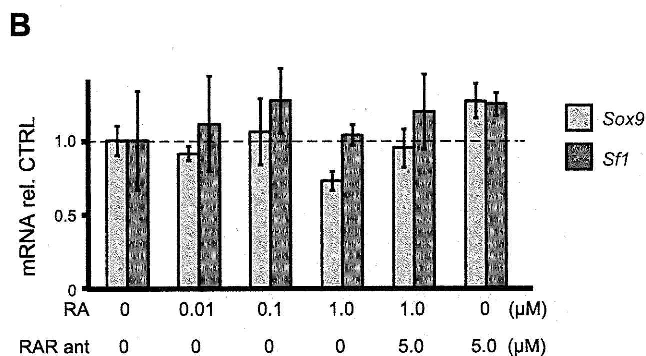


Figure 3. RA is not required for *Cyp26b1* up-regulation by SOX9 or SF1. *A*) qRT-PCR analysis of *Cyp26b1* mRNA levels in TM3 cells treated with RA (0.01, 0.1, and 1.0 µM), RA (1.0 µM) together with the RAR antagonist AGN193109 (RAR ant, 5µM), or AGN193109 alone (5 µM). RA stimulated *Cyp26b1* expression, whereas AGN193109 counteracted this effect. *B*) qRT-PCR analysis of *Sf1* and *Sox9* mRNA levels in TM3 cells treated with RA (0.01, 0.1, and 1.0 µM), RA (1.0 µM) and AGN193109 (5 µM), or AGN193109 alone (5 µM). Neither treatment affected *Sf1* or *Sox9* expression levels. *C*) qRT-PCR analysis of *Cyp26b1* mRNA levels in TM3 cells transfected with *Sox9* or *Sf1* expression vector (1 µg each) and/or treated with 5 µM AGN193109. RAR antagonist did not interfere with *Cyp26b1* up-regulation stimulated by SOX9 or SF1. Data sets represent mRNA expression relative to *Rn18s* (*A, C*) or control (no RA or AGN193109; *B*); means ± SE of 3 biologically independent experiments performed in triplicate. Level of statistical significance: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ *vs.* control (CTRL); unpaired Student's *t* test.

These observations suggest the possibility that RA synthesized by ALDH1A1 mediates the *Cyp26b1* regulation by SOX9 and SF1 observed in our experiments. However, neither exogenous RA nor RAR antagonist affected *Sox9* or *Sf1* expression levels (Fig. 3*B*), and RAR antagonist had no effect on the ability of transfected *Sox9* and *Sf1* expression vectors to up-regulate *Cyp26b1* expression (Fig. 3*C*). Therefore, the effects of SOX9 and SF1 are not mediated by RA action involving RARs and represent an independent mechanism of regulating *Cyp26b1* during testis development.

Cyp26b1 expression requires SOX9 and SF1 *in vivo*

We next sought to determine whether *Cyp26b1* is regulated by SOX9 or SF1 *in vivo*. We have shown previously that *Cyp26b1* expression is impaired in *Ck19*-Cre; *Sox9*$^{flox/flox}$ mice (38), in which the *Sox9* gene is deleted before the time of sex determination (43). However, in those mice, the XY gonads develop as ovaries, in which expression of all male-specific genes is presumably suppressed. To investigate SOX9 action on *Cyp26b1* expression *in vivo* more specifically, we used AMH-Cre: *Sox9*$^{flox/flox}$ mice. In these mice, *Sox9* expression is markedly reduced at 13.5 dpc, after the time of sex determination, and absent by 14.5 dpc. In contrast to *Ck19*-Cre:*Sox9*$^{flox/flox}$ mice, the XY gonads of AMH-Cre: *Sox9*$^{flox/flox}$ mice are phenotypically testes, maintaining normal expression levels of early testis-specific genes, such as *Fgf9* and *Sox8* (19). We found that *Cyp26b1* expression was significantly reduced in AMH-Cre: *Sox9*$^{flox/flox}$ XY gonads at 13.5 dpc (**Fig. 4*A***), supporting our hypothesis that SOX9 is required for positive regulation of *Cyp26b1* expression.

In *Sf1*-knockout mice, regression of the gonads after sex determination precludes analysis of the roles of this gene in gonadal differentiation (44, 45). For this reason, we also studied *Cyp26b1* expression in mice deleted for the gene encoding CBP/p300-interacting transactivator with Glu/Asp-rich carboxyl-terminal domain 2

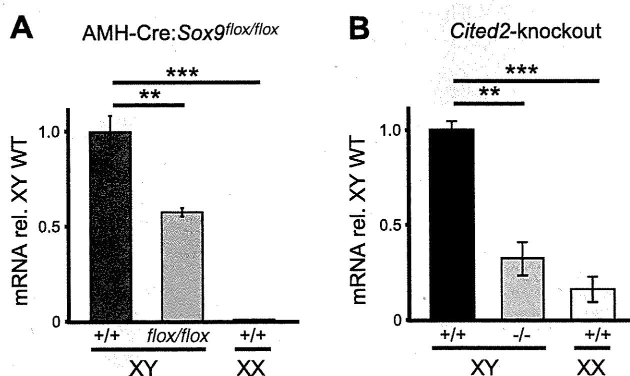


Figure 4. *Cyp26b1* expression is significantly reduced in mice with reduced SOX9 or SF1 expression. *A*) qRT-PCR analysis of *Cyp26b1* expression at 13.5 dpc of WT (+/+) XY (n=4), AMH-Cre:*Sox9*$^{flox/flox}$ (*flox/flox*) XY (n=3), and WT XX gonads (n=5). *B*) qRT-PCR analysis of *Cyp26b1* expression at 12.25 dpc of WT XY (n=3), *Cited2*-knockout (−/−) XY (n=3), and WT XX (n=3) gonads. Data sets represent mRNA expression relative to *Rps29* (means±SE). $**P < 0.01$, $***P < 0.001$ *vs.* WT XY; unpaired Student's *t* test.

(*Cited2*; refs. 20–22). CITED2 is a transcriptional coactivator or repressor that cooperates with WT1 to stimulate *Sf1* transcription (20); in *Cited2*-knockout mice, *Sf1* expression is temporarily reduced (20–22). The delay in testis development in *Cited2*-null mice is caused by the temporal loss of *Cited2*-mediated enhancement of *Sf1* levels, rather than a direct effect by loss of *Cited2* (20). Therefore, *Cited2*-knockout mice are a useful proxy with which to study loss of SF1 function during gonad differentiation. We chose 12.25 dpc as the time point of examination because the reduced *Sf1* expression recovers by 12.5 dpc in fetal gonads (22). Analysis by qRT-PCR showed that in *Cited2*-knockout mice, *Cyp26b1* expression was significantly reduced at 12.25 dpc in fetal testes (Fig. 4*B*). The observations from AMH-Cre:*Sox9*$^{flox/flox}$ and *Cited2*-knockout mice strongly suggest that SOX9 and SF1 up-regulate *Cyp26b1* expression during testicular development *in vivo*.

FOXL2 attenuates *Cyp26b1* up-regulation by SOX9 or SF1

Unlike the situation in developing testes, germ cells in the developing ovaries require exposure to RA to ensure entry into meiosis and initiation of the oogenic pathway (13, 14). Thus, *Cyp26b1* is up-regulated during testis development and down-regulated during ovarian development (ref. 13 and the present study; Fig. 1*A*). In view of our findings that SOX9 and SF1 can up-regulate *Cyp26b1* expression, the lack of *Cyp26b1* expression in the ovary might in theory be explained by a lack of SOX9 and SF1 in that tissue. However, we found that SF1 mRNA is expressed at moderate levels in the fetal ovary (Fig. 1*A*). Moreover, SF1 protein was readily detectable by immunofluorescence in the ovary at 12.5 dpc (**Fig. 5*A***). In view of these observations and because of the critical requirement to suppress CYP26B1 activity in the developing ovary, we reasoned that additional mechanisms might operate in developing ovaries to actively suppress *Cyp26b1* transcription, consistent with the emerging concept that balanced opposing signals act to ensure correct testis or ovarian development (46, 47).

FOXL2 is a forkhead transcription factor expressed mainly in somatic cells of female gonads and in developing eyelids (48). FOXL2 is considered essential for ovarian folliculogenesis and granulosa cell development (26, 48, 49). Furthermore, recent studies have suggested that FOXL2 antagonizes SOX9-dependent pathways (50) and suppresses SF1 target gene activation (51). Therefore, we tested whether FOXL2 actively suppresses *Cyp26b1* induction. First, we compared the temporal expression pattern of *Foxl2* and *Cyp26b1*. In XX gonads, *Cyp26b1* expression decreased from 12.5 dpc and, consistent with our hypothesis, coincided with a dramatic up-regulation of *Foxl2* expression (Fig. 5*B*).

To further investigate potential involvement of FOXL2 in *Cyp26b1* regulation, we transfected TM3 cells with a *Foxl2* expression plasmid together with *Sox9*, *Sf1*, or a combination of both expression plasmids. *Sox9* and

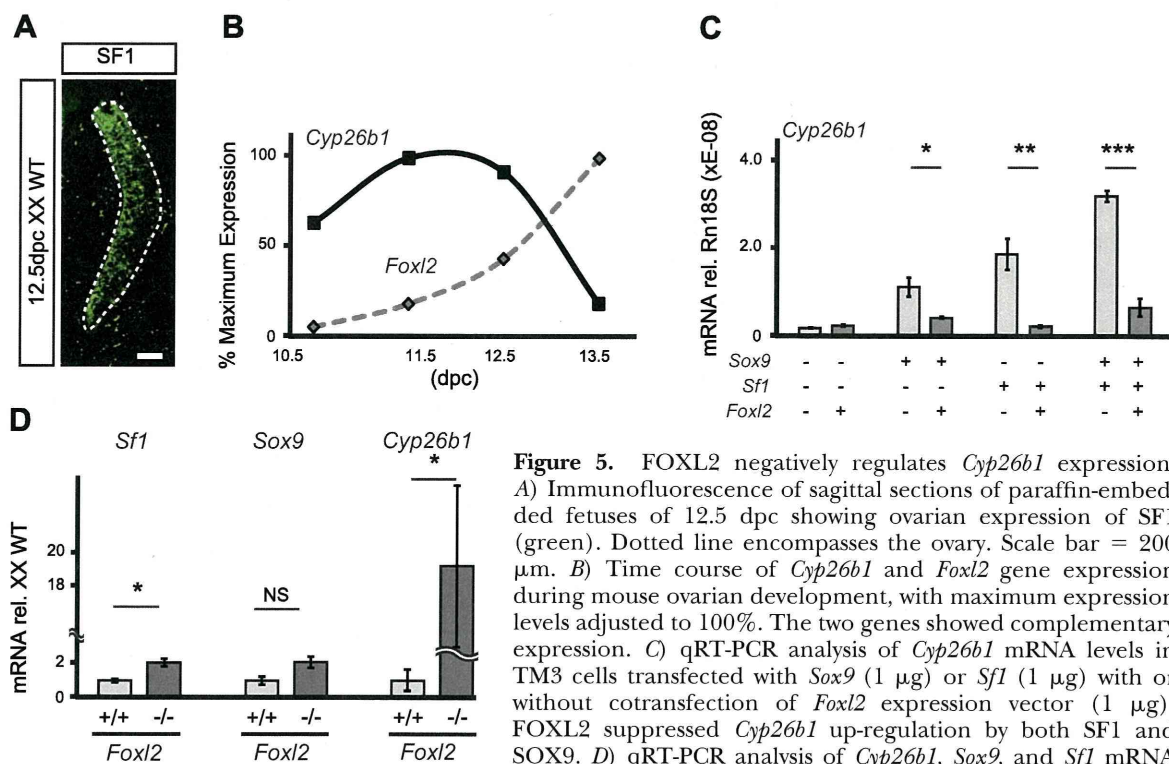


Figure 5. FOXL2 negatively regulates *Cyp26b1* expression. *A*) Immunofluorescence of sagittal sections of paraffin-embedded fetuses of 12.5 dpc showing ovarian expression of SF1 (green). Dotted line encompasses the ovary. Scale bar = 200 μm. *B*) Time course of *Cyp26b1* and *Foxl2* gene expression during mouse ovarian development, with maximum expression levels adjusted to 100%. The two genes showed complementary expression. *C*) qRT-PCR analysis of *Cyp26b1* mRNA levels in TM3 cells transfected with *Sox9* (1 μg) or *Sf1* (1 μg) with or without cotransfection of *Foxl2* expression vector (1 μg). FOXL2 suppressed *Cyp26b1* up-regulation by both SF1 and SOX9. *D*) qRT-PCR analysis of *Cyp26b1*, *Sox9*, and *Sf1* mRNA levels in XX gonads of 13.5 dpc *Foxl2*-null mice. Relative expression levels to *Sdha* of each gene were compared with those of XX wild-type controls. Absence of FOXL2 caused strong up-regulation of *Cyp26b1*. Data sets represent mRNA expression relative to *Rps29* (*B*), *Rn18s* (*C*), or *Sdha* (*D*); means ± SE of 3 biologically independent experiments performed in triplicate. NS, nonsignificant. **P* < 0.05, ***P* < 0.01, ****P* < 0.001; unpaired Student's *t* test.

Sf1 expression constructs do not contain binding sites for FOXL2 in their regulatory regions, and, accordingly, expression levels of these constructs were unaffected by the coexpression of FOXL2 (data not shown). However, FOXL2 strongly suppressed endogenous *Cyp26b1* expression induced by SOX9, SF1, or both together (Fig. 5*C*).

Finally, we analyzed *Cyp26b1* expression in 13.5 dpc XX gonads of *Foxl2*-null mice. We found that *Sox9* and *Sf1* expression levels increased ~2-fold (Fig. 5*D*). In contrast, *Cyp26b1* expression was increased ~20-fold relative to that in controls (Fig. 5*D*). The *Cyp26b1* expression levels in *Foxl2*-knockout ovary were 5-fold lower than those in WT testes (data not shown), as would be expected, because the *Foxl2*-knockout ovaries lack positive regulators such as SOX9, and consistent with the report that germ cells enter meiosis in these mutant ovaries (49). In these experiments, absence of FOXL2 caused up-regulation of *Cyp26b1* to a greater extent than that likely to be caused by up-regulation of *Sox9* and/or *Sf1*. The magnitude of the increase of *Cyp26b1* expression in *Foxl2*-null mice is consistent with relief of active suppression by FOXL2 during ovarian development *in vivo*, consistent with *in vitro* data observed in TM3 cells (Fig. 5*C*).

DISCUSSION

Despite its critical role for germ cell sexual differentiation, the mechanisms of *Cyp26b1* regulation during gonadal development are not known. In the present study, we present cell-based gain-of-function data, together with *in vivo* loss-of-function analyses in knockout mice, implicating the gonadal transcription factors SOX9, SF1, and FOXL2 in regulating *Cyp26b1* during gonadal development.

SOX9 was previously suggested to be acting upstream of *Cyp26b1* (38). We found that *Cyp26b1* was up-regulated in the testicular somatic cell line TM3 after transfection with the *Sox9* expression construct and that *Cyp26b1* expression was significantly reduced in XY gonads of AMH-Cre:*Sox9*$^{flox/flox}$ mice. In contrast to SOX9, SRY did not affect *Cyp26b1* expression levels in TM3 cells, indicating that activation of *Cyp26b1* transcription is not a general property of SOX transcription factors. Taken together, our data support the conclusion that SOX9 positively regulates *Cyp26b1* expression during testicular development.

Previous studies have established that *Cyp26b1* is expressed in both Sertoli and interstitial cells (13). However, *Sox9* expression is limited to Sertoli cells in fetal testes, indicating that some other molecules expressed in interstitial cells must be involved in *Cyp26b1* up-regulation. SF1 was considered a good candidate because *Sf1* is expressed not only in Sertoli cells but also in interstitial Leydig cells and is one of the earliest genes expressed in a male-specific manner in fetal gonads (52). We showed that the spatial expression patterns of *Sf1* and *Cyp26b1* overlapped each other in cells inside and outside testis cords; in TM3 cells, *Sf1* transfection robustly up-regulated *Cyp26b1* expression; and in *Cited2*-knockout mice, a mouse model with impaired expression of *Sf1*, *Cyp26b1* expression was significantly reduced. These data implicate SF1 as a second factor involved in the positive regulation of *Cyp26b1* expression during testicular development. Furthermore, our data suggest that the previously unidentified interstitial cells expressing *Cyp26b1* (13) are Leydig cells. Taken together, our data show that in fetal Sertoli cells, both SOX9 and SF1 contribute to *Cyp26b1* regulation, whereas in fetal Leydig cells, *Cyp26b1* is regulated by SF1 (**Fig. 6**).

Considering the rapid up-regulation of *Cyp26b1* by SOX9 and SF1 in TM3 cells; the similar temporal expression profile of *Cyp26b1*, *Sox9*, and *Sf1* in fetal testes; and the fact that this regulation does not require RA, it seems likely that SOX9 and SF1 directly up-regulate *Cyp26b1* during testicular development. CYP26B1 is expressed in various organs during fetal development, such as limb, hindbrain, and the first and the second branchial arches (17, 53), indicating that the regulation of the *Cyp26b1* gene is complex, and many *trans*-acting factors and *cis*-regulatory sequences are likely to be involved. Substantial homology is maintained between the sequence of mouse *Cyp26b1* intron-1 and the human *CYP26B1* 5′ flanking region (data not shown), suggesting that these sequences may be important for *Cyp26b1* gene regulation. However, neither 3 kb of 5′ flanking sequence nor intron-1 or intron-2 of mouse *Cyp26b1* responded to mSOX9 and mSF1 in our TM3 culture assay (data not shown); the

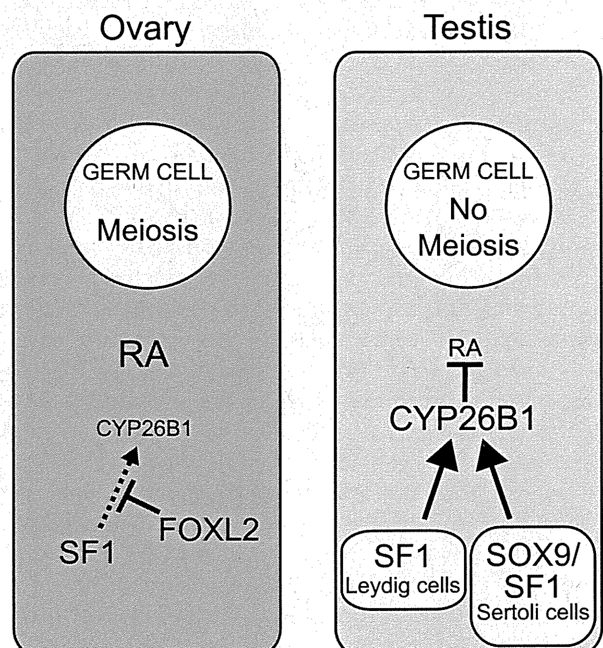


Figure 6. Model for the regulation of *Cyp26b1* expression and germ cell sexual fate. Distribution of RA in gonads is determined by the RA-degrading enzyme, CYP26B1. *Cyp26b1* is expressed in a male-specific manner, and resulting RA levels are high in ovary and low in testes. In testes, *Cyp26b1* is up-regulated by SF1 in Leydig cells and by SF1 and SOX9 in Sertoli cells. Although SF1 could be expected to up-regulate *Cyp26b1* expression in ovaries, this expression is suppressed by the female-specific transcription factor FOXL2. Thus, male and female pathways compete in the regulation of *Cyp26b1*.

gonad-specific enhancer of *Cyp26b1* is therefore presumed to be located some distance from the *Cyp26b1* transcription unit itself. Further studies will be necessary to identify the gonad-specific enhancers of *Cyp26b1* and to specifically address whether SF1 and/or SOX9 directly bind to these elements.

We found that *Sf1* mRNA and protein are expressed in fetal ovaries at 12.5 dpc at higher levels than previously recognized (52); this expression and its functional significance are supported by observations that the ovary regresses after sex determination in *Sf1*-knockout mice (44). Consistent with previous studies (52), we also found lower *Sf1* expression levels at 13.5 dpc. The presence of SF1 in the ovaries suggested that mechanisms might exist in the developing ovary to counter the possible up-regulation of *Cyp26b1* in that tissue.

Our data implicate a third regulatory factor, FOXL2, as part of such a mechanism. FOXL2 is a key transcription factor in ovarian development, and the phenotype of mammalian models of *Foxl2* deficiency varies from XX sex reversal in goats (54) to ovarian failure in mice and humans (26, 48, 49). *Foxl2* is expressed in somatic cells during ovarian development and is involved in the regulation of other female-specific genes, such as *Aromatase*, *Follistatin*, and *Bmp2* (30, 55, 56). It has been reported that FOXL2 represses SF1-induced *Cyp17* transcription by interacting directly with SF1 (51); that observation not only substantiates the concept that FOXL2 can act as a negative transcriptional regulator but also supports our finding that it antagonizes SF1 regulation of *Cyp26b1*. The absence of FOXL2 *in vivo* caused up-regulation of *Cyp26b1* to a greater extent than that likely to be caused by the observed up-regulation of *Sox9* and/or *Sf1*. Therefore, our findings suggest that down-regulation of *Cyp26b1* during ovarian development may occur through a combination of decreasing SF1 levels and up-regulation of *Foxl2* expression.

Suppression of *Cyp26b1* expression by of FOXL2 may provide a failsafe mechanism to ensure that ovarian germ cells are not exposed to RA. Such a mechanism would support and extend our recent findings that fibroblast growth factor 9 (FGF9) acts directly on germ cells to inhibit meiosis (57). Those findings indicate that two independent and mutually antagonistic pathways involving RA in the ovary and FGF9 in the testis determine mammalian germ cell sexual fate commitment (57). In addition to that model, our present study suggests a further level of antagonism between male and female germ cell pathways, namely suppression of *Cyp26b1* expression by FOXL2. A model for this regulatory network is depicted in Fig. 6, providing a framework for further studies of the interaction between somatic and germ cells in fetal gonads and the control mechanisms that underpin development of germ cells. FJ

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FOXL2 and BMP2 Act Cooperatively to Regulate *Follistatin* Gene Expression during Ovarian Development

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Follistatin is a secreted glycoprotein required for female sex determination and early ovarian development, but the precise mechanisms regulating follistatin (*Fst*) gene expression are not known. Here, we investigate the roles of bone morphogenetic protein 2 (BMP2) and forkhead-domain transcription factor L2 (FOXL2) in the regulation of *Fst* expression in the developing mouse ovary. *Bmp2* and *Fst* showed similar temporal profiles of mRNA expression, whereas FOXL2 protein and *Fst* mRNA were coexpressed in the same ovarian cells. In a cell culture model, both FOXL2 and BMP2 up-regulated *Fst* expression. In *ex vivo* mouse fetal gonad culture, exogenous BMP2 increased *Fst* expression, but this effect was counteracted by the BMP antagonist Noggin. Moreover, in *Foxl2*-null mice, *Fst* expression was reduced throughout fetal ovarian development, and *Bmp2* expression was also reduced. Our data support a model in which FOXL2 and BMP2 cooperate to ensure correct expression of *Fst* in the developing ovary. Further, *Wnt4*-knockout mice showed reduced expression of *Fst* limited to early ovarian development, suggesting a role for WNT4 in the initiation, but not the maintenance, of *Fst* expression. **(*Endocrinology* 152: 0000–0000, 2011)**

Although testes and ovaries have quite distinct structures and functions, they arise from the same embryonic organ primordia, the genital ridges. In mammals, testis fate is determined by the action of the Y-linked gene *Sry*, which initiates the differentiation of Sertoli cells (1). Ectopic expression of *Sry* in XX embryos causes testicular development and, therefore, XX sex reversal (2). Ovarian fate has been considered as a default pathway caused by the absence of the *Sry* gene. To date, many testis-specific genes have been discovered, and the mechanisms of testicular development have been studied intensively. In contrast, only a handful of ovarian-specific genes have been identified, and the relationship between theses genes is not yet clear.

Follistatin (*Fst*) is one gene the expression of which is restricted to the developing ovary but not the developing testis (3, 4). FST protein is a single-chain glycoprotein that binds to TGFβ-superfamily molecules such as activin and neutralizes their activity (5). *Fst* is expressed in many organs such as neuronal tissues, kidney, liver, bone, heart, muscle, and skin (5, 6), and *Fst*-null mice die within a few hours after birth due to skeletal deformities, muscle abnormalities, and growth failure, confirming a broad range of biological roles (7).

FST was originally identified as an inhibitor of FSH secretion (8, 9) and was thought to have an important role in folliculogenesis and ovarian function (10, 11). However, *Fst*-null mice also displayed partial XX sex reversal during embryogenesis, suggesting an earlier role for *Fst* in ovarian development (4). For example, *Fst* deficiency causes development of a celomic blood vessel, normally a feature of testis development, in an XX gonad (4). Further, primordial germ cells in *Fst*-null mice enter meiosis normally, but undergo massive apoptosis at 16.5 days post

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Abbreviations: BMP2, Bone morphogenetic protein 2; dpc, days post coitus; FOXL2, forkhead-domain transcription factor L2; P0, newborn; qRT-PCR, quantitative real-time RT-PCR; SMAD, Sma- and Mad-related protein.

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coitus (dpc) (4). It is not clear whether FST acts directly on germ cells to facilitate their development and/or survival, or whether these effects are mediated by action of FST on somatic development of the ovary.

An important question in early ovarian development is how *Fst* expression is regulated. Two candidate molecules have been suggested to up-regulate *Fst* expression, WNT4 and forkhead-domain transcription factor L2 (FOXL2). *Wnt4* is considered one of the master genes for ovarian development. XX $Wnt4^{-/-}$ mice are partially sex reversed as judged by criteria such as formation of the celomic blood vessel. $Wnt4^{-/-}$ mice also show greatly reduced *Fst* expression at 14.5 dpc (4, 12). Homozygous mutation of *WNT4* in humans causes SERKAL syndrome, with fetal lethality and testes or ovotestes in XX fetuses (13). *Wnt4* is expressed in the mesonephric mesenchyme and celomic epithelium overlying the future gonads of both sexes at 9.5 dpc. At 11.5 dpc, *Wnt4* expression is down-regulated in XY gonads, but persists in XX gonads (12).

The other current candidate for the up-regulation of *Fst* expression is FOXL2. FOXL2 is a forkhead transcription factor expressed mainly in somatic cells of female gonads and in developing eyelids (14). The phenotype of mammalian models of *Foxl2* deficiency varies from XX sex reversal in goats (15) to ovarian failure in mice and humans (14, 16, 17). *Foxl2* is considered essential for ovarian folliculogenesis and granulosa cell development independently of *Wnt4* (18). Overexpression of *Foxl2* in mice results in an increase in *Fst* expression in 13.5 dpc XX gonads (19). Conversely, in *Foxl2*-null mice, *Fst* expression is reduced in postnatal ovaries (20). Taken together, these data suggested that *Wnt4* and *Foxl2* act upstream of *Fst* during ovarian development.

Recently, it has been reported that FOXL2 and Sma- and Mad-related protein (SMAD)3 interact and synergistically up-regulate *Fst* expression in pituitary cells (21). SMAD proteins function as intracellular signaling molecules transferring the signal induced by bone morphogenetic proteins (BMPs) at the cell membrane to the nucleus. BMPs are members of the TGF-β superfamily, and among them, *Bmp2* is expressed in an ovary-specific manner during early gonad development. *Wnt4* is thought to act upstream of *Bmp2* in ovarian development (4, 22). Loss of BMP2 function in mice is lethal during embryogenesis before sex is determined (4). However, gonad-specific SMAD1/5 heterozygous double knockout XX mice are infertile (23), suggesting that BMP2 plays an important role in ovarian development. There have been reports of direct interaction between forkhead proteins and SMADs (24–27). On the basis of these observations, we hypothesized that BMP2 might play a role in regulating *Fst* expression during ovarian development.

We report here that the expression of *Bmp2*, *Foxl2*, and *Fst* overlapped during ovarian development *in vivo*, and that BMP2 and FOXL2 cooperatively up-regulated *Fst* expression *in vitro*. BMP2 also up-regulated *Fst* expression in *ex vivo* organ culture experiments, and its up-regulation was cancelled by the BMP antagonist Noggin. In *Foxl2*-null embryos, *Fst* and *Bmp2* expression was significantly reduced, and in *Wnt4*-null mice, *Fst* expression was also reduced at early stages. These observations indicate that WNT4 may have a role in up-regulating *Fst* expression early in ovarian development, and that FOXL2 and BMP2 are required to maintain *Fst* expression. This is the first report to identify a molecular function for BMP2 during ovarian development.

Materials and Methods

Animals and cell lines

Mouse embryos were collected from timed matings of the Swiss Quackenbush and CD1 outbred strain, with noon of the day on which the mating plug was observed designated as 0.5 dpc. Embryos were sexed at 11.5 dpc using an X-linked green fluorescent protein marker (28) and at 12.5–16.5 dpc by gonadal morphology. Protocols and use of animals were approved by the Animal Welfare Unit of the University of Queensland.

Transfection into KK1 cells

Murine ovarian granulosa cell tumor-derived KK1 cells (29) were cultured in DMEM (Invitrogen, Carlsbad, CA)/F12 medium (Life Technologies, Inc., Gaithersburg, MD) with 10% fetal bovine serum at 37 C in 5% CO_2. KK1 cells were plated at 1.5×10^5 per well in six-well plates 24 h before transfection. Cells were transfected with 2 μg or 4 μg of plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Total amount of transfected plasmid was standardized using the empty expression vector, pcDNA3.1(+) (Invitrogen, V795-20) as control. Recombinant human BMP2 (355-BM) and recombinant mouse Noggin (1967-NG/CF) were purchased from R&D systems (Minneapolis, MN) and reconstituted in PBS. *Foxl2* cDNA was amplified by RT-PCR from mouse embryonic gonad mRNA using 5'- and 3'-untranslated region primers and ligated into p-GEMT easy vector (Promega Corp., Madison, WI). To generate *Foxl2* expression vector, coding sequence of *Foxl2* was excised using *Eco*RI, blunted, and subcloned into blunted *Kpn*I and *Eco*RI sites of pcDNA3.1 expression vector (Promega).

Quantitative real-time RT-PCR (qRT-PCR)

For transfected cells, total RNA was collected 48 h after cell transfection. For tissue analysis, embryonic gonads without mesonephroi were dissected in ice-cold PBS at the appropriate stages. Total RNA from cells and tissue was isolated using the RNeasy Mini Kit (QIAGEN, Chatsworth, CA) including deoxyribonuclease treatment. Total RNA (500 ng; KK1 or 300 ng; gonads) was used for the synthesis of cDNA using SuperScript III (Invitrogen) and random primers (Invitrogen), according to

TABLE 1. Primers used for qRT-PCR analysis

Gene	Gene description	Primers (forward)	Primers (reverse)
SYBR qPCR			
Rn18s	18S rRNA	GATCCATTGGAGGGCAAGTCT	CCAAGATCCAACTACGAGCTTTTT
Rps29	Ribosomal protein S29	TGAAGGCAAGATGGGTCAC	GCACATGTTCAGCCCGTATT
Fst	Follistatin	GCAGCCGGAACTAGAAGTACA	ACACAGTAGGCATTATTGGTCTG
Foxl2	Forkhead box L2	GCTACCCCGAGCCCGAAGAC	GTGTTGTCCCGCCTCCCTTG
Wnt4	Wingless-type MMTV integration site family member 4	CTGGACTCCCTCCCTGTCTTT	CATGCCCTTGTCACTGCAA
BMP2	BMP 2	CGGACTGCGGTCTCCTAA	GGGGAAGCAGCAACACTAGA
BMPR1a	BMP receptor type 1a	CAGTTTTATCTAGCCACATCTCTGA	GGGAGGCTTCCTTACAGAACA
BMPR2	BMP receptor type 2	GAGCCCTCCCTTGACCTG	GTATCGACCCCGTCCAATC
Taqman qPCR			
Sdha	Succinate dehydrogenase complex, subunit A, flavoprotein	Mm01352366_m1	
Fst	Follistatin	Mm00514982_m1	
Bmp2	BMP 2	Mm01340178_m1	

MMTV, Mouse mammary tumor virus.

manufacturer's instructions. cDNA samples were diluted 1:4, and 1 μl used in each 25 μl qRT-PCR, containing SYBR green PCR master-mix (Applied Biosystems, Foster City, CA). Transcript levels were analyzed on an ABI Prism-7500 Sequence Detector System over 40 cycles of 95 C for 15 sec and 60 C for 1 min in a two-step thermal cycle, preceded by an initial 10-min step at 95 C. qRT-PCR primers are listed in Table 1. The average and SEM of three biological replicates measured in triplicate was calculated. *Rps29* (*Ribosomal protein S29*) served as the housekeeping gene to standardize qRT-PCR data based on minimum variability at each stage during gonadal development (30). We used unpaired Student's *t* test to demonstrate statistical significant difference between the given sample and the control.

In situ hybridization and immunohistochemistry

In situ hybridization and immunohistochemistry were performed sequentially, and each procedure was performed as described previously (31–33). Briefly, sections of paraformaldehyde-fixed, paraffin-embedded embryos were dewaxed, rehydrated, incubated in proteinase K, refixed with 4% paraformaldehyde, acetylated, and prehybridized. Hybridization was performed overnight at 60 C. After 2 h blocking, antidigoxigenin antibody at 1:2000 dilution in blocking solution was added, and sections were incubated overnight at 4 C. After washing, sections were equilibrated in NTM 100 mM Tris (pH9.5), 100 mM NaCl, 50 mM MgCl2 buffer and incubated in color solution. After *In situ* hybridization, immunohistochemistry was performed by washing in PBS, stimulating antigen retrieval by boiling for 8 min, and incubation with 0.3% H_2O_2. Thereafter, sections were washed in PBTx, blocked with 10% sheep serum, and incubated with primary antibody overnight at 4 C. Biotinylated secondary antibody was hybridized for 1 h. For color reaction, sections were incubated with streptavidin-horseradish peroxidase for 30 min at room temperature. Polyclonal anti-FOXL2 antibody was generated as described by Cocquet *et al.* (34) and used at 1:2000 dilution.

Ex vivo organ culture

Mouse gonads were collected from 11.5 dpc X-linked green fluorescent protein embryos. Four to six gonads were used in each group. First, gonads were incubated in culture medium (DMEM + 10% fetal calf serum) with recombinant BMP2 (500 ng/ml) and/or recombinant Noggin (2 μg/ml) for 1 h at 37 C. Thereafter, the gonads were incubated on filter membrane (Millipore filters, HAWPO1300) floating on culture medium containing BMP2 (500 ng/ml) or recombinant Noggin (2 μg/ml) at 37 C in 5% CO_2 for 48 h. Total RNA was collected using the RNeasy Mini Kit (QIAGEN) with deoxyribonuclease treatment as described above.

Knockout mouse analysis

The generation of *Wnt4*-, *Foxl2*-, and double-knockout mice was previously reported (12, 16, 35). These mice were maintained on a mixed C57B6/J/129/SVJ genetic background. For each genotype, gonads were separately processed and analyzed independently. In addition to the samples of 13.5 dpc and newborn (P0), we collected samples from *Foxl2*-null mice at 16.5 dpc, and *Wnt4*-null mice at 15.5 dpc. Total RNA was obtained from dissected gonads by enzymatic extraction (Melt; Ambion, Austin, TX) followed by linear RNA amplification using Pico Ovation (NuGEN). qRT-PCR (TaqMan) was performed using an ABI 7900HT system (Applied Biosystems). The average and SEM of three biological replicates measured in triplicate were calculated. *Sdha* was used as the housekeeping gene to standardize the data as reported previously (35).

Results

Fst and *Bmp2* have similar temporal expression profiles in fetal ovaries

To elucidate the relationships between *Fst* and other known ovarian genes, we performed a detailed time course expression analysis from 11.5 dpc to P0 using qRT-PCR analysis. As reported previously, *Fst*, *Wnt4*, *Foxl2*, and *Bmp2* were expressed in an ovarian-specific manner (Fig. 1A). In contrast, the genes encoding the receptors for

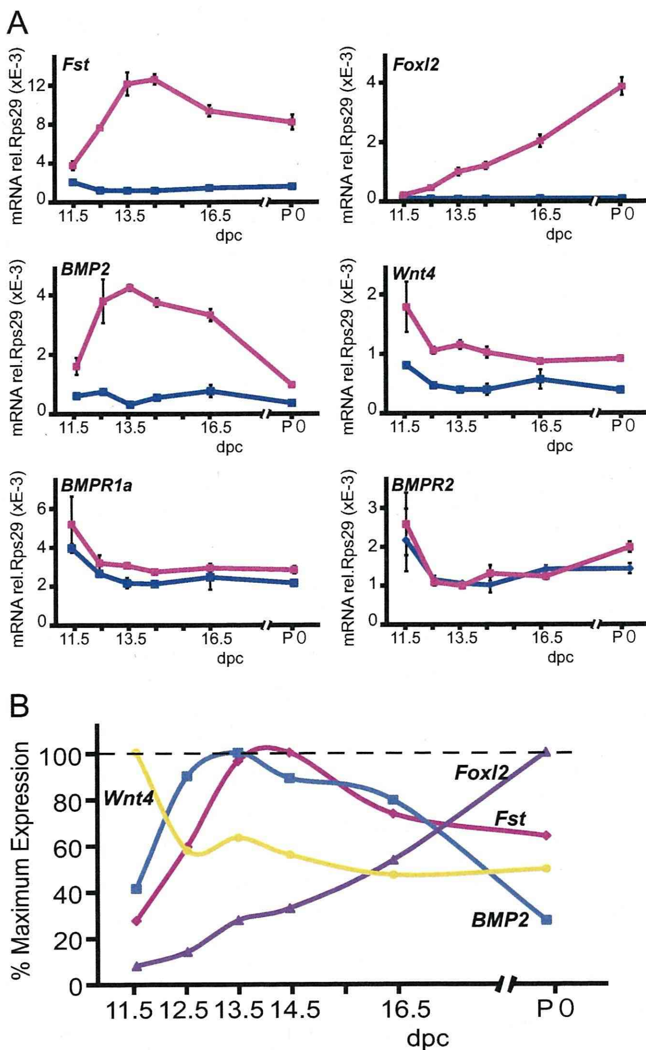

FIG. 1. Time course of marker gene expression during mouse gonadal development. A, Expression of female marker genes and BMP receptor genes expression in fetal gonads. Data sets represent mRNA expression relative to *Rps29* (mean ± SEM of three biologically independent experiments performed in triplicate). The *red and blue lines* indicate female and male, respectively. B, Expression of female marker genes during ovarian development. Data from panel A were combined into one graph with maximum expression levels adjusted to 100%.

BMPs, *Bmpr1a* and *Bmpr2*, did not show sexually dimorphic expression (Fig. 1A).

To better compare the time course of these ovarian marker genes, we normalized the maximum expression levels of each gene to 100% (Fig. 1B). *Fst* expression was detectable at 11.5 dpc at low levels, and increased thereafter until 13.5 dpc before decreasing again. The level of *Wnt4* expression was already at a maximum at 11.5 dpc and decreased over the period investigated, consistent with the possibility that *Wnt4* might play a more prominent role in initiating, rather than maintaining, *Fst* expression. In contrast, *Foxl2* expression was first detectable at 12.5 dpc and continued to increase until P0, consistent with a later role in regulating *Fst* expression. The profile of *Bmp2* expression was very similar to that of *Fst* at all time points examined, consistent with the hypothesis that BMP2 has an important role in regulating *Fst* expression.

Fst and FOXL2 are expressed in the same cells in fetal ovaries

Wnt4 and *Bmp2* encode secreted factors, whereas *Foxl2* encodes a transcription factor, and so the hypothesis that *Foxl2* might be involved in *Fst* regulation requires that both genes/proteins are expressed in the same cells. Therefore, we next investigated the spatial distribution of *Foxl2* and *Fst* expression by histological analysis. Due to the lack of a FST antibody that has sufficient sensitivity to detect expression in fetal gonads, we combined *in situ* hybridization (ISH) for *Fst* mRNA with immunohistochemistry for FOXL2 protein. In 13.5 dpc XX gonads, most FOXL2-positive cells also expressed *Fst* (Fig. 2), supporting the suggested involvement of FOXL2 in regulating *Fst* expression in the developing mouse ovary.

FOXL2 and BMP2 cooperatively up-regulate endogenous *Fst* expression *in vitro*

To further investigate the potential involvement, and relative contributions, of BMP2 and FOXL2 in the regulation of *Fst* expression, we carried out a series of experiments using the cell line KK1 (29, 36), derived from ovarian tumor cells of transgenic mice expressing simian virus 40 T-antigen. KK1 cells express *Cyp19* and produce cAMP in response to human chorionic gonadotropin, FSH, and LH, suggesting that they are analogous to ovarian granulosa cells. KK1 cells were able to be transfected at high efficiency in our hands (Fig. 3A). We found that transfection and expression of *Foxl2* in KK1 cells up-regulated endogenous *Fst* expression in a dose-dependent manner (Fig. 3B). Expression of another female-specific gene, *Rspo1*, did not change as a result of *Foxl2* transfection (data not shown), indicating that *Fst* up-regulation is not the result of global increases in transcription. Similarly, treatment of the cells with exogenous BMP2 protein resulted in increased *Fst* expression (Fig. 3C). Together, these data support a model in which FOXL2 and BMP2 might be involved in *Fst* regulation in the fetal ovary.

To investigate potential cooperative action of FOXL2 and BMP2 in stimulating *Fst* expression, we combined BMP2 treatment with *Foxl2* transfection. Individually, *Foxl2* transfection and BMP2 treatment up-regulated *Fst* expression approximately 3- and 2-fold, respectively. Combined treatment of *Foxl2* and BMP2 resulted in a 10-fold up-regulation of *Fst* expression (Fig. 3D), demonstrating a cooperative and mildly synergistic effect of FOXL2 and BMP2 on *Fst* expression.