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Retinoic Acid Signaling Regulates Sonic Hedgehog and Bone Morphogenetic Protein Signalings During Genital Tubercle Development

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Abstract

Retinoic acid (RA) plays pivotal roles in organogenesis, and both excessive and reduced amounts of RA cause developmental abnormalities. Reproductive organs are susceptible to teratogen toxigenicity, and the genital tubercle (GT) is one such representative organ. The physiological function of endogenous RA signaling and the mechanisms of RA-induced teratogenicity are poorly understood during the GT development. The objective of this study is to understand the developmental and teratogenic roles of RA during GT development by analyzing genetically modified mouse models. We found dynamic patterns of gene expression for the RA-synthesizing enzyme, *Raldh2*, and for the RA-catabolizing enzyme, *Cyp26b1*, during GT development. *Rarb*, an indicator gene for RA signaling, starts its expression in the prospective corpus cavernosum

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penis and in the urethral plate epithelium (UE), which plays central roles during GT development. Excessive RA signaling in *Cyp26b1*$^{-/-}$ mutants leads to abnormal extents of cell proliferation and differentiation during GT development, and also upregulates expression of growth factor signalings. They include Sonic hedgehog (Shh) signaling and Bone morphogenetic protein (Bmp) signaling, which are expressed in the UE and its bilateral mesenchyme. RA signaling positively regulates *Shh* and *Bmp4* expression during GT development as testified also by the experiment of RA administration and analyses of loss–of-function of RA signaling mutants. Thus, RA signaling is involved in the developmental cascade necessary for UE formation and GT development.

Keywords

retinoic acid; *genital tubercle*; Sonic hedgehog (Shh); Bone morphogenetic protein (Bmp); *urethral plate epithelium*

INTRODUCTION

Retinoic acid (RA), an active derivative of vitamin A, exerts crucial functions in vertebrate development (Batourina et al., 2002; Niederreither and Dollé, 2008). Both excessive and reduced RA signaling cause developmental abnormalities (Niederreither and Dollé, 2008).

The supply of vitamin A in vivo is regulated by the blood stream. Additionally, RA is a diffusible molecule among tissues. However, it has been reported to function in a spatiotemporal manner (Duester, 2008). The regulation of RA activity depends on both the conversion of vitamin A to biological active RA by oxidation and by the catabolism of RA into an inactive form. Many enzymes are in fact involved in such processes. Among those enzymes, the RA-synthesizing enzyme, *Raldh*, and the RA-catabolizing enzyme, *Cyp26*, are expressed in spatiotemporal-specific patterns during embryonic development (Yashiro et al., 2004; Duester, 2008; Niederreither and Dollé, 2008). It has been suggested that the restricted expression of these two kinds of RA-metabolizing enzymes regulates region-specific RA signaling in the embryo (Duester, 2008).

The genital tubercle (GT) is a representative tissue for reproductive organ formation. Many researchers study GT development in the fields of developmental biology and pharmacology (Pask et al., 2005, 2008). The urethral plate epithelium (UE), an endoderm-derived tissue (Haraguchi et al., 2001; Seifert et al., 2008), develops at the ventral midline of the GT. GT possesses a unique developmental process that involves tissues derived from all three germ layers and the UE plays a central role in GT development (Haraguchi et al., 2000, 2001).

RA signaling has been suggested to function in organogenesis, including that of the brain, spinal cord, heart, eye, limb buds, and genitourinary tract (Duester, 2008; Mendelsohn et al., 1994). However, due to early lethality and severe disorganization of the entire caudal body of several RA signaling mutants (Abu-Abed et al., 2001; Niederreither and Dollé, 2008; Ribes et al., 2009), few studies have analyzed RA function during GT development. RA administration has been utilized as a good model system for teratology and toxicology studies. Because the GT is susceptible to the toxicity of certain external factors, many studies have assessed such effects on the GT (Jiang et al., 2007; Nakata et al., 2009).

Exposure of mouse embryos to excessive amounts of RA at E8.5 to E9.5 induces agenesis of the UE on the ventral side of the GT (Ogino et al., 2001). Human genetic studies have also linked GT agenesis and defects in urethra formation to abnormal RA levels in patients with cytochrome P450 oxidoreductase deficiency (Fukami et al., 2010). It is essential, therefore, to investigate the mechanisms underlying the teratogenic effects of RA during GT development.

Many growth factor signaling pathways have been reported to be involved in GT embryonic development, including Sonic hedgehog (Shh) (Haraguchi et al., 2001; Seifert et al., 2009a) and Bone morphogenetic protein (Bmp) signaling pathways (Suzuki et al., 2003; Chiu et al., 2010). During organogenesis, RA signaling regulates Shh signaling during brain development, caudal body trunk formation, and prostate budding (Ribes et al., 2006; Vezina et al., 2008; Ribes et al., 2009). RA signaling regulates Bmp signaling during prostate development, the formation of craniofacial structures and early gastrulation (Zhu et al., 1999; Halilagic et al., 2007; Vezina et al., 2008). However, RA regulates Shh or Bmp signaling in different ways under aforementioned developmental contexts.

In this study, we investigated the developmental and teratogenic roles of RA during GT development. We suggest that *Shh* and *Bmp4* are possible targets of RA signaling during GT development and RA-induced teratogenicity.

MATERIALS AND METHODS

Mice

The mutant alleles employed in this study were Cyp26b1$^{-/-}$ (Yashiro et al., 2004), RARaDN^{Flox} (Rosselot et al., 2010), Gli1^{CreERT2} (Ahn and Joyner, 2004; Miyagawa et al., 2009), and Isl1^{MerCreMer} (Laugwitz et al., 2005). All experimental procedures and protocols were approved by the committee on animal research at Kumamoto University and Wakayama Medical University. Noon on the day when a vaginal plug is detected is designated as E0.5.

The Tamoxifen (TM)-inducible Cre recombinase system removes the floxed sequence of the target genome (Feil et al., 1997). TM (Sigma Chemical Co., St. Louis, MO) was dissolved in sesame oil (Kanto Chemical, Tokyo, Japan) at a final concentration of 10 mg/ml and administered at 100 mg/kg body weight (intraperitoneally [i.p.]) to pregnant mice. Isl1$^{MerCreMer/+}$:RARaDN$^{Flox/-}$ and Gli1$^{CreERT2/+}$:RARaDN$^{Flox/-}$ males were mated with RARaDN$^{Flox/Flox}$ females, and only mutant embryos with homozygous RARaDN$^{Flox/Flox}$ alleles were analyzed. The Isl1$^{MerCreMer/+}$:RARaDN$^{Flox/Flox}$ mutants were treated with TM at E9.5, and Gli1$^{CreERT2/+}$:RARaDN$^{Flox/Flox}$ animals were treated at E10.5. Male embryos were used for all experiments unless otherwise indicated.

RA Treatment

Pregnant Crij:CD1 mice were given a single dose of All-trans-RA (100 mg/kg; Sigma-Aldrich) by gastric intubation via oral gavage at E14.5. The fetuses were harvested 2, 4, 6, and 8 hr after RA administration. RA was dissolved in DMSO. Control animals were given an equivalent volume of DMSO.

Section and Whole-Mount In Situ Hybridization for Gene Expression Analysis

Section in situ hybridization for gene expression was performed as described previously (Miyagawa et al., 2009). Each experimental set for section in situ hybridization was conducted strictly in the same condition between the control and the mutant or RA-treated embryos, including sample fixation, paraffin section preparation, and the in situ hybridization procedures. Three pairs of mice were analyzed for each genes on *Cyp26b1*$^{-/-}$ mutant analysis (Fig. 3); two pairs of mice were analyzed for each genes in RA administration analysis (Fig. 4) and RA loss-of-function mutants analysis (Fig. 5). Whole-mount in situ hybridization was performed with digoxigenin-labeled probes using standard procedure (Haraguchi et al., 2000). The following probes were used for in situ hybridization: *Rarb* (P. Chambon), *Raldh2* (P. Chambon), *Cyp26b1* (H. Hamada), *Hoxd13* (P. Dolle), *Bmp4* (C.M. Jones), *Shh* (C. Shukunami), *Ptc1* (J. Motoyama), *Meis2* (K. Backs), and *Hoxa13* (H.S. Stadler).

Histological staining, BromodeoxyUridine Incorporation, and Immunohistochemical Analysis

Hematoxylin and Eosin staining and X-gal staining were performed by standard procedures as previously described (Haraguchi et al., 2007).

Pregnant females were injected (i.p.) with 100 mg of 5-BromodeoxyUridine (BrdU; Sigma)/kg body weight at E13.5 or E14.5. One hour after injection, embryos were collected and fixed.

Immunohistochemistry procedures were performed as previously described (Miyagawa et al., 2009). Deparaffinized sections were autoclaved at 121°C for 1 min in citrate buffer (pH 6.0) for antigen retrieval. The sections were incubated at a 1:100 dilution for anti-Laminin antibody (L9393; Sigma), a 1:500 dilution for anti-SHH antibody (clone H-160, SC9024; Santa Cruz Biotechnology), and a 1:300 dilution for anti-pSmad1/5/8 (#9511s; Cell signaling). For SHH and pSmad1/5/8 immunohistochemistry, both Vectastain ABC (Vector Laboratories, Burlingame, CA) and TSA (NEL700001KT; PerkinElmer Inc., Waltham, MA) kits were employed to amplify the signals. For BrdU immunostaining, sections were additionally treated with 2.4 N HCl for 15 min to denature the DNA after antigen retrieval.

Statistical Analyses

Statistical analyses were performed using the Student's *t* test followed by the *F*-test. $p<0.05$ is considered statistically significant.

RESULTS

Expression of Genes Involved in RA Signaling is Detected During GT Development

Retinaldehyde dehydrogenase 2 (*Raldh2*) is one of the key RA-synthesizing enzyme during embryonic development. The initiation of GT outgrowth begins at E10.5. *Raldh2* is expressed in the caudal ventral region adjacent to the GT during initial outgrowth before E11.5 (Fig. 1A and A′). Expression of *Raldh2* is gradually shifted from the outside of the GT to the inner midline of the GT. Such expression is specifically restricted to the UE at E12.5

(Fig. 1B and B′). Formation of the UE is an essential morphogenetic process during GT development (Yamada et al., 2003). The UE plays essential roles that affect the development of its bilateral mesenchyme and, hence, the development of the GT (Haraguchi et al., 2000, 2001). Expression levels of *Raldh2* in the UE gradually increase at E13.5 and E14.5 (Fig. 1C, C′, D, and D′).

Cytochrome P450 26b1(*Cyp26b1*), a RA-catabolizing enzyme, is initially expressed in the mesenchyme adjacent to the distal ectodermal epithelium of the GT at E11.5 (Fig. 1E and E′), and becomes broadly expressed in the GT mesenchyme at E12.5 (Fig. 1F and F′). Such expression remains in the distal portion of the GT. However, its proximal expression is gradually restricted to the mesenchyme on the dorsal side of the UE at E13.5 and E14.5 (Fig. 1G, G′, H, and H′).

Retinoic acid receptor beta (*Rarb*) is one of the RA downstream target genes (Sucov et al., 1990). *Rarb* is expressed in the caudal region adjacent to the proximal GT and the mesenchyme beneath the ectodermal epithelium in the lateral side of the GT at E11.5 and E12.5 (Fig. 1I, I′, J, and J′). Such expression gradually shifts to the UE and the prospective corpus cavernosum penis of the GT at E13.5 and E14.5 (Fig. 1K, K′, L, and L′).

Abnormal Cell Proliferation and Differentiation in the GTs of *Cyp26b1*$^{-/-}$ Mutants

To understand the role of RA signaling during GT development, we analyzed the phenotypes of *Cyp26b1*$^{-/-}$ mutants during GT development. The mutants display hyperplastic GTs in both male and female embryos at E18.5 (Fig. 2A and B). The development of several GT accessory structures is impaired in *Cyp26b1*$^{-/-}$ mutants, including the hair follicles under the preputial genital skin and the preputial glands (Fig. 2C and D). During normal development, condensed mesenchyme in the proximal GT develops at E18.5 (Fig. 2C). Such condensed mesenchyme corresponds to the corpus cavernosum penis in adult mice (Murakami, 1987). *Cyp26b1*$^{-/-}$ mutants fail to develop such a prospective cavernous body (Fig. 2D). Ectopic blood vessels were observed surrounding the UE (Fig. 2C′ and D′). Epithelia isolating the glans and prepuce in the control GTs are absent in the mutants (Fig. 2C, C′, D, and D′).

In order to analyze the cause and onset of late embryonic stage phenotypes, we analyzed the phenotypes of *Cyp26b1*$^{-/-}$ mutants at earlier embryonic stages. Laminin is an essential component of basal membrane for blood vessel (Laurie et al., 1984). Blood vessel structures, indicated by laminin immunostaining, developed ectopically in the dorsal and bilateral mesenchyme of the UE at E14.5 in the mutants (Fig. 2E and F). Regarding the hyperplastic phenotype of the GTs at E18.5, BrdU incorporation was performed to examine cell proliferation (Fig. 2G and H). The rate of cell proliferation in the bilateral mesenchyme of the UE is not different between the mutants and control littermates at E13.5, but significantly higher in the mutants at E14.5 (Fig. 2I). Cell death was also examined by Terminal deoxynucleotidyl transferase dUTP nick-end labeling (Tunel) staining. There is an increased rate of apoptosis in the distal glans of the GTs at E14.5. A thinner and sharpened shape of the distal glans of the GTs was observed at E16.5, but the proximal–distal lengths of the GTs are rather similar between the mutants and controls (data not shown).

All together, *Cyp26b1*$^{-/-}$ mutants display abnormal degrees of cell proliferation and differentiation processes during the embryonic development of the GT.

Altered Expression of Homeobox Genes and Growth Factors in the GTs of *Cyp26b1*$^{-/-}$ Mutants

Cyp26b1 is one of the most important enzymes that catabolizes RA during development. Inactivation of such enzyme causes excessive RA signaling in affected organs, such as the limbs (Yashiro et al., 2004). Consistently, there is ectopic RA signaling in the GTs of the mutants, indicated by upregulated *Rarb* expression in the dorsal and bilateral mesenchyme of the UE as well as in the mesenchyme of the prospective corpus cavernosum penis in the mutants (Fig. 3A and B).

The GT is one of the appendages from the trunk of the body, like the case of the limb. The developmental programs of the GT show some shared aspects with those of the limb (Dollé et al., 1991; Yamada et al., 2003). Both *Hoxa13* and *Hoxd13* show decreased expression in the limbs of *Cyp26b1*$^{-/-}$ mutants (Yashiro et al., 2004). Human patients possessing a *Hoxa13*mutation and the corresponding mouse mutants display appendicular phenotypes, including the limbs and the GTs (Kondo et al., 1997; Morgan et al., 2003; Tüzel et al., 2007). Thus, we investigated expression patterns of such homeobox genes during GT development. *Hoxa13* is expressed in the UE and its surrounding mesenchyme. *Hoxd13* is also expressed in the UE and in a broader part of the mesenchyme surrounding the UE compared with *Hoxa13*. Both *Hoxa13* and *Hoxd13* show upregulated expression in GT mesenchyme at E14.5 (Fig. 3C–F). *Meis2* is another homeobox gene that shows distally expanded expression patterns in the limb mesenchyme of *Cyp26b1*$^{-/-}$ mutants (Yashiro et al., 2004). Its expression is detected in ectodermal epithelium and the ventral bilateral mesenchyme of the UE in the proximal GT at E14.5 in control embryos (Fig. 3G). Such mesenchymal expression of *Meis2* in the corresponding region is prominently decreased in *Cyp26b1*$^{-/-}$ mutants (Fig. 3H).

Cyp26b1$^{-/-}$ mutants show multiple defects in both cell differentiation and proliferation in the GT. We further examined the possibility that certain growth factor activities may be altered under excessive RA signaling in the mutants. *Shh* signaling plays pivotal roles in both the initial outgrowth of the GT and its patterning (Haraguchi et al., 2001; Seifert et al., 2009a). *Patched1* (*Ptc1*) is expressed downstream of *Shh* signaling, suggesting its activity. *Ptc1* expression in the bilateral mesenchyme of the UE is slightly upregulated in *Cyp26b1*$^{-/-}$ mutants at E13.5 (data not shown). The upregulated expression of *Ptc1* in the mutant becomes more prominent at E14.5 (Fig. 3M and N). Such enhanced *Shh* signaling is supported by an increased *Shh* gene expression at both the mRNA and protein levels (Fig. 3I–L). *Bmp* signaling plays important roles in the growth and differentiation of the GT (Suzuki et al., 2003; Wu et al., 2009). *Bmp4*, a *Bmp* signaling ligand, is normally expressed in the bilateral mesenchyme of the UE. In *Cyp26b1*$^{-/-}$ mutants, the expression of *Bmp4* is upregulated in the bilateral mesenchyme at E14.5 (Fig. 3O and P). Phosphorylated Smad1/5/8, downstream of Bmp signaling transcriptional factors, are detected more prominently in the ventral region of mutant GTs at E14.5 (Fig. 3Q and R). Thus, expression of the *Shh* and *Bmp* signaling factors are increased in the *Cyp26b1*$^{-/-}$ mutants.

Prompt Alterations of *Bmp4* and *Shh* Expression are Induced by RA Administration

Expression of several factors involved in *Shh* and *Bmp* signaling are increased in the ventral GT region in *Cyp26b1*$^{-/-}$ mutants. To examine whether such changes in gene expression patterns are induced by excessive RA signaling and how early such alterations can be detected, we performed in vivo RA administration and examined the effects. We investigated the kinetics of gene expression changes at various times after RA administration. Expression levels of *Rarb* as well as of *Shh* and *Bmp4* are increased 2 hr after RA administration (Fig. 4A, B, F, G, P, and Q). The increased expression level of *Rarb* peaks 4 hr (Fig. 4A–E), *Shh* level peaks 6 hr (Fig. 4F–J), and *Bmp4* level peaks 2 hr (Fig. 4P–T) after RA administration. *Ptc1* expression in the bilateral mesenchyme of the UE is unaltered 4 hr after RA administration, but is upregulated 6 hr after RA administration (Fig. 4K–O). *Cyp26b1* expression is upregulated prominently in the GT mesenchyme and ectodermal epithelium 6 hr after RA administration (Fig. 4U and V). *Meis2* expression in the ventral bilateral mesenchyme of the UE is prominently reduced 6 hr after RA treatment (Fig. 4W and X). The consequences of gain-of-function experiments in RA signaling done by RA administration and achieved in *Cyp26b1*$^{-/-}$ mutants indicate similar effects of downstream gene expression.

Impaired RA Signaling Reduces *Shh* and *Bmp4* Expression in the GT

Multiple phenotypes in the GTs of *Cyp26b1*$^{-/-}$ mutants indicate that excessive RA signaling leads to dysregulation of GT development. To examine whether the upregulated expression of *Shh* and *Bmp4* in *Cyp26b1*$^{-/-}$ mutants is derived by consequences of abnormal RA signaling, loss-of-function mutants were examined to identify the physiological function of RA signaling. By employing the Creloxp recombination system, we conditionally activated the expression of the dominant-negative RA receptor (RARaDN^{Flox}) (Rosselot et al., 2010) to obtain loss-of-function RA signaling phenotypes. The Isl1^{MerCreMer} allele shows induced Cre recombinase expression in the UE (Fig. 5A). Expression level of *Rarb* in the UE decreases in Isl1^{MerCreMer}: RARaDN mutants compared with controls (Fig. 5B and C). *Shh* expression in the UE is also reduced (Fig. 5D and E), consistent with the decreased expression level of *Ptc1* in the bilateral mesenchyme of the UE (Fig. 5F and G).

The Gli1^{CreERT2} allele has been utilized to modulate the mesenchymal expression of target genes in the GT (Miyagawa et al., 2009). By employing the Gli1^{CreERT2} driver strain, induction of mesenchymal expression of RARaDN was achieved. As a result, *Rarb* expression in the GT mesenchyme is reduced (Fig. 5H and I). *Bmp4* expression in the bilateral mesenchyme of the UE is also reduced in contrast to *Ptc1* expression, which is unaltered (Fig. 5J–M).

Altogether, experiments on both excessive and reduced RA signaling indicate its positive regulation on *Shh* and *Bmp4* expression in the GT.

DISCUSSION

RA signaling for organogenesis has been analyzed for decades. It is known to regulate various organogenesis processes. Administration of RA to pregnant animals induces

multiple abnormalities, including abnormalities in GT development (Abu-Abed et al., 2001; Ogino et al., 2001; Lee et al., 2010). However, functional analysis of RA signaling has not been performed during GT development due to early lethality and severe caudal disorganization in RA signaling mutants, including *Cyp26a1*$^{-/-}$ and *Raldh2*$^{-/-}$ (Niederreither et al., 1999; Abu-Abed et al., 2001; Ribes et al., 2009). Comprehensive analysis of RA actions during GT development is therefore required.

In this study, we revealed dynamic changes in the expression patterns of genes in the RA signaling pathway from E12.5 to E14.5, after the initiation of GT outgrowth. *Raldh2* switches its expression to the UE. *Cyp26b1* shows gradually restricted expression in the mesenchyme on the dorsal-bilateral side of the UE. Following such expression pattern changes in RA-metabolizing enzymes, *Rarb* starts to be expressed in the UE. Regarding such emerging expression profiles of RA signaling factors in the UE and surrounding tissues, RA signaling might be involved in the formation of the UE, which regulates the development of the GT.

Abnormal RA Signaling Leads to Multiple Defects During GT Development in *Cyp26b1*$^{-/-}$ Mutants

The distal UE (DUE) was originally demonstrated to be essential for GT outgrowth (Haraguchi et al., 2000). Conditional knockout of several Fgf family genes suggested a redundancy of Fgf genes. Such observation raised a question about the significance of DUE for GT outgrowth (Lin et al., 2009; Miyagawa et al., 2009; Seifert et al., 2009b). *Fgf8* is expressed in the DUE in control embryos and was suggested to be a marker for the DUE (Haraguchi et al., 2000, 2001; Seifert et al., 2009b). It is similarly expressed in *Cyp26b1*$^{-/-}$ mutants at E10.5, indicating that the initiation process of GT outgrowth is unaffected in the mutants (data not shown). Both the limb and GT are developing appendages from the body trunk. Changes in the endogenous distribution of RA following genetic ablation of *Cyp26b1* result in truncated limb formation, accompanied by increased cell death and delayed chondrocyte maturation (Yashiro et al., 2004). Slightly increased cell apoptosis was also observed in *Cyp26b1*$^{-/-}$ mutants in the distal GT at E14.5, which might contribute to a thinner and sharpened shape of the distal glans of the GT observed at E16.5 (data not shown). However, the proximal–distal length of the GT in the mutants was not affected as observed at E16.5 (data not shown). Despite increased apoptosis in the distal glans, the proximal GT shows a prominent hyperplastic phenotype at E18.5. Such hyperplasia is consistent with the increased cell proliferation detected in the bilateral mesenchyme of the UE at E14.5. Cell proliferation was significantly reduced with conditional ablation of *Shh* expression in the UE (Seifert et al., 2010) (data not shown), suggesting that *Shh* may positively regulate cell proliferation. Increased expression level of *Shh* in the *Cyp26b1*$^{-/-}$ mutant might contribute to the hyperplastic phenotype of the GT.

There are defects in differentiation in various regions of the GT in the *Cyp26b1*$^{-/-}$ mutant, including the formation of ectopic blood vessels on the dorsal and lateral sides of the UE. *Hoxa13* was shown to regulate vascularization in the development of the glans penis (Morgan et al., 2003; Shaut et al., 2007). Its expression is increased in the mesenchymal cells surrounding the UE of *Cyp26b1*$^{-/-}$ mutants, which might be involved in abnormal

blood vessel formation in mutant GTs. Ablation of the cytochrome P450 oxidoreductase gene, which is an electron donor for all cytochrome P450 enzymes including *Cyp26*s, leads to defects in vasculogenesis (Otto et al., 2003). Such a phenotype is induced by the increased level of RA in the mutants (Ribes et al., 2007). Considering the ectopic blood vessel formation observed in the GT with excessive RA signaling in *Cyp26b1*$^{-/-}$ mutants, vasculogenesis might be a susceptible developmental process affected by excessive RA signaling.

Several differentiation defects other than ectopic blood vessel formation were observed in the GTs of *Cyp26b1*$^{-/-}$ mutants. Such abnormalities include impaired condensation of the prospective cavernosum penis, failure of the formation of hair follicles and preputial glands, and agenesis of the epithelia that separate the glans mesenchyme from the preputial tissues. In fact, expression of *Bmp* and *Shh*, as well as several homeobox genes, were altered in mutant GTs. Excessive RA signaling in *Cyp26b1*$^{-/-}$ mutants exerts teratogenic effects on the differentiation and growth processes of the GT.

Alterations in GT gene expression are quite different from those in the limbs of *Cyp26b1*$^{-/-}$ mutants. Expression of both *Hoxa13*and *Hoxd13* is upregulated in the proximal GT, whereas their levels are reduced in the distal limbs of the mutants. *Meis2* is another homeobox gene used as a proximal marker of limb development. Its expression is expanded to the distal limbs of the mutant, however, reduced in the ventral bilateral mesenchyme of the UE in mutant GTs. RA affects the expression of the same genes in different ways depending on the developmental context.

Shh and *Bmp4* are Possible Target Genes of RA Signaling During GT Development

Excessive RA signaling in *Cyp26b1*$^{-/-}$ mutants upregulates Shh and Bmp signaling factors in mutant GTs. Administration of teratogenic doses of RA confirmed a prompt increase in *Shh* and *Bmp4* expression in the GTs. *Rarb* expression is regulated by RA signaling via the binding of the heterodimeric RA receptor to RA (Sucov et al., 1990), and maternal administration of teratogenic doses of RA induces prominent upregulation of *Rarb* expression in E11.5 limbs within 3 hr, reaching a peak of expression after 6 hr (Harnish et al., 1992). Increased expression levels of *Rarb* are prominent 2 hr after RA treatment in the GT at E14.5, reaching a peak in the GT 4 hr after RA administration. Notably, expression levels of *Shh* and *Bmp4* are also increased 2 hr after RA administration. The responding timing of these genes after RA treatment is comparable with that of *Rarb*, the direct target gene of RA signaling. This report is the first showing that *Shh* and *Bmp* genes can be immediate targets of RA signaling during GT formation. Thus, alterations in *Shh* and *Bmp4* expression would be primary molecular events inducing growth and differentiation abnormalities of the GT in *Cyp26b1*$^{-/-}$ mutants.

Shh is expressed in the UE, and *Bmp4* is expressed in the bilateral mesenchyme of the UE. Downregulation of *Shh* was achieved by conditionally inactivating RA signaling in the UE; similarly, downregulation of *Bmp4* was achieved by conditionally inactivating RA signaling in the GT mesenchyme. The in situ downregulation of *Shh*and *Bmp4* with tissue-specific inactivation of RA signaling suggests that RA signaling may regulate the expression of *Shh* and *Bmp4* within the tissue compartments where they are expressed.

Expression of *Bmp4* was shown to be positively regulated by Shh signaling during GT development (Haraguchi et al., 2001; Seifert et al., 2010). It is possible that upregulated Shh signaling promotes *Bmp4* expression in *Cyp26b1*$^{-/-}$ mutants. However, increased *Bmp4* expression was induced 2 hr after RA treatment when the mesenchymal expression of *Ptc1* was unchanged, indicating that RA regulation of *Bmp4* expression in the mesenchyme may not be mediated through *Shh* signaling in the GT. *Bmp4* expression is reduced with conditional inactivation of RA signaling in the bilateral mesenchyme of the UE in Gli1$^{CreERT2/+}$: RARaDN$^{F/F}$ mutants. In the corresponding areas of such mutants, expression level of *Ptc1* is not different from that of control embryos. Hence, RA signaling presumably regulates *Bmp4* expression independent of *Shh* signaling.

Teratogenic mechanisms induced by abnormal RA signaling are suggested by experiments adapting RA administration and the use of genetically modified mouse models in this study. Both experimental systems identified potential downstream target genes of *Shh* and *Bmp4* during GT formation. It is known that insufficient maternal supplies of Vitamin A or dysregulation of RA in genetically deficient patients induce various developmental abnormalities, including those of the genitourinary system. This study offers important information on the downstream targets of RA signaling and may provide insights for further studies investigating RA-induced teratogenicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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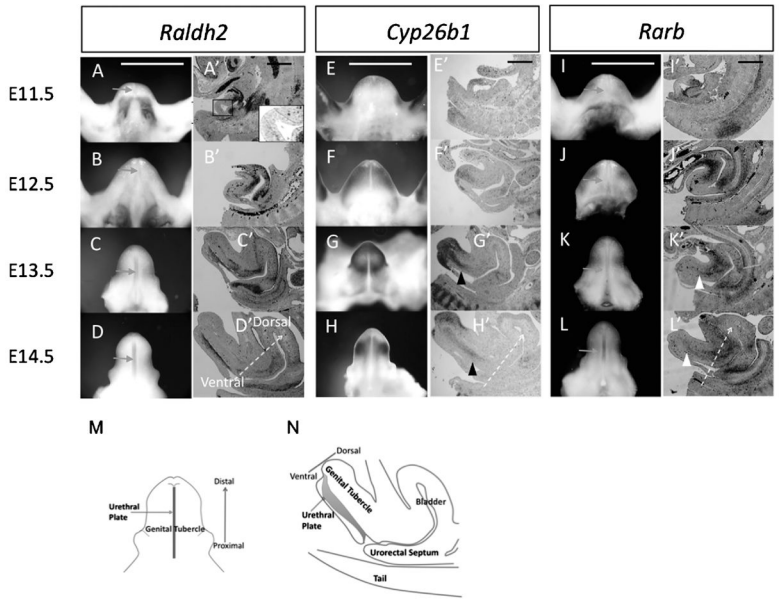

Fig. 1.

RA signaling activities are detected during GT development. **A**–**L** are ventral views of GT; **A′**–**L′** are midline sagittal sections of the caudal urogenital region. A–D and A′–D′: *Raldh2* is expressed in the caudal ventral region adjacent to the GT at E10.5, but not in the urethral plate epithelium (UE, enlarged view in A′) (A and A′); its expression begins in the UE from E12.5 (B and B′), becoming stronger in the UE at E13.5 and E14.5 (C, C′, D and D′). Arrows in A–D indicate the UE. The areas in between the dashed lines in A′–D′ indicate the UE. E–H and E′–H′: *Cyp26b1* is expressed in the mesenchyme adjacent to the distal ectodermal epithelium of the GT at E11.5 (E and E′), becomes broadly expressed in the mesenchyme at E12.5 (F and F′); and its expression becomes restricted to the mesenchyme dorsal of the UE in the proximal region and in the distal mesenchyme of the GT at E13.5 and E14.5 (G, G′, H, and H′). Black arrowheads indicate *Cyp26b1* expression in the proximal GT. I–L and I′–L′: *Rarb* is expressed in the caudal region adjacent to the proximal GT at E11.5 and E12.5 (I, I′, J, and J′). Its expression begins in the UE and mesenchyme of the prospective corpus cavernosum penis in the proximal GT areas at E13.5 to E14.5 (K, K′, L, and L′). White arrowheads indicate *Rarb* expression in the prospective corpus cavernosum penis of the GT. Scale bar, 400 μm. Arrows with dashed stems in D′, H′, and L′ indicate the ventral–dorsal direction of the GT. M: schematic picture for ventral view of E14.5 GT. N: schematic picture for midline sagittal view of E14.5 GT. GT, genital tubercle; RA, retinoic acid.

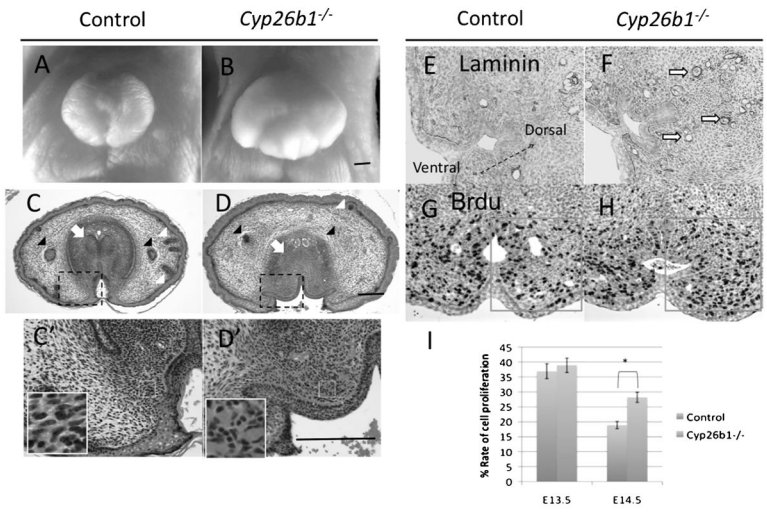

Fig. 2.

Abnormal cell proliferation and differentiation in the GTs of *Cyp26b1*$^{-/-}$ mutants. **A** and **B**: Frontal view of an E18.5 female GT in a control embryo and *Cyp26b1*$^{-/-}$ mutant. The size of the GT in the mutant is enlarged compared with that of the control. **C**, **C′**, **D**, and **D′**: Transverse section of the proximal GT in an E18.5 female; C′ and D′ are enlarged views of the framed area (dashed black line) in C and D. The development of the preputial gland (black arrowhead) and hair follicles (white arrowheads) under the genital skin is impaired in the *Cyp26b1*$^{-/-}$ mutants; mesenchymal condensation of the prospective corpus cavernosum penis is abnormal in the mutants (white arrow); ectopic blood vessels (area indicated by white frame) develop in the bilateral and dorsal mesenchyme of the UE in the mutants (C′ and D′); epithelium (black arrow) between the glans and prepuce is absent in the mutant (C, C′, D, and D′). **E** and **F**: Blood vessels indicated by Laminin immunostaining developed ectopically (hollow arrow) in the dorsal and bilateral mesenchyme of the UE at E14.5 in the mutant. To show the images of the mesenchyme on the dorsal side of the UE, the picture is displayed with an angled orientation, indicated by arrows. **G** and **H**: BrdU immunostaining of an E14.5 GT section. **I**: Cell proliferation assay. Framed area in G and H was counted, and the ratio of BrdU-positive cells was compared between the control and the mutant at E13.5 ($n = 6$) and E14.5 ($n = 6$). The asterisk (*) indicates significant statistical differences. All images of the cross-section are placed ventral side down unless otherwise indicated. Arrow with dashed stem in E indicates the ventral–dorsal direction of the GT. Scale bar, 200 μm. GT, genital tubercle; UE, urethral plate epithelium; BrdU, 5-BromodeoxyUridine.

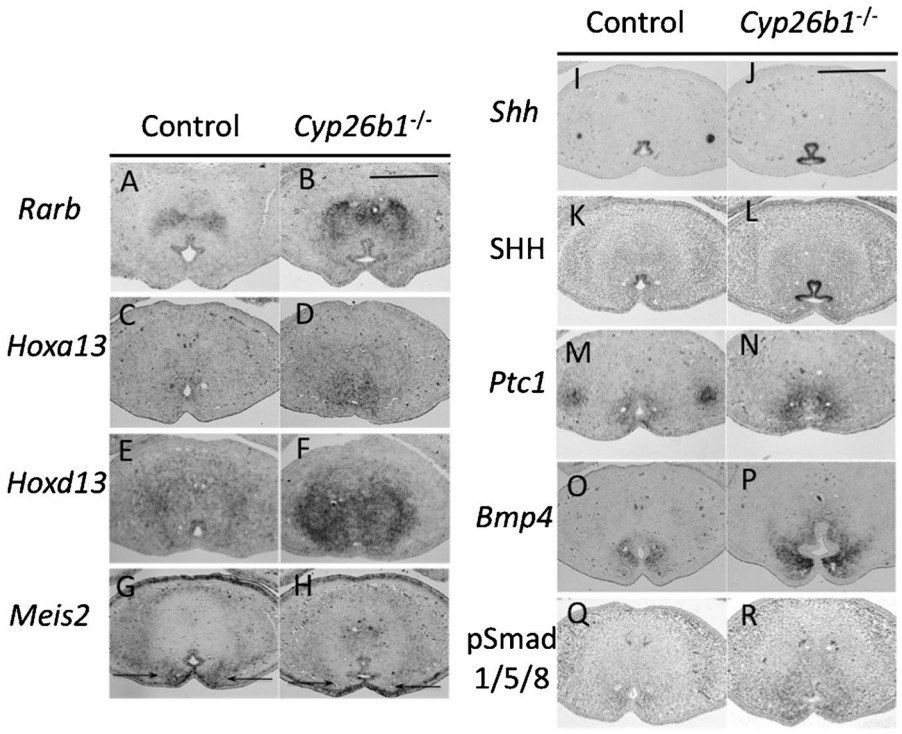

Fig. 3.

Altered expression of the homeobox genes, Shh and Bmp signaling factors in the GTs of *Cyp26b1*$^{-/-}$ mutants. **A** and **B**: *Rarb* expression is upregulated in the dorsal and bilateral mesenchyme of the UE as well as in the prospective corpus cavernosum penis in *Cyp26b1*$^{-/-}$ mutants. **C** and **D**: Expression level of *Hoxa13* is upregulated in the mesenchyme around the urethra in the *Cyp26b1*$^{-/-}$ mutants compared with control. **E** and **F**: Expression level of *Hoxd13* is increased in the mesenchyme of the GT. **G** and **H**:*Meis2* expression level is decreased in the ventral bilateral mesenchyme of the UE (indicated by black arrows) in *Cyp26b1*$^{-/-}$ mutants. **I**–**L**: *Shh* expression level in the UE is increased in the mutant, both in mRNA (I and J) and in protein (K and L) levels. **M** and **N**: *Patched1* (*Ptc1*) expression is upregulated in the bilateral mesenchyme of the UE in the mutant. **O** and **P**: *Bmp4* expression is upregulated in the bilateral mesenchyme of the UE in the mutant. **Q** and **R**: pSmad1/5/8 expression is upregulated in the ventral region of mutant GTs. All images of the GT section are placed ventral side down. Scale bar, 400 μm. UE, urethral plate epithelium; GT, genital tubercle; *Shh*, *Sonic hedgehog; Bmp*, *bone morphogenetic protein*.

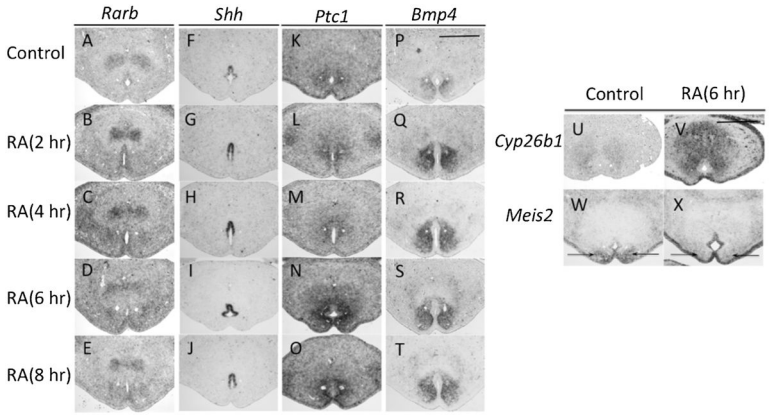

Fig. 4.

Prompt alterations of expression for *Shh* and *Bmp4* induced by RA administration. **A**–**E**: *Rarb* expression level is upregulated 2 hr after RA administration (A and B). Such upregulated expression continued for 4 hr (C), and decreased 6 hr (D and E) after RA treatment. **F**–**I**: *Shh* expression level is increased 2 hr (F and G) after RA treatment, sustained at high levels for 6 hr (H and I), and slightly decreased 8 hr after RA treatment (**J**). **K**–**O**: *Ptc1* expression is unaltered within 4 hr of RA treatment (K–M), then prominently increased 6 and 8 hr after RA treatment (N and O). **P**–**T**: *Bmp4* expression is upregulated 2 hr after RA treatment, and sustained with increased level until 8 hr after RA treatment. **U** and **V**: *Cyp26b1* expression is upregulated in the GT mesenchyme and ectoderm epithelium 6 hr after RA administration. **W** and **X**: *Meis2* expression in the ventral bilateral mesenchyme of the UE (indicated by black arrows) is reduced 6 hr after RA treatment. All images are placed ventral side down. Scale bar, 400 μm. GT, genital tubercle; RA, retinoic acid; UE, urethral plate epithelium.

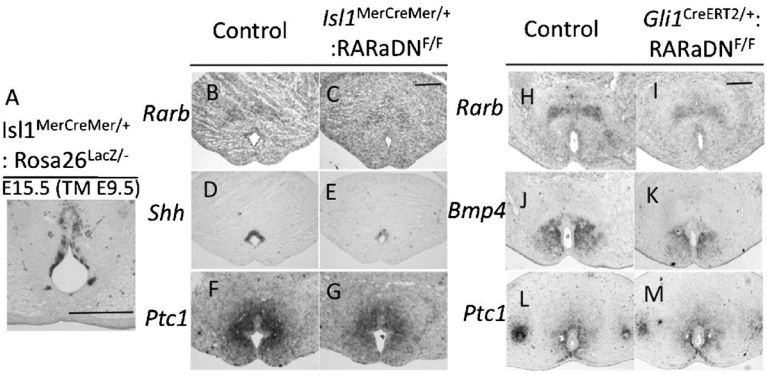

Fig. 5.

Impaired RA signaling reduces *Shh* and *Bmp4* expression levels in the GT. **A**: The Isl1^{MerCreMer} mouse line shows Tamoxifen (TM)-induced Cre expression in the UE. **B** and **C**: *Rarb* expression in the UE is reduced in the Isl1$^{MerCreMer/+}$:RARaDN$^{F/F}$ mutant. **D** and **E**: *Shh* expression in the UE is reduced in the Isl1$^{MerCreMer/+}$:RARaDN$^{F/F}$ mutant. **F** and **G**: *Ptc1* expression in the bilateral mesenchyme of the UE is reduced in the Isl1$^{MerCreMer/+}$:RARaDN$^{F/F}$ mutant. **H** and **I**: *Rarb* expression in the GT mesenchyme is reduced in the Gli1$^{CreERT2/+}$:RARaDN$^{F/F}$ mutant. **J** and **K**: *Bmp4* expression in bilateral mesenchyme of the UE is reduced in the Gli1$^{CreERT2/+}$: RARaDN$^{F/F}$ mutant. **L** and **M**: *Ptc1* expression in the bilateral mesenchyme of the UE is unaltered in the Gli1$^{CreERT2/+}$: RARaDN$^{F/F}$ mutant. All images of the cross-section are placed ventral side down. Scale bar, 200 μm. GT, genital tubercle; RA, retinoic acid; UE, urethral plate epithelium; *Bmp*, *bone morphogenetic protein*.