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The Role of Sonic Hedgehog-Gli2 Pathway in the Masculinization of External Genitalia

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During embryogenesis, sexually dimorphic organogenesis is achieved by hormones produced in the gonad. The external genitalia develop from a single primordium, the genital tubercle, and their masculinization processes depend on the androgen signaling. In addition to such hormonal signaling, the involvement of nongonadal and locally produced masculinization factors has been unclear. To elucidate the mechanisms of the sexually dimorphic development of the external genitalia, series of conditional mutant mouse analyses were performed using several mutant alleles, particularly focusing on the role of hedgehog signaling pathway in this manuscript. We demonstrate that hedgehog pathway is indispensable for the establishment of male external genitalia characteristics. Sonic hedgehog is expressed in the urethral plate epithelium, and its signal is mediated through glioblastoma 2 (Gli2) in the mesenchyme. The expression level of the sexually dimorphic genes is decreased in the glioblastoma 2 mutant embryos, suggesting that hedgehog signal is likely to facilitate the masculinization processes by affecting the androgen responsiveness. In addition, a conditional mutation of Sonic hedgehog at the sexual differentiation stage leads to abnormal male external genitalia development. The current study identified hedgehog signaling pathway as a key factor not only for initial development but also for sexually dimorphic development of the external genitalia in coordination with androgen signaling. (***Endocrinology*** **152: 2894–2903, 2011)**

Differentiated male and female external genitalia enable highly efficient internal fertilization. External genitalia exhibit substantial sexual dimorphisms in many animal species. In mice, anatomical sexual dimorphisms of the genital tubercle (GT) (embryonic external genitalia) are first visible at embryonic day (E)16.5 (1). The urethral fold and the prepuce develop more prominently in male than that of female GT. As a result, male urethra forms a tubular structure within the glans. In addition, the prospective corporal body is bilaterally segmented in the male GT mesenchyme. The male-specific GT differentiation is established by androgen secreted from the testis. In contrast, female external genitalia display few of such characteristic processes due to a lack of androgen stimulation. Among reproductive organ formation, male and female external genitalia arise from the common anlage and differentiate by the respective hormone environment from midembryogenesis in mice. Although significant progress of our understanding for its developmental processes has been achieved by genetic analyses on mice, molecular mechanisms of sexually dimorphic development of the GT are not yet known.

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Abbreviations: Dhh, Desert hedgehog; Dkk, Dickkopf; E, embryonic day; Fkbp5, Fk506 binding protein; Gli, glioblastoma; GT, genital tubercle; KO, knockout; Ptc, Patched; Sfrp, secreted frizzled-related protein; Shh, Sonic hedgehog; Smo, Smoothened; TM, tamoxifen; TP, testosterone propionate; UPE, urethral plate epithelium; Wg, wingless; Wnt, Int and Wg in Drosophila.

During the past few decades, disorders of sexual development are among the most common human birth defects. Hypospadias, in which the urethral meatus is located at the ventral side of the penis, has been described as a malformation with a high prevalence as human birth defects (2, 3). In most cases, the etiology of such disorders is still obscure. Thus, a better understanding of the developmental mechanisms of GT will shed light on the causative mechanisms of genital malformations.

Initially, both male and female GT are morphologically identical. The GT as a common anlage develops from the cloacal region starting at E10.5 (1). The after outgrowth is the consequence of mesenchymal swelling around the cloaca. The outgrowing GT is accompanied with the formation of urethral plate epithelium (UPE), the future urethral epithelium, in the ventral (lower) side. These developmental events are completely androgen independent. During these processes, Sonic hedgehog (Shh) expressed in the endodermal epithelium plays an essential role in the regulation of epithelial-mesenchymal interactions (4–6). Shh induces the expression of various genes in the bilateral mesenchyme to the urethra and triggers signal cascade for initial GT outgrowth. Accordingly, *Shh* knockout (KO) mice display a complete GT agenesis (4, 6).

The hedgehog signal is mediated through its membrane receptor complex consisting of Patched (Ptc) and Smoothened (Smo). In the absence of hedgehog ligands, Ptc blocks Smo activity, and glioblastoma (Gli) transcriptional factor is inactivated by proteolytic processing. Hedgehog binding to Ptc unleashes Smo activity, which promotes transcriptional activation of the Gli. In vertebrates, three distinct Gli family proteins, Gli1, Gli2 and Gli3, are involved in the transcription response to the hedgehog signaling (7, 8). Mutant mice analyses have revealed that Gli1 and Gli2 function primarily as activators, whereas Gli3 acts mainly as a repressor, although some overlapping roles among Gli proteins are also reported (9–12). Loss of *Gli2* or *Gli3* is embryonic lethal, whereas *Gli1* is dispensable for normal development. Gli1 itself is a target of hedgehog signal; hence, it appears to participate in a positive-feedback loop in the hedgehog pathway. It is widely accepted the hedgehog pathway controls multiple developmental processes. However, the contribution of Shh and each Gli family protein for the GT sexual development remains unclear.

Reciprocal interactions between epithelium and mesenchyme in the urogenital organs have been shown by tissue graft experiments. For instance, mesenchymal androgen receptor is necessary for the androgen-dependent epithelial cell differentiation of the prostate (13, 14). In contrast, the epithelium is required for differentiation and spatial organization of the smooth muscle (15, 16). In addition, the inductive effect of the epithelium on mesenchymal growth and differentiation has been demonstrated for GT development (17, 18), although such epithelium-derived factor is still not identified. Despite the previous description of hormonal (androgen) control of the sexual development (19), the involvement of nongonadal and locally produced masculine factors is not yet known. In this article, we demonstrated that hedgehog pathway is indispensable for the masculinization processes of male external genitalia. We showed that Shh expressed in the UPE regulates GT mesenchymal differentiation through Gli2. Genetic disruption of hedgehog signal led to a hypospadias-like phenotype. Intriguingly, conditional gene inactivation of *Shh* at the sexual differentiation stage induced a female-like structure of the male GT. These results reveal newly identified functions of Shh-Gli signaling for sexually dimorphic development during embryogenesis.

Materials and Methods

Mouse

The mutant alleles used herein were *Gli1* (12), *Gli2* (10), *Gli3* (20), *Shh* (21), Shh^{lox} (22), *CAGGS-CreERTM* (23), *Del5-LacZ* (24), and *R26-SmoM2* (25). All experimental procedures and protocols were approved by the Committee on the Animal Research at Kumamoto University. Embryos for each experiment were collected from more than three independent pregnant females. Noon on the day when a vaginal plug was detected was designated as E0.5.

The tamoxifen (TM)-inducible Cre recombinase system removes the floxed sequence of the target genome (26). TM (Sigma, St. Louis, MO) was dissolved in sesame oil (Kanto Chemical, Tokyo, Japan) at a final concentration of 10 mg/ml. Two milligrams of TM per 40 g body weight was administrated (ip) to the pregnant mice. Under these conditions, no overt teratologic effects nor sexual disorders of the reproductive organs were observed (24).

For androgen treatment, pregnant mice were administrated (ip) a daily single injection of 100 mg/kg body weight testosterone propionate (TP) (Wako, Osaka, Japan) for 5 d starting from E13.5 and killed 24 h later after the last injection (E18.5).

The concentration of testosterone in the testes of the mice at E18.5 was measured by Clinical Pathology Laboratory, Inc. (Kagoshima, Japan) using a chemiluminescent immunoassay (27). More than seven embryos per group were collected, and the average relative testosterone contents were evaluated for further analysis. Error bars represent the SE. A statistical analysis was performed using Student's *t* test or Welch's *t* test followed by F-test; differences with $P < 0.05$ were considered to be significant.

Histology, LacZ staining, immunohistochemistry, and *in situ* hybridization for gene expression analysis

Hematoxylin and eosin staining and LacZ staining were performed by standard procedures as previously described (24). For immunohistochemistry, paraformaldehyde-fixed, paraffin-em-

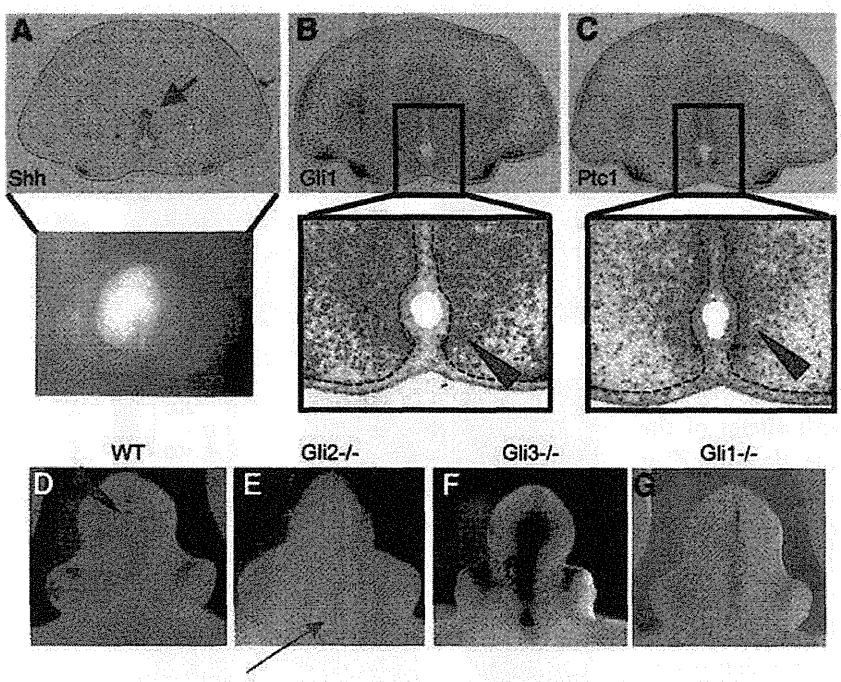

FIG. 1. Expression pattern of hedgehog signaling genes in the male GT at E15.5. A, *Shh* is expressed in the UPE (*arrow* in the cross-section). B and C, *Gli1* (B) and *Ptc1* (C), representative hedgehog-responsive genes, are expressed in the mesenchyme adjacent to the UPE (*arrowheads* in the highly magnified pictures). *Dotted lines* indicate the boundary between the epithelium and mesenchyme of the developing GT. D–G, The LacZ staining pattern of the *del5-LacZ* line with several *Gli* mutant allelic backgrounds at E15.5. In the wild-type mouse embryos (WT), the LacZ signals are detected in the mesenchymal region adjacent to the UPE (D, *arrowhead*). An *asterisk* indicates the preputial gland. The *Gli2* KO embryos exhibit a hypoplasic GT and groove-like structure in the ventral GT (*arrow*) without LacZ signals derived from the *del5-LacZ* allele (E). The ablation of *Gli3* leads to enhanced LacZ signals, although lacking overt morphological GT defects (F). The *Gli1* mutant GT does not show apparent GT phenotypes, and the LacZ signal is detected almost unaltered (G). No sexually dimorphic expression pattern is observed, and the representative images from male embryos are shown.

bedded sections were treated for antigen retrieval (microwave treatment 10 min in citrate buffer; pH 6.0). The sections were incubated at a 1:100 dilution of anti-p450Scc antibody (American Research Products, Belmont, MA). Immunofluorescence analysis was performed with Alexa Fluor 546 antirabbit IgG (Invitrogen, Carlsbad, CA) and counterstained with Hoechst 33342 (Sigma).

For *in situ* hybridization, the sections were deparaffinized, rehydrated, incubated in 1 μg/ml proteinase K for 7 min at 37 C, and refixed with 4% paraformaldehyde for 10 min at room temperature. After washing in PBS containing 0.1% Tween 20, overnight hybridization was performed in a buffer (50% formamide, 5× saline sodium citrate, 50 μg/ml yeast tRNA, 1% sodium dodecyl sulfate, and 50 μg/ml heparin) with 1 μg/ml probe at 68 C. After washing with 5× saline sodium citrate and 50% formamide for 1 h at 68 C, the slides were incubated in blocking solution [10% blocking reagent (Roche, Mannheim, Germany) in 100 mM maleate buffer and Tris-buffered saline with Tween 20 (140 mM NaCl, 2.7 mM KCl, 0.1% Tween 20, 25 mM Tris-HCl; pH 7.5)] for 2 h. Antidigoxigenin antibody (Roche) in a blocking solution was added to the slides and incubated for 1 h. After washing with Tris-buffered saline with Tween 20, the sections were equilibrated in NTMT buffer [100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween 20 and 100 mM Tris-HCl (pH 9.5)], including 2 mM levamisole (Sigma) and incubated in color solution [3.5 μg nitroblue tetrazolium chloride (Sigma) and 1.75 μg 5-bromo-4-chloro-3-indolyl-phosphate (Sigma) per milliliter of NTMT buffer]. The templates used in this study were kindly provided from J. Motoyama (*Gli1* and *Ptc1*), C. Shukunami (*Shh*), C. Niehrs (*Dkk2*), and B. Capel (*Scc*). The template of *Sfrp1* was obtained by standard RT-PCR procedures. The primer sequences were: secreted frizzled-related protein (Sfrp)1, TTC TAC ACC AAG CCC CCG CAG, GAT GGG CCC CAG CTT CAA GG. The preparation of the digoxigenin-labeled probes was performed according to the manufacturer's instructions (Roche).

Quantitative RT-PCR analysis

The changes in gene expression were confirmed and quantified using the 7500 real-time PCR system (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Total RNA (1 μg), isolated with ISOGEN (Nippongene, Tokyo, Japan) from the mesenchyme adjacent to the UPE of GT (blue area shown by figure 3A), was used in RT-PCR reactions carried out with SuperScript III (Invitrogen) and SYBR Green master mix (Applied Biosystems). The relative RNA equivalents for each sample were determined by standardization with the ribosomal protein L8 levels. More than three pools of samples per group were tested in triplicate, and the average relative RNA equivalents per sample were analyzed further. Error bars represent SE. The statistical comparisons among the experimental groups were assessed by ANOVA. When F ratios were significant ($P < 0.05$), Scheffé *post hoc* tests between two groups were done, and $P < 0.05$ was considered as statistically significant. The primer sequences were: Dickkopf (Dkk)2, TGT CTG AAG CAC AGG CTG GAT, CTT CTG GAG CCT CTG ATG GC; Sfrp1, AAG GAG AGG CAG AAT CCT TTC A, TTT CCA AAC CGG CCA ACA; and ribosomal protein L8, ACA GAG CCG TTG TTG GTG TTG, CAG CAG TTC CTC TTT GCC TTG T.

Results

Expression pattern of hedgehog pathway genes

The expression pattern of *Shh*, *Gli1*, and *Ptc1* was examined in the GT at E15.5, the critical time points to the onset of androgen-induced sexually dimorphic development of the GT (28). *Shh* mRNA was specifically expressed in the UPE (Fig. 1A, *arrow*). In contrast, *Gli1* and *Ptc1*, representative hedgehog-responsive genes, were expressed in the mesenchyme adjacent to the UPE (Fig. 1, B and C, *arrowheads*), indicating that Shh mediates epithe-

lial-mesenchymal interactions during GT sexual differentiation. Sexually dimorphic expression was not observed among these genes (data not shown). The mesenchymal differentiation has been shown to be tightly associated with GT masculinization (28);, the function of hedgehog signaling pathway was, therefore, investigated.

Gli2 as a main regulatory gene mediating hedgehog signaling during GT development

In vertebrates, three distinct Gli family proteins mediate Shh signal transduction. To determine the role of each Gli transcription factor in the GT development, several *Gli* mutants were analyzed by crossing with alleles of the hedgehog-responsive indicator mouse line, *del5-LacZ*, at E15.5 (24). In the wild-type embryos, the hedgehog responsiveness was detected in the mesenchyme adjacent to the UPE (Fig. 1D; the red arrowhead indicates the *del5-LacZ*-derived mesenchymal signals). The expression pattern derived from the *del5-LacZ* allele was basically similar to the endogenous *Gli1* and *Ptc1* expressing region (data not shown). This LacZ signal was completely lost associated with the GT hypoplasia and cleft at the proximal ventral midline in the *Gli2* mutants (Fig. 1E). The LacZ signal in the *Gli3* mutants driven by the *del5-LacZ* allele was augmented, indicating that Gli3 appears to function as a hedgehog signal repressor (Fig. 1F). However, the ablation of Gli3 does not affect GT development *per se* (Fig. 1F). In the case of *Gli1* mutants, no apparent phenotypes were observed with almost the same level and pattern of the LacZ signal as wild-type GT (Fig. 1G). Hence, these data suggest that Gli2 is a major regulator mediating hedgehog signal during sexually dimorphic development.

Differential GT abnormalities in Gli2 KO male and female embryos

To investigate the Gli2 function for the GT masculinization, *Gli2* mutants were analyzed at E18.5, when sexual dimorphisms is apparent. In the wild-type embryos, morphological sexual dimorphisms are apparent with a well-developed prepuce and tubular urethra in the male GT (Fig. 2A). The more drastic genital defect in the Gli2 mutant male could be due to an open urethral groove-like structure and preputial fusion defects (Fig. 2A; serial sections covering the proximal to the distal GT region all showed such abnormalities). Notably, this male mutant GT phenotype was much more prominent than the degree of the female mutant GT (Fig. 2A). Desert hedgehog (Dhh), the other hedgehog ligand, could affect fetal Leydig cell differentiation, which plays a crucial role in androgen production (29, 30). The more drastic genital defect in the *Gli2* male mutant GT can be due to the abnormal testis

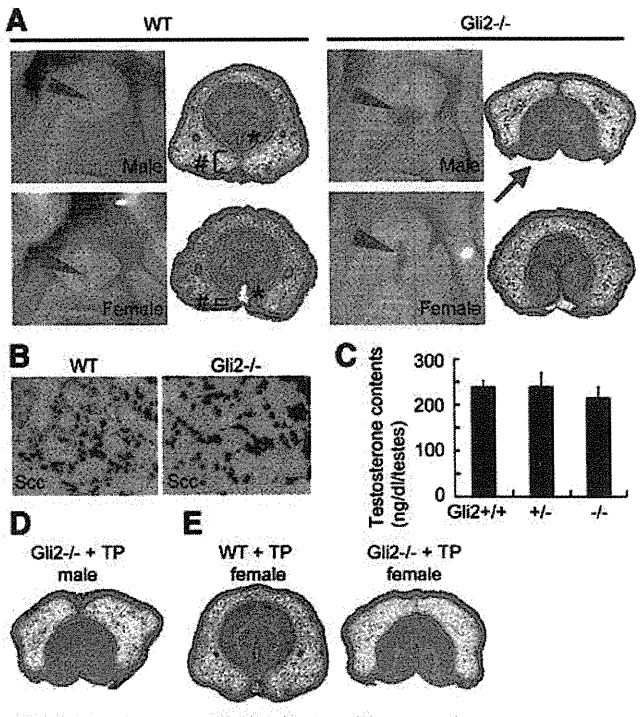

FIG. 2. Requirement of Shh-Gli2 signal for masculine processes. A, Wild-type (WT) male GT at E18.5 shows a well-developed prepuce (#) and a tubular urethra (*). In the *Gli2* KO mutant embryonic GT, the urethra uncovered by epidermal epithelium is shown by methyl *green staining* (shown by *arrowheads*). The male mutant GT exhibits more severe phenotypes than those of the female mutants. The histological section shows that the urethral epithelium is exposed to the outer surface of the *Gli2* KO male embryos (*arrow*). B and C, *Gli2* male mutant GT does not show defects in androgen signaling pathway. The expression of *p450Scc* is not changed in the *Gli2* mutant testis at E16.5 (B). No significant differences of the testosterone content are observed irrespective of *Gli2* genotypes. D and E, Intrauterine androgen treatment experiments for the *Gli2* mutants. TP treatment does not recover the GT phenotype of the *Gli2* male mutants (D). The wild-type female embryonic GT treated with TP is masculinized displaying a similar morphology to the male GT (E, compare with wild-type male and female GT in A), but mutant female GT does not show masculine phenotypes (E).

development and/or androgen production. To exclude such a possibility, the testis of the *Gli2* KO embryos was examined. *p450Scc*, a marker gene for Leydig cell differentiation, was expressed normally (Fig. 2B). In fact, the testosterone content in the testis was not altered among *Gli2*-wild-type, heterozygote and homozygote mouse embryos (Fig. 2C). Furthermore, the expression of *5α-reductase* in the GT and the length of the anogenital distance, a reliable marker for masculinization and fetal androgen exposure, were not altered (data not shown). *Gli2* male mutants treated with TP at E13.5-E17.5 failed to recover the GT defects (Fig. 2D). These results indicate that the more severe phenotype of the *Gli2* male mutant GT is not caused by androgen deficiency.

Wild-type female GT was masculinized when embryos were treated with TP (Fig. 2E, compare with the wild-type

GT in Fig. 2A) (31, 32). However, *Gli2* mutant female GT was not masculinized with the TP treatment (Fig. 2E), also suggesting a requirement of Gli2 for GT masculinization.

Decreased responsiveness for androgen signaling in the Gli2 mutant GT

To examine how hedgehog signal affects GT masculinization, the responsiveness to androgen was analyzed in the *Gli2* mutants. *Mafb* and *Fkbp5*, representative androgen-responsive genes that are expressed in the GT mesenchyme, showed a sexually dimorphic expression (Fig. 3A) (33). Notably, the degree of the differential gene expression between male and female GT was decreased in the *Gli2* mutant embryos, indicating that the responsiveness of androgen was indeed decreased. *Dkk2* and *Sfrp1* have been demonstrated as feminized marker genes in the GT (28). Their expression level was increased in the *Gli2* mutant male than that of wild-type male GT (Fig. 3B), indicating that gene expression status of the *Gli2* male mutant GT shifts toward female-like pattern. On the other hand, androgen did not seem to affect the hedgehog signal responsiveness in the GT, because the *Gli1* expression was not altered in such embryos (Fig. 3A).

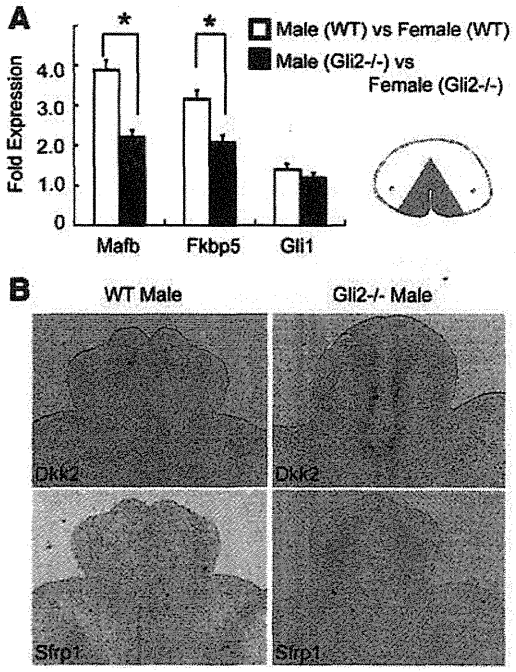

FIG. 3. Impaired masculine characteristics in the male *Gli2* mutant GT. A, The fold expression of *MafB* and *Fkbp5* between male and female GT is decreased in the *Gli2* mutant GT. *Gli1* expression does not show sexually dimorphic expression pattern. RNA was isolated from the mesenchyme adjacent to the UPE (*blue area* in A). The relative RNA equivalents for each sample were determined by standardization with ribosomal protein L8 levels. Statistical significance is indicated by *asterisks*, with $P < 0.05$. B, The expression of *Dkk2* and *Sfrp1* is up-regulated in the *Gli2* mutant GT in comparison with those of the wild-type (WT) male GT at E15.5. Their expression pattern is shown by the representative transverse sections.

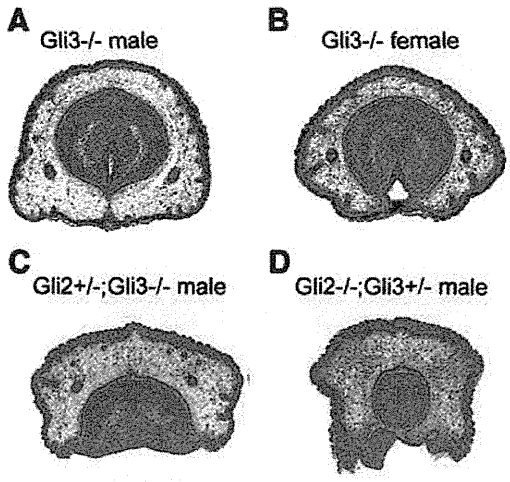

FIG. 4. Morphological analysis of compound mutants for *Gli2* and *Gli3* alleles. A and B, *Gli3* mutant male (A) and female GT (B) exhibit normal sexually dimorphic development at E18.5. C, The GT of the $Gli2^{+/-};Gli3^{-/-}$ embryos shows a groove-like structure similar to the phenotype of the *Gli2* homozygotes. D, The $Gli2^{-/-};Gli3^{+/-}$ embryos display drastic GT defects with underdeveloped glans, which does not contain the urethra.

The roles of Gli3 in the GT development

In contrast to the drastic phenotype of the *Gli2* mutants, the *Gli3* mutant GT developed morphologically normal and showed a proper sexual differentiation (Fig. 4, A and B), whereas a few mutant embryos exhibited a developmental defect with a lack of dorsal part of the GT (two out of 16 embryos, one male and one female) (data not shown). A genetic compensation among several *Gli* genes has been reported during organogenesis in developmental context-dependent manners (10, 11). Thus, *Gli2* and *Gli3* compound mutant embryos were analyzed in the current study. *Gli2* heterozygotes ($Gli2^{+/-}$) and double heterozygotes ($Gli2^{+/-};Gli3^{+/-}$) were phenotypically normal during development and adult life (data not shown). Loss of *Gli3* alleles ($Gli2^{+/-};Gli3^{-/-}$) led to severe defects in the prepuce and urethral formation similar to the phenotype of the *Gli2* homozygous background ($Gli2^{-/-}$) (Fig. 4C, compare with Fig. 2A). Furthermore, additional loss of a *Gli3* allele in the *Gli2* homozygotes ($Gli2^{-/-};Gli3^{+/-}$) induced drastic defects in the GT development with underdeveloped glans without urethral epithelium (Fig. 4D). Although Gli3 basically functions as a hedgehog signal repressor in the normal GT development (see Fig. 1F), it appeared to also possess an activator function and partially compensate for the hedgehog signal transduction when *Gli2* allele was mutated.

Shh signaling is required for masculinization of the GT

The *Shh* mutants display a complete GT agenesis as described previously (Fig. 5A) (4, 6, 34). To examine whether Shh signal relayed by Gli2 plays an important

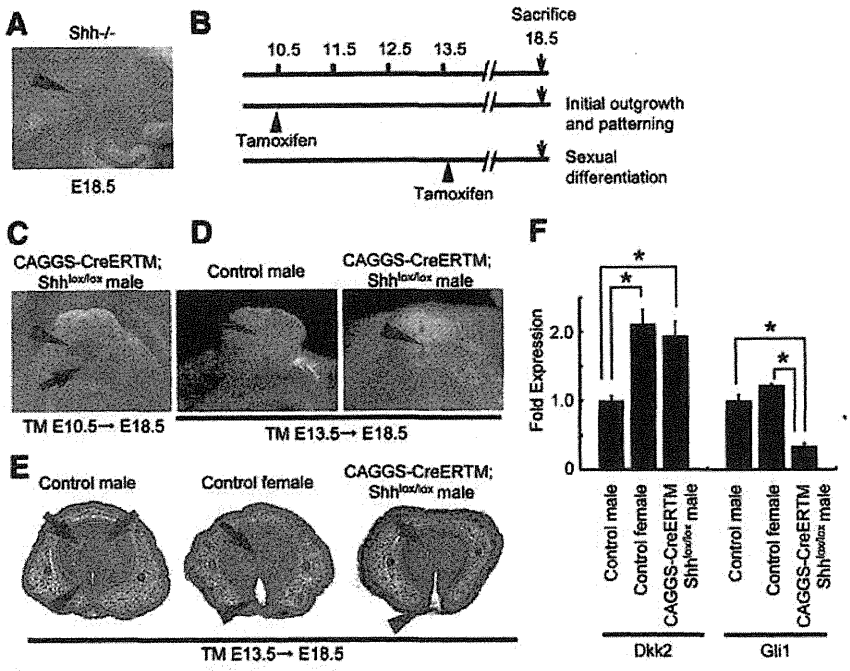

FIG. 5. Requirement of Shh in both initial development and sexual differentiation of the GT. A, The *Shh* null embryos show a GT agenesis and a persistent cloaca. B, TM treatment timeline; TM is treated at E10.5 or E13.5 to investigate the effect of Shh for the initial development and sexual differentiation of the GT, respectively. C, Conditional *Shh* KO embryos treated with TM at E10.5 display a severe lower (ventral) GT abnormality with a groove-like structure (*arrowheads*). An *arrow* indicates a cleft in the scrotal region. D and E, Male mutant embryos treated with TM at E13.5 display the abnormal masculinization phenotypes of the GT. Note the slit-like defect in the ventral midline of the mutant GT (D, *arrowhead*). The urethral fold is unfused, resulting in an impaired tubular urethra (E, *arrowhead*), and the prospective corporal body is underdeveloped (E, *arrows*). F, *Dkk2* expression is increased, whereas *Gli1* expression is decreased in the mutant male GT. The relative RNA equivalents for each sample were determined by standardization with ribosomal protein L8 levels. Statistical significance is indicated by *asterisks*, with $P < 0.05$.

role in the process of male sexual differentiation, *Shh*-conditional mutant analyses were employed. *Shh*-floxed mice were mated with the *CAGGS-CreERTM* line (23) to conditionally inactivate the hedgehog signal. The TM was treated at E10.5 and E13.5, just before the GT protrusion and the sexual differentiation, respectively, and the corresponding GT phenotype was analyzed (Fig. 5B). The conditional mutant mice treated with TM at E10.5 displayed a severe lower (ventral) defect with a groove-like structure in the GT (Fig. 5C), possibly due to impaired initial outgrowth and early patterning. On the other hand, the conditional male mutant embryos treated with TM at E13.5 exhibited demasculinized GT phenotypes. Their prepuce remained open showing a slit in the ventral midline of the GT (Fig. 5D, *arrowhead*), resulting in a failure to form a tubular structure (Fig. 5E, *arrowhead*). In addition, although control male GT showed the condensation of a bilaterally segmented prospective corporal body, such a structure was undifferentiated in the mutant male GT resembling the female GT (Fig. 5E, *arrows*). In addition, *Dkk2* expression as a feminized marker was increased in the male mutants (Fig. 5F). As expected, *Gli1* expression was decreased in the mutant GT (Fig. 5F). The androgen production was normal judged by their anogenital distance, indicating that androgen signal was not affected in the mutants.

Accelerated prepuce development in the female GT of gain-of-function mutants for hedgehog signaling

To test whether hedgehog signal activation could induce the masculine phenotype of the female GT, a constitutively activated *Smo* allele (*SmoM2*) was conditionally expressed using the *CAGGS-CreERTM* allele (25, 35). After TM treatment to mice at E13.5, the phenotype of *CAGGS-CreER;R26-SmoM2* female embryos was examined at E18.5. The mutant female GT showed a well-developed prepuce, particularly in the ventral side, in comparison with that of the control female embryos (Fig. 6A, *arrow*). However, the urethral fold was not fused in the GT ventral midline similar to the normal female GT (Fig. 6, A and B). In addition, the expression level of the sexually dimorphic genes, *MafB* and *Dkk2*, was not altered between control and mutant female GT (data not shown). These results suggest that the activation of hedgehog signal alone could partially masculinize the female GT but not sufficient for complete sexual reversal of the GT in the current experimental condition.

It has been reported that conditionally activated hedgehog pathway in the embryonic ovary can ectopically induce the expression of steroid synthesis enzyme and produce androgen (36). However, p450Scc expression was barely detected in both mutant and control ovary, in contrast to the high level expression in the testis (Fig. 6C). The internal reproductive organ development and the length of anogenital distance were not affected in the current conditional mutant mouse embryos (data not shown). These data indicate that the well-developed prepuce is not attributed to the ectopic androgen production in the female mutant embryos.

Discussion

External genitalia formation consists of a series of complex developmental processes. Among such processes, the

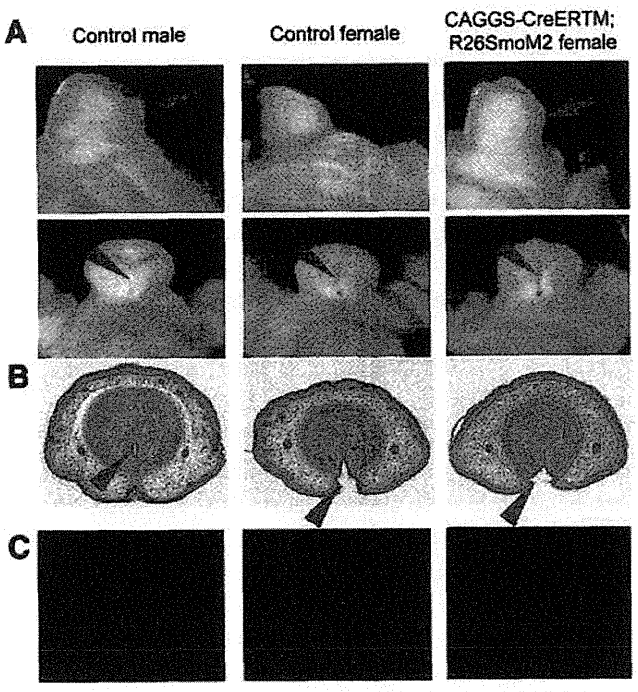

FIG. 6. Prepuce hyperplasia by constitutively activated hedgehog signal in the female GT. A and B, The female GT of *R26SmoM2* mutant embryos shows a prepuce hyperplasia, particularly in its ventral side (*arrow*). In the mutant embryos, a slit-like structure is observed in the ventral midline of the GT (A, *arrowhead*) as a consequence of improper incorporation of the urethra into the glans region (B, *arrowhead*). C, No significant differences of p450Scc expression are observed between mutant and control ovaries, whereas high level of its expression is detected in the control male testis.

establishment of sexual dimorphisms is a unique developmental program during embryogenesis. It has been shown that androgen signaling hormonally establishes the male sexual characteristics (19). However, its mediating genes, often termed as effector genes, involving for the masculine process have been poorly understood. The presence of effectors of androgen signaling has been assumed, although the elucidation of such genes has been barely performed. The secreted growth factor, *Shh*, is one of the major developing regulators during embryogenesis and coordinately interacts with various signaling molecules to promote cell proliferation, survival, and differentiation. Hedgehog signaling is essential for the protrusion and initial outgrowth of the GT; hence, mice with a target deletion of *Shh* allele show a GT agenesis (4, 6, 34, 37). In addition, it was reported that the extent of GT outgrowth is correlated with the duration of Shh signaling, with longer Shh exposures leading to more extensive outgrowth (38). We here demonstrate that hedgehog signaling affects not only organ growth but also mesenchymal cell differentiation cooperatively with androgen signaling. This would be a novel function of hedgehog signaling mediating sexually dimorphic development in the male GT.

Epithelial-mesenchymal interactions mediated by growth factors are required for the genital organ development. Previous tissue graft experiments have demonstrated the inductive effect of the epithelium on the mesenchymal growth and differentiation in the GT (17, 18). Various growth factors have been shown to function during initial GT outgrowth and patterning (1). However, epithelium-derived developmental regulators and its functions, particularly during late organogenesis for sexually dimorphic development, are still not determined. *Shh* expressed in the endodermal epithelium elicited mesenchymal gene expression during GT sexual differentiation. Mutant mice for *Gli2* and *Shh* showed abnormal male external genitalia formation. Based on the current results, it is suggested that Shh signaling has a key role in not only initial GT development but also during sexual differentiation of the external genitalia. *Shh* is therefore a possible candidate for the epithelium-derived factor that regulates mesenchymal growth and differentiation for male external genitalia. Although hedgehog signaling is necessary for male GT development, the activation of hedgehog signaling is not sufficient for induction of complete sex-reversal GT in the female embryos. A constitutively activated *Smo* (*SmoM2*) expression induced a hyperplasic prepuce in the ventral side, but failed to achieve urethral fusion in the female GT. Thus, additional factors may be necessary for induction of the GT masculinization, which involve various growth factors, including bone morphogenetic protein, fibroblast growth factor, and Int and Wg (wingless) in Drosophila (Wnt) signalings like the case for prostate development (39–42).

It has been recently demonstrated that Shh possesses multiple functions for anogenital and urogenital organ formation (28, 43–45). Although there are some conflicting data (46, 47), androgen can induce *Shh* expression in the embryonic prostate (48, 49). However, this induction of *Shh* expression by androgen may not be elicited in the developing GT. The *Gli1* and *Ptc1* expression levels were not altered between male and female GT. Thus, hedgehog signaling does not appear to be a downstream target of androgen of this developmental context. On the other hand, androgen responsiveness was decreased in *Gli2* mutants when compared for sexually dimorphic gene expression between male and female GT. FK506 binding protein (Fkbp5) is one of the molecular chaperones that maintain a stimulatory effect on the androgen receptor-mediated transcriptional activity (50, 51). The mutants for *Fkbp4*, another gene belonging to the same family with *Fkbp5*, display the decreased level of androgen signal sensitivity, which results in a GT hypoplasia (52). These results are in agreement with the current observation that the androgen responsiveness was down-regulated in the *Gli2* mutants.

Notably, female marker genes, *Dkk2* and *Sfrp1*, were abundantly expressed in the *Gli2* male mutants in comparison with those of the wild-type male GT. Increased level of Wnt-inhibitory genes, such as *Dkk2* and *Sfrp1*, can lead to down-regulation of Wnt/β-catenin signaling, which activity is necessary for masculine process for GT development (28). These observations suggest that hedgehog signal may facilitate masculine processes upon modulating the androgen responsiveness in the mesenchyme. Androgen treatment to the *Gli2* female mutant embryos did not induce GT masculinization. This also supports the notion that androgen is not sufficient for induction of male GT development in the absence of hedgehog signaling.

In contrast to severe phenotypes of *Gli2* mutants, GT in the *Gli3* mutants were morphologically normal and showed proper sexual differentiation, even though the *del5*-derived LacZ signal was augmented. Thus, the role of Gli3 as a hedgehog repressor during GT development remains unknown, so far. However, double mutants analysis revealed that Gli3 can compensate for Gli2 function as a hedgehog activator when *Gli2* alleles are mutated. The compensatory role of Gli3 has been known particularly in the developing foregut and hindgut, such as trachea, esophagus, lung, and anorectal organ (11, 44). To our knowledge, the current result would be the first case showing a redundant role of Gli2 and Gli3 function in the reproductive organ. Most double homozygotes ($Gli2^{-/-}$; $Gli3^{-/-}$) die around E10.5 (10, 11), and a few survivor did not exhibit GT protrusion like the case of *Shh* KO mutants (Miyagawa, S., and G. Yamada, unpublished data). This also suggests a redundant role of Gli2 and Gli3 function throughout GT development.

Hedgehog pathway-mediated embryonic masculinization can also be attributed to androgen production through Dhh, the other member of the mammalian hedgehog family ligand. Inactivation of the *Dhh* gene leads to defects of fetal Leydig cell differentiation, resulting in the insufficient production of androgen and male-to-female sex-reversal in both the internal and external reproductive organs in such mice (30, 53). Although Gli2 has been suggested to be a primary activator of Shh-mediated transcription, androgen production and testicular development is normal in the *Gli2* mutant embryos. Dhh-mediated hedgehog signaling in the testis is possibly compensated by the Gli3 protein. Recently, conditionally expressed SmoM2 driven by *SF1-Cre* transgenic mice ectopically express steroid synthesis-related gene expression, such as *Cyp17*, which is not expressed in the normal embryonic ovary (36). In contrast, the current constitutively activated SmoM2 driven by *CAGGS-CreERTM* allele did not elicit excessive androgen production judged by *p450Scc* expression (54, 55). These observations may suggest the existence of the critical time point of hedgehog-mediated Leydig cell differentiation. *SF1-Cre* allele induces the target gene recombination in the undifferentiated stage of steroid synthesizing cells, whereas the current mutant embryos were conditionally expressing *SmoM2* at later stages.

Hypospadias is one of the most frequent birth defects often considered as disorders of masculinization. There is a controversy about the causative mechanisms of hypospadias. Both experimentally and clinically, any defects in androgen pathway cause urogenital defects, including hypospadias (1). Hypospadias is generally considered as caused by multifactorial factors; thus, familial cases for the hypospadias are rather rare among total patients. Moreover, only a small portion of heritable hypospadias can be attributed to such defects (56). The mutations of several growth factors, including hedgehog signaling, potentially induce early embryonic defects or lethality in severe cases. Hence, the contribution of growth factor signaling to the sexual differentiation has been generally unclear. Taking an advantage of conditionally mutant analyses, the current results shed light on hedgehog signaling as a newly identified crucial regulator for external genitalia masculinization and its possible relation with urogenital disorders.

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Molecular mechanisms of induction of persistent changes by estrogenic chemicals on female reproductive tracts and external genitalia☆

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ABSTRACT

The effects of environmental endocrine-disrupting chemicals (EDCs) are a great and growing concern for human and animal development and life. The reproductive organs are considered as a primary target of EDCs, yet the effects on reproductive organs can extend to other body systems. Perinatal diethylstilbestrol (DES)-exposed mice exhibit various reproductive organ abnormalities. The perinatal DES-exposure model has allowed insight into our understanding of the mechanisms of persistent reproductive organ abnormalities elicited by exposure to estrogens and/or estrogenic EDCs. The persistent changes in the vagina of neonatally DES-exposed mice result from sustained expression of growth factors by ligand-independent transcriptional activation of the estrogen receptor. Developmental regulatory genes, such as Wnt and Hox genes, are also targets of DES during fetal stages and altered gene expression can induce malformations of the reproductive organs. In this review, we focus on the development of female reproductive tracts and external genitalia, and discuss the recent progress in understanding the disruptive effects of estrogens and EDCs on these organs.

1. Introduction

The mammalian female reproductive tract provides the sites for gamete fertilization, implantation and subsequent development of the embryo and delivery of the fetus. The female reproductive tract is derived from the Müllerian duct and urogenital sinus. During normal mouse development, the Müllerian duct forms as a small invagination of the surface epithelium of the mesonephros, located adjacent to the cranial end of the Wolffian duct. The Müllerian duct extends caudally towards the urogenital sinus. Once the Müllerian duct forms, it differentiates into oviduct, uterus, cervix and the upper part of the vagina, whereas the urogenital sinus gives rise to the lower part of the vagina and urinary tract. In males, the Müllerian duct regresses under the action of anti-Müllerian hormone, which is secreted from Sertoli cells in the testis. Androgens are also secreted from Leydig cells in the testis, and consequently the Wolffian duct is maintained, resulting in its differentiation into epididymis, vas deferens and seminal vesicle. Thus, reproductive organ development depends on gonadal development and its secretion of hormones at the correct times and amounts during development [1–3].

Recent studies have shown various forms of sex determination in vertebrates. Sex is genetically determined in the medaka (fish) by the presence or absence of the Y chromosome specific gene DMY and estrogens facilitate and maintain sex differentiation of the ovarian cells and the following female pathway [4–6]. Administration of exogenous estrogens shortly after fertilization causes male to female sex-reversal, with the formation of a functional ovary and reproductive capabilities [7–9]. Successful induction of sex-reversal by sex steroid hormones has been also achieved in various fish species and amphibians [10–12]. In the chicken, sex reversal can be induced experimentally, at least in part, by injecting eggs with estrogens, or by inhibiting estrogen production [13,14], indicating a critical role for estrogen in avian sex determination. Some reptiles, including crocodilians and some turtles and lizards, exhibit temperature-dependent sex determination; sex depends on the temperature at which the eggs are incubated. Incubation temperature can modify the expression and activity of aromatase in the red-eared slider turtle and American alligator [15–17]. In addition, administration of exogenous estrogens to an egg can override the effects of male incubation temperature on sexual differentiation [18,19], suggesting that endogenous estrogen mediates ovarian development as a downstream signaling event in response to environmental temperature. Thus, in these animals, estrogen is critical for gonadal sex differentiation (ovary formation), and the subsequent female reproductive tract development.

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In contrast, the relative importance of sex steroid hormones in sex determination apparently seems to diminish in mammals compared with other vertebrates. Estrogen signaling is indeed required for complete ovarian differentiation and maintenance in adult mice [20–23]. Intriguingly, estrogen receptors (ERs) are necessary to repress the transdifferentiation of an adult ovary to a testis, probably cooperating with forkhead transcription factor Foxl2 [24,25]. However, there is no evidence that endogenous estrogens affect sex determination and ovary formation in mammals. Studies using knockout and mutant mice for ERs and aromatase genes have revealed no fundamental effects of endogenous estrogens on anatomical/morphological development in the reproductive tract s during embryogenesis and neonatal stage [20–23]. Notwithstanding this observation, ERs have been already expressed in developing mammalian reproductive organs from early stages and thus, they respond to estrogenic signals and can be the targets of endocrine-disrupting chemicals (EDCs). Therefore, administration of exogenous estrogens or estrogenic environmental signals that mimic hormones in animals can disrupt its normal development. One of the best-studied cases is a synthetic estrogen, diethylstilbestrol (DES). Beginning in the 1940s, DES was routinely prescribed to pregnant women for the prevention of miscarriages. To date, it is well-known that *in utero* exposure to DES induces vaginal clear-cell adenocarcinoma and various malformations in the reproductive tracts in young women [26–30]. Furthermore, in males exposed *in utero* to DES, reproductive organ abnormalities, including hypospadias, are found more frequently than in non-exposed controls [30–32]. Sexually dimorphic development of external genitalia depends on sex hormone environment during embryogenesis and thus can be strongly affected by the EDCs [33–36].

Potential endocrine disruptive effects in wild animals and humans exposed to EDCs during development have been summarized previously [37,38]. Despite this, the molecular mechanisms underlying EDC action remain largely unknown. Animal studies have shown that experimental exposure to estrogens/estrogenic chemicals induces misregulation of the endocrine systems and developmental sequences during embryogenesis. The perinatal mouse model has been used to understand molecular mechanisms of EDC-induced abnormalities in reproductive organs. In particular, DES effects were well recognized and firmly documented as it significantly alters the developing organism and results in persistent effects in the adult. In this review, we focus on the effects of DES on the female reproductive tracts and external genitalia.

2. Estrogen independent activation of ERα and growth factor signalings in mouse vagina exposed neonatally to DES

Estrogen acts via intracellular ERs that are members of the nuclear receptor superfamily of transcription factors. Upon ligand binding, ERs enhance the rate of transcriptional initiation by recruiting and assembling transcription regulatory complexes to the promoter regions of its target genes. Thus, estrogens exhibit acute and transient actions in target organs. In the adult female reproductive tracts, administration of estrogens in the adult increases organ weight and promotes cell proliferation and differentiation, whereas estrogen withdrawal induces rapid involution of uteri and vaginae resulting in atrophy. These specific and reversible effects of estrogens are important in maintaining homeostasis and are required for normal health and reproduction. In contrast, long-term exposure to estrogens induces an imbalance in cell proliferation and increases the risk of cancer of the reproductive organs in rodents and in humans [39,40]. It is also well-known that *in utero* exposure to DES causes vaginal clear-cell adenocarcinoma in a subset of exposed females including humans [28]. This is rare type of tumor, but its epidemiology revealed a clear association with early exposure to DES *in utero*. In addition, as the generation of women exposed to DES become older, concern has arisen about their health risks, because it has been hypothesized that *in utero* DES exposure could also influence the incidence of breast cancer, squamous neoplasia of the cervix, vaginal cancer and potentially other pathologies of the reproductive system [27,41–43]. Like humans, perinatal female mice exposed to DES develop estrogen-independent persistent cell proliferation, stratification and cornification of the vaginal epithelium, resulting in hyperplastic lesions and vaginal cancer later in life [44–46]. Although the evidence for endocrine disruption in humans resulting from exposure to EDCs is limited, animal studies have shown that perinatal exposure of various EDCs reproducibly induces estrogen-independent abnormal phenotypes in vagina. For instance, neonatal exposure of bisphenol A, an EDC exhibiting a weak estrogenic activity, also induces such malformations in mouse vagina. Thus, the animal DES model has been used to advance our knowledge of the potential risk of the carcinogenetic effects of estrogens, including developmental effects of EDCs [47]. Thus, the animal DES model has been used to advance our knowledge of the potential risk of the carcinogenetic effects of estrogens, including developmental effects of EDCs [48,49].

The proliferation and differentiation of mouse vaginal epithelial cells are strongly regulated by ovarian estrogens. The vaginae of ovariectomized mice show an atrophied epithelium of 2-3 cell layers, but estrogen administration rapidly induces epithelial cell proliferation, stratification and superficial cornification. In the uterus and vagina, mitogenic effects of estrogen are mediated by stromal ERα, as shown by recombination experiments with ERα mutant-stroma and wild type-epithelium [50,51]. These data indicate that such stroma-derived growth factors stimulate epithelial cell proliferation and differentiation during normal activity. In fact, previous studies have shown that several growth factors, including EGF-like growth factors, are expressed in the female reproductive organs upon estrogenic stimulation [52–54]. Intriguingly, the vagina in ovariectomized mice exposed DES neonatally also expresses EGF-like growth factors at high levels, even in the absence of endogenous estrogen [55–57]. Furthermore, EGFR and erbB2, receptors for EGF-like growth factors, are activated in such vagina. Serine residues located in the N-terminal activation function (AF-1) domain of ERα were identified as downstream targets of the erbB signaling pathway [57]. In the neonatal DES-exposed mouse vagina, the AF-1 domain of ERα is also phosphorylated even after ovariectomy. It has been shown that phosphorylation of ERα induces transcription activity in a ligand-independent manner through AF-1 [58]. Thus, persistent phosphorylation of ERα via erbB signaling could activate growth factor expression, resulting in formation of an auto-activation loop, which can contribute to the formation of cancerous lesions later in life (Fig. 1). Intriguingly, this activation loop of ERα-EGF-like growth factors-erbBs seems to be established only in the epithelium but not through the stroma, because high expression of EGF-like growth factors is detected in the epithelium only in vaginae from neonatally DES-exposed mice [57]. Failure of the regulatory interactions between the epithelium and stroma could be one of the mechanisms for aberrant activation of vaginal epithelial cell proliferation. In addition to erbB signal transduction, IGF-I signaling also appears to contribute to abnormalities in vagina exposed to DES perinatally [59]. It is currently unknown how the ERα transactivation induced by only AF-1 is maintained in vagina from mice exposed neonatally to DES. Specific modulators that change ERα function could provide further insight into this question.

Although the precise mechanisms have not been established explaining how such an activation loop with persistent gene expression is elicited, one of the possibilities might be an alteration of methylation of specific genes (Fig. 1). It has been shown that prenatal DES exposure alters methylation patterns in the promoter of

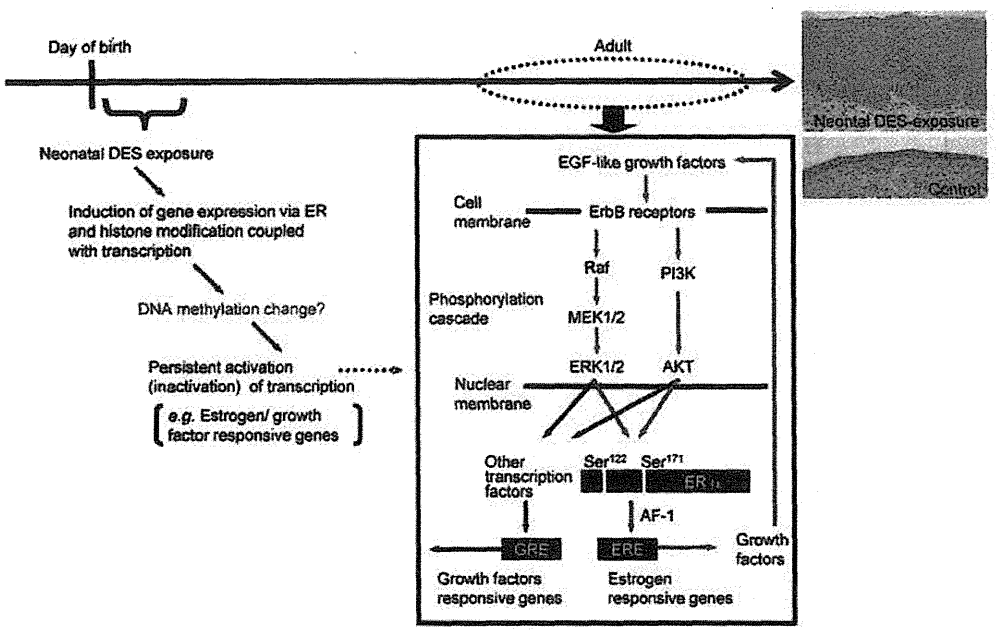

Fig. 1. Hypothetical model for the estrogen-independent estrogen receptor and growth factors activation pathway in neonatally DES-exposed mouse vagina. Histological sections show ovariectomized mouse vagina exposed neonatally to DES or vehicle alone.

some estrogen-responsive genes, including the demethylation of the c-fos and lactoferrin genes in the Müllerian duct [60,61]. c-Fos is a growth promoter that can predispose cells to becoming tumors. Hypomethylation of the nucleosomal binding protein 1 (Nsbp1) gene and its subsequent elevated expression is also reported after neonatal exposure to either DES or genistein [62]. Neonatal estrogen – or bisphenol A – exposure in rats induces hypomethylation of phosphodiesterase type 4 variant 4 (PDE4D4) gene, resulting in elevated expression of this gene in the prostate [63]. PDE4D4 is a crucial regulator for cAMP degradation and suggests a correlation between its expression and development of prostatic intraepithelial neoplasia. Thus, epigenetic changes could lead to altered gene expression and hence to altered tissue differentiation and formation, which could produce an increased susceptibility to disease and dysfunction later in life.

It was reported that DNA methylation changes can induce transgenerational effects, further exacerbating the potential role of EDCs that affect this pathway [64]. Increased level of lactoferrin gene in the uterus is detected in neonatally DES-exposed mice and also in their pups which never received DES-exposure [65]. Furthermore, the pups of prenatally DES-exposed mice have higher risks of reproductive organ abnormalities including tumors [66,67]. The preliminary evidence of the risk of reproductive dysfunction in daughters whose mothers were exposed DES *in utero* is reported [68–70], but further follow-up is needed.

3. Morphological defects of female reproductive tracts by DES

EDCs act in specific ways in tissues to disrupt normal developmental sequences. When EDCs affect relatively early periods of organogenesis, they can lead to congenital anomalies. DES acts both as a carcinogen and teratogen in the developing fetuses and neonates in mammals. For example, DES causes the boundary between the oviduct and uterus (the uterotubal junction) to be lost, resulting in infertility or subfertility [71,72]. DES-exposed mice also exhibit a malformed oviduct that lacks coils, and uterine abnormalities including hypoplasia, epithelial cell stratification, disorganized myometrial muscle and reduced uterine glands [45,73].

The Müllerian duct differentiates into the oviduct, uterus, cervix and upper part of vagina, along an anterior to posterior pattern. The Abdominal B (AbdB) Hox genes are expressed in a nested fashion throughout the Müllerian duct (Fig. 2). Hoxa9 is expressed in the oviductal or cranial part of the Müllerian duct but not in the more caudal region. Hoxa10 expression exhibits a sharp boundary at the junction between the presumptive uterus and oviduct. Hoxa11 is also expressed in the uterus as well as extending caudally in the cervix. Hoxa13 expression is caudally restricted primarily to the vagina in mice [74]. Analyses in knockout mice revealed that AbdB Hox genes play an instructive role on cellular identities along undifferentiated axes. In female Hoxa10 mutants, the anterior part of the uterus exhibits an anterior transformation to the oviduct and abnormalities of the uterotubal junction, and stratification of the distal uterine epithelium [75]. Loss of Hoxa11 results in a narrowing of the entire uterus, which makes it difficult to assess the uterotubal junction, and decrease in the number of uterine glands [76,77]. Importantly, although not identical, these phenotypes are

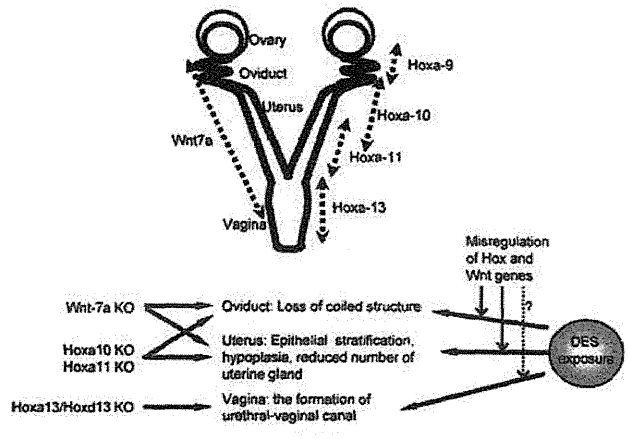

Fig. 2. Expression pattern of Hox genes and Wnt7a in female reproductive tracts during embryogenesis. Mutant mice for those genes exhibit various defects similar to those of prenatal DES-exposed mice.

similar to those of prenatally DES-exposed mice (Fig. 2). In fact, DES exposure can alter the expression of the Hox genes in the Müllerian duct. *In utero* DES exposure shifts Hox9 expression from the oviduct to the uterus and decreases both Hoxa10 and Hoxa11 expression in the embryonic uterus [78,79]. In addition to Hox genes, prenatal DES exposure also reduced Wnt7a expression in the Müllerian duct [80]. Wnt7a plays critical roles in epithelial–mesenchymal interactions during embryonic development [81]. Wnt7a is expressed throughout the entire Müllerian duct in embryos, whereas after birth it becomes restricted to the oviductal and uterine epithelium. Wnt7a mutants exhibit a lack of oviductal coiling and uterine gland formation, and exhibit a multilayered uterine epithelium [82]. It is therefore likely that abnormalities in the prenatally DES-exposed mouse oviduct and uterus are associated with misregulation of morphogenetic genes such as Wnt7a and the Hox genes (Fig. 2). Although mutant mouse studies provide an insight into complex gene networks during female reproductive organ formation, the involvement of endogenous estrogens in the expression of these genes has not been understood, because ER mutant mice do not show distinct altered phenotypes of Müllerian duct formation and differentiation. In contrast, it is obvious that altered expression of Wnt7a and AbdB Hox genes produced by DES exposure are mediated through ERα, because ERα mutant mice fail to induce such abnormalities [73]. Recently, it has been shown that the frequency of DNA methylation in the Hoxa10 intron is higher in prenatally DES-exposed mice when compared with controls [83]. Hoxa10 is associated with a variety of aspects of cellular physiology and women's health. Although altered methylation by DES exposure in humans has not been reported, down-regulation of Hoxa10 expression and aberrant methylation of Hoxa10 gene is associated with endometrial carcinoma and endometriosis in humans [84–86].

The urogenital sinus, which differentiates into the urinary tract, lower part of vagina and clitoris, is also affected by DES. Neonatal DES exposure induces female hypospadias, the formation of a common urethral-vaginal canal accompanied by a wide cleft clitoris [87]. Compound-induced mutation of Hoxa13 and Hoxd13 results in a common canal of the urinary tract and vaginal lumen [88], although the relationship between female hypospadias induced by DES and Hox genes expression has not been elucidated. Intriguingly, dihydrotestosterone (DHT), or non-aromatizable androgens, also induce female hypospadias [89]. These results indicate that fetal and neonatal stages showing active morphogenesis and development are more sensitive to endocrine disruptive stimulation than are adults.

4. Hormone-dependent development of external genitalia

Prenatally DES-exposed humans and laboratory animals exhibit a range of reproductive organ malformations including hypospadias, microphallus, retained testes and many aspects of the testicular dysgenesis syndrome [90–94]. Hypospadias (in which the urethral meatus is located on the ventral side of the penis) is one of the most frequent human birth defects. The increasing prevalence of hypospadias in humans has been hypothesized to be the result of exposure to EDCs/estrogens during fetal development [95–98]. In laboratory animals, fetuses exposed to DES or 17α-ethinylestradiol (EE2; a synthetic estrogen used in contraceptive pills) exhibit hypospadias-like phenotype with a failure of preputial development in male mice [94]. In addition, permanent dysmorphogenesis of the penis has been observed in adult rats treated neonatally with DES [99].

The sexual dimorphic development of external genitalia depends on the presence or absence of androgens and is, therefore, strongly affected by hormonal environment during embryogenesis. Flutamide, which inhibits androgens binding to the androgen receptor, demasculinize the external genitalia in male rodent offspring [100,101]. Likewise, the fungicides vinclozolin and procymidone, and pesticides DDT and its metabolite *p,p'*-DDE induce defects of androgen pathway with sufficient potency to induce cleft phallus and ambiguous genitalia [102–105]. In contrast, mechanisms of DES and other estrogens-induced external genitalia malformation has not been clarified. ERs are endogenously expressed in the embryonic external genitalia [93,106,107]. Further, ERα mutant mice are resistant to estrogens-induced penile abnormalities [108] indicating that estrogen-exposure could directly perturb male genitalia development.

The external genitalia are typically sexually dirmorphic organs and arise through dichotomous differentiation of common precursor tissues [109,110]. It is well documented that androgen plays a central role in such processes as epithelium-mesenchymal interactions [36,111]. Recently, canonical Wnt signal was shown to regulate the masculinization of external genital cooperatively with the androgen signal [34]. The bilateral mesenchyme adjacent to the urethral plate epithelium displayed sexually dimorphic activity of the Wnt signal. Loss- and gain-of-function mutants of β-catenin display altered sexual development of the external genitalia, suggesting that the Wnt signaling pathway functions as a locally expressed masculine effector [34]. Mutation of the Hoxa13 gene, which is associated with hand-foot-genital syndrome, causes genital abnormalities including hypospadias [112–114]. The Hoxa13 mutant mouse shows reduced expression of androgen receptors, suggesting a requirement of Hoxa13 for masculinization of external genitalia [115]. As discussed above, ERs signaling can affect the cross-talk with developmental factors such as Wnt and Hox genes. Therefore, estrogen signals could affect such signals as observed in the Müllerian duct.

5. Conclusion

Despite a large number of reports over the last decade on the mechanisms of DES and EDCs on reproductive organ development, much still needs to be understood. An emerging paradigm, the fetal origin of adult disease, is a new framework for considering the effects of EDCs on human and animal life. The plasticity of development in the perinatal period is evolutionarily advantageous for adaptation to the prenatal and early postnatal environment. However, the modern environment has environmental factors, including EDCs that easily influence fetal and neonatal development, resulting in permanent changes in cell differentiation and morphology. The instructive roles of estrogen signaling during embryogenesis are also important issues as an understanding of the precise mechanisms through which EDCs affect developing animals can also help us to understand how normal development is accomplished.

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Letter

Neonatal exposure to 2,3,7,8-tetrachlorodibenze-p-dioxin increases the mRNA expression of prostatic proteins in C57BL mice

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ABSTRACT — The effects of neonatal exposure to low doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on prostatic secretory protein expression were investigated. Male C57BL mice were treated with TCDD at 10, 100, or 1,000 ng/kg body weight at postnatal day (PND) 6. At PND42, the ventral, dorsolateral, and anterior prostatic lobes were dissected and the mRNA expression of prostatic proteins including spermine-binding protein, serine protease inhibitor Kazal type 3, prostate secretory protein 94 (PSP94), immunoglobulin binding protein-like protein (IgGBPLP), experimental autoimmune prostatitis antigen proteins, and peroxiredoxin-6 (Prdx6) was measured by quantitative PCR. There was no significant difference in the weight of the prostatic lobes between the control and TCDD-treated groups. The expression of PSP94 and Prdx6 in the ventral prostate and IgGBPLP in the dorsolateral prostate at PND42 was significantly increased by neonatal TCDD treatment in a dose-dependent manner, while no changes were noted in other prostatic secretions. These data suggest that neonatal exposure to TCDD may have effects on the neonatal differentiation of the prostate and results in the hyper-expression of some prostatic proteins later in life.

Key words: 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), Prostatic secretion, Mouse prostate, Neonatal effects

INTRODUCTION

The developing male reproductive system of laboratory rodents is highly sensitive to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Mably *et al.*, 1992; Roman and Peterson, 1998; Theobald *et al.*, 2000). Its toxic effects include a decrease in the weight of the testis and accessory sex organs, degeneration of germ cells, and decreased spermatogenesis. The adverse effects of maternal exposure to TCDD on the development of the prostate gland have been studied extensively in rats and mice. In Holtzman rats, a single maternal dose of 64 ng/kg body weight (bw) of TCDD caused a significant decrease in ventral prostate (VP) weight (Mably *et al.*, 1992). More recently, it was reported that androgen receptor (AR) mRNA expression was reduced in the VP of Holtzman rats following maternal treatment with as low as 12.5 ng/kg bw TCDD (Ohsako *et al.*, 2001). In the mouse, the C57BL/6J strain appears to be sensitive to TCDD, in which the maternal administration of 5 μg/kg bw TCDD suppressed the development of the VP in the offspring, while the weight of the dorsolateral prostate (DLP) and anterior prostate (AP) decreased by approximately 50% (Lin *et al.*, 2002a). Exposure to TCDD during only the lactational period also resulted in offspring with lower prostate weights, but with less severe changes (Lin *et al.*, 2002b).

Although the previous studies have been clearly demonstrated that TCDD affect the development of the prostate morphologically, it is important to examine the effect on the prostatic function, production of prostatic proteins. We recently reported the identification of the major proteins secreted from the mouse prostate (Fujimoto *et al.*, 2006). The secreted proteins included spermine-binding protein (SBP), serine protease inhibitor Kazal type 3

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(SPI-KT3), prostate secretory protein 94 (PSP94), glucose-regulated protein, 78kDa (GRP78), peroxiredoxin-6 (Prdx6), probasin, experimental autoimmune prostatitis antigen protein (EAPA2), and immunoglobulin binding protein-like protein (IgGBPLP). The expression profile of these proteins would be useful for studying prostatic function and may also provide markers for evaluating the effects of environmental chemicals on the prostate. In the present study, we investigated the effects of neonatal exposure to low doses of TCDD on the mRNA expression of prostatic proteins as well as AR in the prostate.

MATERIALS AND METHODS

Animal experiments

The animal experiments were conducted under the approval of the Animal Experiment Committee of the National Institute of Health Sciences (NIHS). All experiments involving TCDD-treated animals were carried out following the rules for the use of TCDD set by NIHS. Five-day-old male C57BL mice were purchased from Charles River Japan Co. and maintained with free access to a basal diet and tap water. At postnatal day (PND) 6, the animals were divided into 4 groups (n = 6, each group): control and 3 TCDD-treated groups. TCDD (Cambridge Isotope Laboratories, Inc., Andover, MA, USA) in corn oil (50 μl) was injected intraperitoneally (ip) at doses of 0, 10, 100, or 1,000 ng/kg bw). At PND42, the animals were killed under ether anesthesia, since our previous study indicated that the mRNA expression of prostatic proteins is matured at PND42 (Fujimoto *et al.*, 2006). The prostatic lobes were dissected under a microscope, then immediately fixed in RNAlater Solution (Life technologies, Grand Island, NY, USA).

Quantification of mRNA by real-time RT-PCR

Total RNA was prepared from prostatic tissues using an RNA isolation kit (NucleoSpin RNA II; Machery-Nagel GmbH & Co. KG, Düren, Germany). An ABI Prism 7500 (Applied Biosystems/Life Technologies Co., Carlsbad, CA, USA) was employed for the RT-PCR based quantification of prostatic protein mRNAs as described previously (Fujimoto *et al.*, 2006). All mRNA levels were normalized with reference to β-actin mRNA.

Statistical analysis

Statistical comparisons were made by Dunnett's multiple comparison test.

RESULTS

Body and prostate lobe weights

There was no significant difference in body weight between the control and 3 TCDD-treated groups at PND42 (Table 1). There was no significant change in the weight of either the VP, DLP, or AP.

Expression of prostatic protein and AR mRNAs

SBP and SPI-KT3 were preferentially expressed in the VP, while probasin, EAPA2, and IgGBPLP expression was localized in the DLP and AP (Table 2). PSP94 was expressed in both the VP and DLP, while GRP78 and Prdx6 were expressed in all prostatic lobes. The effects of neonatal treatment of TCDD on mRNA expression were evident for PSP94, Prdx6, and IgGBPLP. The effects were lobe specific; that is, neonatal TCDD increased the expression of PSP94 and Prdx6 mRNA in the VP as well as IgGBPLP mRNA in the DLP in a dose-dependent manner. Neonatal TCDD exposure did not change the expression of AR mRNA in the VP or DLP, but decreased its expression in the AP.

DISCUSSION

Maternal exposure to TCDD reportedly causes irreversible changes to the reproductive systems of offspring, including reduced sperm count and reduced size of the reproductive organs. The development of the male reproductive organs in rodents, in particular the prostate gland, has been recognized as a sensitive target to

Table 1. Weight of body and prostatic lobes at PND42

Treatment	body weight (g)	VP (mg/g bw)	DLP (mg/g bw)	AP (mg/g bw)
control	16.8 ± 0.28	0.20 ± 0.03	0.21 ± 0.02	0.30 ± 0.02
TCDD 10	16.9 ± 0.37	0.19 ± 0.02	0.23 ± 0.01	0.22 ± 0.06
TCDD 100	17.7 ± 0.25	0.30 ± 0.08	0.20 ± 0.02	0.34 ± 0.03
TCDD 1000	18.9 ± 0.38	0.24 ± 0.08	0.24 ± 0.01	0.31 ± 0.03

Mean ± S.E.M. (n = 6). Male C57BL mice were treated with TCDD (10, 100, or 1,000 ng/kg bw) at postnatal day (PND) 6 and sacrificed at PND42.