European Journal of Human Genetics (2008) 16, 36–44

www.nature.com/ejhg

ARTICLE

Abnormal urethra formation in mouse models of Split-hand/split-foot malformation type 1 and type 4

Kentaro Suzuki[1], Ryuma Haraguchi[1], Tsutomu Ogata[2], Ottavia Barbieri[3], Olinda Alegria[4], Maxence Vieux-Rochas[4], Naomi Nakagata[1], Masataka Ito[5], Alea A Mills[6], Takeshi Kurita[7], Giovanni Levi*[,4] and Gen Yamada*[,1]

[1]*Center for Animal Resources and Development (CARD), Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan;* [2]*Department of Endocrinology and Metabolism, National Research Institute for Child Health and Development, Tokyo, Japan;* [3]*Department of Experimental Medicine, University of Genova, IST, Genova, Italy;* [4]*CNRS UMR5166–MNHN, Evolution des Régulations Endocriniennes, Paris, Cedex 05, France;* [5]*Department of Developmental Anatomy and Regenerative Biology, National Defense Medical College, Tokorozawa, Japan;* [6]*Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA;* [7]*Division of Reproductive Biology Research, Northwestern University, Chicago, IL, USA*

Urogenital birth defects are one of the common phenotypes observed in hereditary human disorders. In particular, limb malformations are often associated with urogenital developmental abnormalities, as the case for Hand–foot–genital syndrome displaying similar hypoplasia/agenesis of limbs and external genitalia. Split-hand/split-foot malformation (SHFM) is a syndromic limb disorder affecting the central rays of the autopod with median clefts of the hands and feet, missing central fingers and often fusion of the remaining ones. SHFM type 1 (SHFM1) is linked to genomic deletions or rearrangements, which includes the distal-less-related homeogenes DLX5 and DLX6 as well as DSS1. SHFM type 4 (SHFM4) is associated with mutations in *p63*, which encodes a p53-related transcription factor. To understand that SHFM is associated with urogenital birth defects, we performed gene expression analysis and gene knockout mouse model analyses. We show here that *Dlx5*, *Dlx6*, *p63* and *Bmp7*, one of the p63 downstream candidate genes, are all expressed in the developing urethral plate (UP) and that targeted inactivation of these genes in the mouse results in UP defects leading to abnormal urethra formation. These results suggested that different set of transcription factors and growth factor genes play similar developmental functions during embryonic urethra formation. Human SHFM syndromes display multiple phenotypes with variations in addition to split hand foot limb phenotype. These results suggest that different genes associated with human SHFM could also be involved in the aetiogenesis of hypospadias pointing toward a common molecular origin of these congenital malformations.
European Journal of Human Genetics (2008) **16,** 36–44; doi:10.1038/sj.ejhg.5201925; published online 19 September 2007

Keywords: hypospadias; split-hand/foot malformation; dlx; p63; Bmp7; urethra

***Correspondence: G Yamada, Graduate School of Medical and Pharmaceutical Sciences, Center for Animal Resources and Development, Kumamoto University, Honjo 2-2-1, Kumamoto 860-0811, Japan.**
Tel: +81 96 373 6569; Fax: +81 96 373 6560; or G Levi, CNRS UMR5166–MNHN, Evolution des Régulations Endocriniennes, 7, rue Cuvier, 75231 Paris, Cedex 05, France.
Tel/Fax: +33 1 40793621;
E-mail: gensan@gpo.kumamoto-u.ac.jp or glevi@mnhn.fr
Received 13 June 2007; revised 7 August 2007; accepted 8 August 2007; published online 19 September 2007

Introduction

External genitalia develop tubular or groove-like epithelial structures for uresis and sperm ejaculation/intake. The embryonic external genitalia, the genital tubercle (GT), develops from the posterior embryonic region as a bud-anlage.[1,2] At earlier embryonic stages (before E (embryonic)

16 in mouse development), the external genitalia of male and female fetuses are indistinguishable as a common undifferentiated GT. Epithelial differentiation is one of the important processes for reproductive organ development.[3] During GT development, embryonic epithelial structures differentiate progressively to form the urethra. An epithelial groove or fold (the urethral groove is evident for the case of human GT) appears on the ventral side of GT. In the distal region of GT, the urethral fold (UF) forms a solid plate of epithelia known as the urethral plate (UP). The UP canalizes and extends the UF distally into the male glans region. Later, a UF develops further toward the midline and finally fuses in the midline to generate the tubular penile urethra[1] and the progressive formation of the UF leading to midline fusion from proximal to distal regions of the GT has been reported.[4] Within the GT, we have identified a transient epithelial structure located at the distal end of UP termed distal urethral epithelia (DUE).[5,6] The DUE expresses several growth factors, including Fgf (fibroblast growth factor), Bmps (bone morphogenetic proteins) and Sonic hedgehog (Shh).[5–9] The proper spatio-temporal expression of these molecular signals in the DUE is critical for the regulation of normal GT development[5,6,10] and for the differentiation of the UP to the urethra.[1,11,12]

Split-hand/foot malformation (SHFM) is a congenital limb malformation with median clefts of the hands and feet, and aplasia and/or hypoplasia of the phalanges. The aetiogenesis of SHFM is not well understood, however, defects in the development and/or differentiation of the apical ectodermal ridge (AER) are most probably involved.[13,14] Five different forms of SHFM exist in humans associated with different genetic anomalies.[15] SHFM1 (MIM 183600) is associated with genomic lesions on chromosome 7q21 in a minimal region, which includes the distal-less-related homeogenes DLX5 and DLX6.[16,17] The double knockout of *Dlx5* and *Dlx6* (*Dlx5/6* D-KO) in the mouse leads to ectrodactyly in the hindlimbs[18,19] with defective development of the middle portion of the AER. Dlx genes code for homeodomain transcription factor homologues to insect distal-less and play a key role in the control of appendage development.[20–22] In mammals, there are six *Dlx* genes organized into three tail-to-tail bigenic clusters, Dlx1/2, Dlx5/6 and Dlx3/7.[22–24] Dlx genes are expressed in craniofacial primordia, in the developing brain, ectodermal placodes, and limbs, where they are both expressed in the AER[19,21,25] and in the underlying mesenchyme.

SHFM4 (MIM 605289) is caused by mutations in *p63*, a gene coding for a transcription factor homologous to p53 and p73.[26] Mutations of *p63* are also associated with other autosomal, dominant, human syndromes, including ectrodactlyly–ectodermal dysplasia and Cleft lip; EEC (MIM 604292). p63 plays a major role in the control of epithelial morphogenesis[13,14] controlling the expression of stratification markers. *p6*3 knockout mice (*p63* KO) show severe defects affecting their skin, limbs, craniofacial skeleton, hair and mammary gland and in general fail to form normal ectodermal structures with profound defects in squamous epithelial lineages. It has been shown that both p63 and Dlx5 and Dlx6 (Dlx5/6) play a critical role in the control of AER development.[13,14,18,19]

It has been known that there is a frequent association of limb and urogenital birth defects. SHFM has been known as showing urogenital defects; however, it has been not known whether such urogenital defects correspond to the essential SHFM symptoms or they are just among additional phenotype variations as part of the associated disorders. In this study, we analyzed for the first time the urethral defects and abnormalities during urethral epithelialization in both *p63* KO and *Dlx5/6* D-KO embryos. Furthermore, we performed an analysis in mice lacking *Bmp7*, a putative downstream gene of *p63*. The similarity in the UP defects observed in these three mutants suggests a possibility that the integration of different sets of transcription factor genes, *Dlx5/6* and *p63*, with epithelially expressed *Bmp7* may constitute developmental regulators for urethra formation. The implications of the current findings regarding the SHFM phenotypes are discussed. Furthermore, a possible association between hypospadias and SHFM1 and SHFM4 is suggested.

Materials and methods

Mouse embryos

The targeted mutant mice for *Dlx5/6* and *Bmp7*^{LacZ} have been described previously.[18,27] The *p63* mouse line was described previously.[14] The embryonic samples were collected at E10.5–E18.5 and GTs were isolated as described previously.[6] Wild-type or heterozygous littermates were collected as controls. All experiments were performed more than three times. Each KO mice showed urethral defects (abnormal marker expression and histology) more than 80, 70 and 60% as the frequency for Bmp7, Dlx5/6 and p63 gene knockout mice, respectively. Approximately 40% of the p63 KO specimens showed more severe phenotypes.

Histology and immunohistochemistry

Mouse GTs were fixed overnight in 4% paraformaldehyde (PFA)/PBS, dehydrated in methanol, embedded in paraffin 6 μm serial sections that were prepared for hematoxylin and eosin staining, immunohistochemistry and for *in situ* hybridization of gene expression. Antibodies for KERATIN 1 (COVANCE AF109) and KERATIN 14 (COVANCE AF64) were used.

In situ hybridization for gene expression analysis

Section *in situ* hybridization was performed on 6 μm paraffin sections of GTs. After sectioning, samples were rehydrated, bleached, treated with proteinase K (WAKO)

and post-fixed in 4% PFA/PBS. Hybridization with digoxigenin (DIG)-labeled antisense probes was performed at 65°C for more than 12 h. After hybridization, the slides were washed as follows: (1) 65°C incubation for 30 min in 2× SSC and 50% formamide; (2) 65°C incubation for 10 min in 2×SSC, 50% formamide, 1:1 in TBST buffer (0.5 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and (3) room temperature for 5 min in TBST buffer. The DIG-labeled probe was detected with an anti-DIG AP (alkaline phosphatase)-coupled Fab fragment (Roche) and subsequent BM-purple (Roche) treatment. The sections were mounted with Kaiser solution (Merck). Whole-mount *in situ* hybridization was performed by standard procedures with probes for *Bmp7*, *Dlx5*, *Dlx6*, *Keratin8*, *Keratin14*, *p63* and *Fgf8*, which were kindly provided by Drs M Yoshida, H Shibuya, Z Zhao, L Casanova, K Yamanishi, A Bradley and BL Hogan, respectively.

Results

Epithelial differentiation during the UP to urethra formation in the embryonic GTs

Proper regulation of epithelial differentiation during UP to urethra formation is one of the key processes during GT formation.[2,11,12] The extent of epithelial differentiation was compared between distal and proximal UP epithelia. It has been shown that progressive epithelial differentiation occurs from proximal site of GT toward its distal region. Morphologically, such proximal regions of the GTs display non-fused UP showing UF connecting further toward the cloaca (Figure 1g). In contrast, distal part of the UP is characterized as containing the midline-fused UP (Figure 1d). Each part represents different degrees of epithelial differentiation. During GT formation, expression of several keratin were detected along with the epithelialization of UP. *Keratin 8* (*K8*) is a marker of simple epithelia, expressed before epithelia stratification. *Keratin 14* (*K14*) is a differentiation marker, expressed in epithelia that have committed to initiate a stratification program. *K14* and *K8* are expressed rather complementary during UP formation (Figure 1; data not shown). In the distal part of the GT, *K8* was expressed in immature UP epithelia (Figure 1b), whereas *K14* expression was detected in the ectodermal surface epithelia and in more differentiated epithelia, that is, mainly in part of the urethral 'seam' (Figure 1c; data not shown). By contrast, the proximal UP epithelia was characterized as expressing restricted *K8* expression in immature epithelia adjacent to the inner prospective urethral lumen (Figure 1e). Such proximal UP epithelia show progressed degree of differentiation composed with thick *K14*-positive epithelia in the ventral margin adjacent to the ventral midline seam (Figure 1f). In line with such differential *K8* expression in E12.5–E14.5 GT, the epithelia of the distal UP was uniformly monostratified, whereas more proximal urethal regions were multilayered displaying more evident signs of differentiation (Figure 1d and g; data not shown).

p63 is expressed in the developing UP and its inactivation causes defective urethra formation

p63 has been characterized as one of the major genes involved in the regulation of epithelial differentiation. In the view of the highly dynamic epithelial differentiation processes taking place during the UP to urethra formation, we examined the pattern of *p63* expression during GT development and analyzed any phenotypic lesions in GT development of *p63* KO (Figure 2). At E10.5, *p63* was expressed in the cloacal membrane (CM; Figure 2a; an arrow), whereas it was mostly expressed in the DUE at E11.5–E12.5 and also in the UP (Figure 2b and c). p*63* KO GTs at E14.5 showed severe abnormalities, the entire disruption of the UP to urethra formation process without

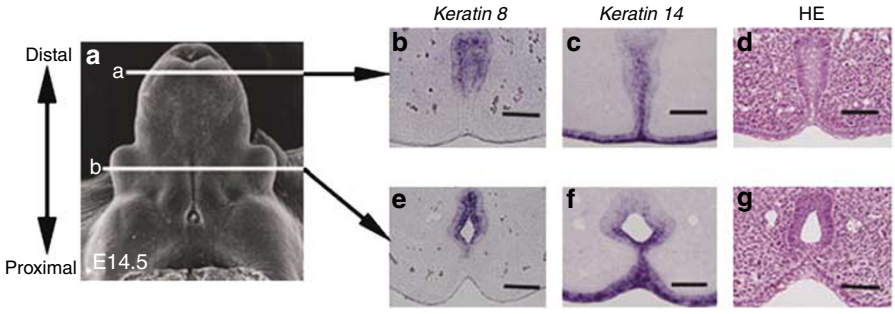


Figure 1 Epithelial differentiation of UP of the embryonic GTs. (**a**) A SEM picture of an embryonic GT at E14.5. (**b**, **c**, **e**, **f**) Distal UP epithelia express simple epithelial marker, *K8*, in the entire UP except for the ventral margin of the UP (**b**). In contrast, more proximal UP epithelia are composed by stratified epithelia adjacent to the *K8* expression site in the inner part of the UP (**e**). *K14*, differentiated epithelial marker, is expressed in the more differentiated epithelia. Proximal UP epithelia show progressed degree of differentiation composed with thick *K14*-positive epithelia compared with distal UP epithelia (**c** *vs* **f**). In line with such differential *K8* and *K14* expression, histological sections show uniform columnar epithelia in the distal UP (**d**) and stratified layers of the epithelia at the more proximal UP (**g**). The location of white line (**a**), (**b**) indicated the distal GT region and the proximal GT region, respectively (**a**). Scale bars: 60 μm in (**b**–**g**).

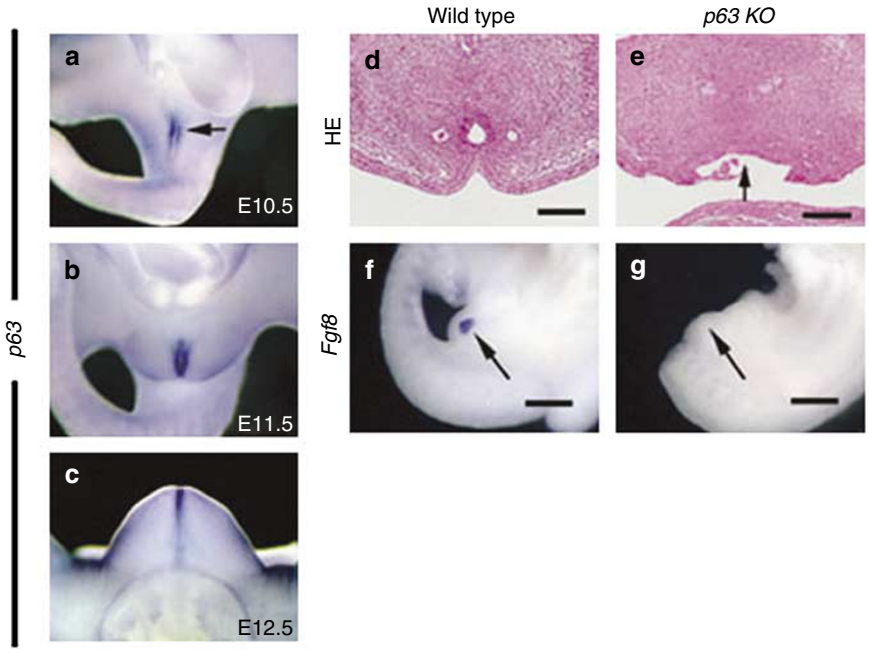


Figure 2 *p63* is expressed during the UP formation and urethral defects are observed in *p63* KO embryos. (**a**–**c**) *p63* is expressed in the CM at E10.5 (an arrow in **a**), and it is also subsequently expressed in the DUE at E11.5 and in the UP at E12.5. (**d** and **e**) *p63* KO GTs show ventral GT abnormalities with an aberrant ventral groove at E14.5 (an arrow in **e**). (**f** and **g**) *Fgf8* expression is significantly reduced in *p63* KO in comparison to wild type at E11.0 (arrows). The tail region was removed in the specimen (**g**). Scale bars: 100 μm in (**d**, **e**), 200 μm in (**f**, **g**).

midline fusion (Figure 2d and e; an arrow in e). GT bud formation *per se* was recognized in *p63* KO embryos. However, the expression of *Fgf8*, one of the signals regulating GT development, was reduced in the DUE of *p63* KO embryonic GTs at E11.0 (Figure 2f and g; the GTs are marked by arrows and the tail is removed in the mutant specimen).

Bmp7 plays a role in UP development; a possible regulation of *Bmp7* expression by p63

Bmp7 is one of the important epithelial Bmps involved in organogenesis. The distal signaling epithelia of the GT, the DUE, expresses several growth factors, including *Fgfs* and *Bmp7*.[5,6,8,10] It has been reported that Bmp7 plays a role as an epithelial regulator for differentiation[28] and it has been identified as one of the candidate downstream genes of *p63*.[29] Although its role in GT formation has not yet been clarified, it may regulate GT apoptosis.[8,10] Given the possible regulation by p63, we examined the expression of *Bmp7* in GTs of *p63* KO embryo. Epithelial expression of *Bmp7* was reduced in *p63* KO GTs (Figure 3b). This reduction of *Bmp7* expression in *p63* KO GTs prompted us to analyze the extent of epithelial differentiation in the UP of *Bmp7* KO embryos. *Bmp7* KO embryonic GTs also displayed severe UP defects (Figure 3c and d) in contrast to the proper UP and the ventral midline seam of the wild-type GTs. To further examine the degree of UP epithelial differentiation, *K8* expression was examined in *Bmp7* KO embryos. In *Bmp7* KO embryos, *K8* expression persisted in the ventral margin of the developing UP and was not lost, as it is observed during the normal progression of urethra formation (Figure 3e and f; such expression was also observed at several stages (data not shown)). The phenotypic similarity of the UP lesions observed in *p63* and *Bmp7* KO embryos suggests the possibility that *Bmp7* may be one of the downstream genes of *p63* during embryonic UP formation.

Dlx5 and *Dlx6* are expressed during GT formation and regulate urethra formation

We examined the expression of *Dlx5* and *Dlx6* during GT formation. The expression of *Dlx5* could be detected at E10.5 in the CM before the emergence of the GT bud (Figure 4a). Subsequently, *Dlx5* was expressed in the developing UP and in the GT mesenchyme (Figure 4b) at E11.5. From E 12.5 onwards, its expression was restricted and mainly in the distal mesenchymal region of GT examined up to E14.5 (Figure 4c; data not shown). The expression pattern of *Dlx6* was similar to that of *Dlx5* (data not shown). As a result, *p63* and *Dlx5/6* were expressed in an overlapping manner during the development of the UP. This dynamic *Dlx5/6* expression pattern prompted us to examine their roles during GT development.

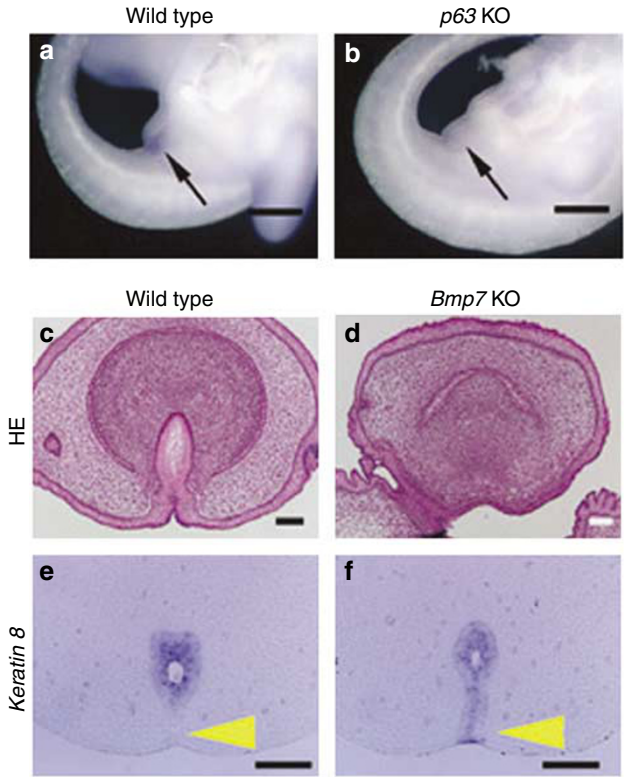


Figure 3 Downregulation of *Bmp7* expression in *p63* KO embryos; *Bmp7* KO embryos display defects in the UP formation. (**a** and **b**) *Bmp7* expression in the DUE is reduced in *p63* KO GTs at E11.0 (arrows in **a** and **b**). (**c** and **d**) A cross-section of the GT at E17.5. UP is present at the ventral midline in wild type (**c**). In contrast, *Bmp7* KO GTs lack such UP formation (**d**). (**e** and **f**) *Bmp7* KO UP shows remaining *K8* expression in the ventral margin in contrast to the wild-type (yellow arrowheads) at E12.5. Scale bars: 200 μm in (**a**, **b**), 100 μm in (**c**–**f**).

Dlx5/6 D-KO embryos showed severe UP defects during the UP to urethra formation (Figure 4d and e; note the defects were observed along the whole proximo-distal (P-D) aspect of the GT). The prospective future urethra orifice is located at the distal tip of the developing normal UP (Figure 4d; an arrow head). In contrast, *Dlx5/6* D-KO GTs displayed an abnormal urethra orifice formation adjacent to the scrotum (Figure 4e; an arrow head). Mutant GTs showed defective urethra formation at E18.5 forming a groove-like structure in the ventral GT (Figure 4f and g; the arrows show the male tubular urethra and the ventral midline seam in f in contrast to the ventral groove-like structure in g). Such urethral defects were also prominent in female GTs (data not shown). *Fgf8* expression was reduced in *Dlx5/6* D-KO embryos (Figure 4h and i). Functional compensation has been known between Dlx gene family members such as the case for *Dlx5* and *Dlx6*.[30] Single *Dlx5* KO embryos displayed no genital abnormalities (data not shown).

Defects of the epithelial differentiation in the UP of *Dlx5/6* D-KO embryos

To examine the extent of epithelial differentiation, Keratin expression was examined in wild-type and *Dlx5/6* D-KO embryos. The immature epithelial marker *K8* was not expressed in the normal ventral margin of the UP at E12.5 (Figure 5a). In contrast, in *Dlx5/6* D-KO embryos, high levels of *K8* expression persisted in the ventral margin of the UP epithelia (Figure 5b). In contrast to wild-type GTs, *Dlx5/6* D-KO embryonic GTs showed reduction of K14 and K1 expression at the ventral GT (Figure 5c–f; arrows). These results indicate that in the absence of *Dlx5* and *Dlx6*, the epithelia of the UP do not differentiate properly during urethra formation.

Discussion

SHFM and hypospadias by the mutation of p63 and Dlx5/6

SHFM, also known as ectrodactyly or lobster-claw deformity, is characterized by patterning defects of the central digital rays.[31] SHFM appears as genetically distinct pathologies which share, however, relatively similar clinical manifestations. Five human SHFM disease loci have been genetically mapped to chromosomes.[17,26,32–34] The corresponding murine models for *p63* inactivation (for SHFM4) and *Dlx5/Dlx6* double inactivation (for SHFM1) show defects in limb development of varying severity. Often SHFM appears as a syndromic malformation in which limb defects are accompanied by craniofacial, urogenital and ectodermal abnormalities. Although association with craniofacial and ectodermal abnormalities could be explained from the known functions of the disease genes, the significance of the urogenital symptoms as part of the clinical signs of SHFM remained unelucidated.[35]

In this study, we have shown the similarity in UP phenotypes of two mouse mutants associated to SHFM suggesting the necessity to investigate further the presence of urogenital defects in human SHFM-associated symptoms. In fact, some clinical cases have been reported the presence of urogenital defects, such as hypospadias in SHFM.[36,37] However, the frequency of such urogenital defects remains unclear as most of the reports mentioned such symptoms only marginally. In the case of SHFM1, no prominent reports have so far described for external genitalia defects.[38] Hence, a more careful examination of external genital phenotypes seem important in the view of our results.

Hypospadias results from the failure of the formation or fusion of the UFs. The failure of prospective UF fusion corresponds to the position of the abnormal opening of the urethra. In this study, we found that *Dlx5/6* D-KO, *p63* KO and *Bmp7* KO GTs displayed abnormal location of urethra orifice toward the scrotum. The frequency of hypospadias among total birth varies depending on the regions, but it is

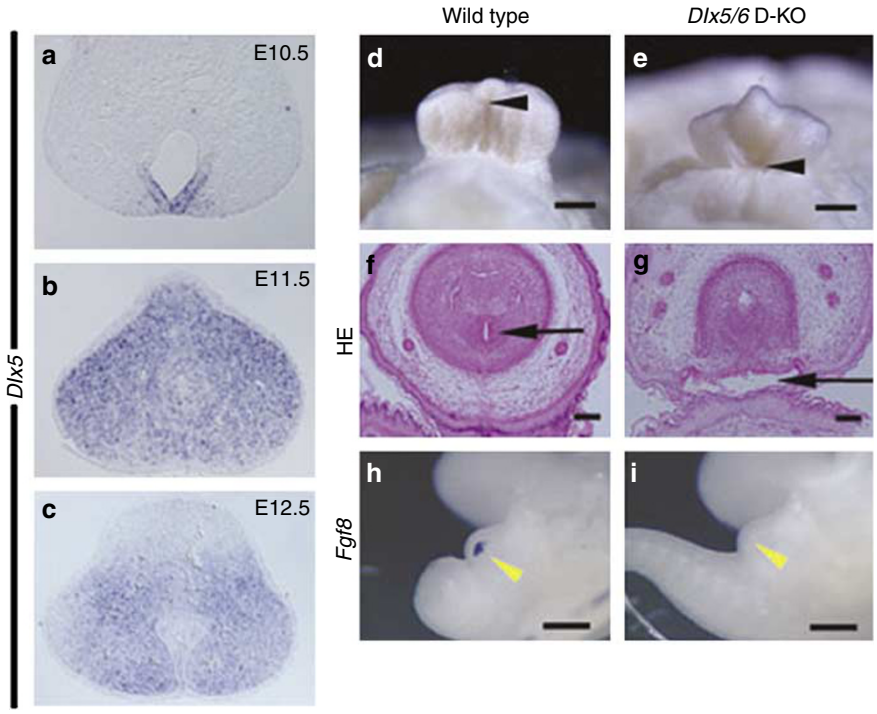


Figure 4 *Dlx5* is expressed during the UP formation and *Dlx5/6* D-KO embryos show urethral defects. (**a**–**c**) *Dlx5* is initially expressed broadly in the CM at E10.5 before GT bud emergence (**a**). *Dlx5* is expressed proximodistally (p–d) uniformly in UP and mesenchyme at E11.5 (**b**). From E12.5 onwards, its expression is restricted in the distal region of GT (**c**). (**d** and **e**) The prospective urethra orifice is located at the distal tip of the developing UP in the wild-type GTs at E18.5 (**d**). In contrast, the urethra orifice of *Dlx5/6* D-KO is located abnormally at the developing proximal UP (**e**). The arrowheads indicate the location of the urethra orifice. (**f** and **g**) A cross-section of the external genitalia at E18.5. The urethra of wild type is canalized in the glans (**f**). *Dlx5/6* D-KO GTs show defects in the formation of the urethra (**g**). The arrows indicate the position of the urethra (wild type) and the aberrant ventral groove (*Dlx5/6* D-KO embryos). (**h** and **i**) Marked decrease of *Fgf8* expression at E11.5 in the DUE (yellow arrowheads). Scale bars: 200 μm in (**d**, **e**, **h**, **i**), 100 μm in (**f**, **g**).

generally high in USA, Norway and Denmark often reaching to approximately 0.4% of the total birth.[39,40] As abnormalities during the course of UP to urethra formation may likely lead to hypospadias in newborns,[2] the current study suggests that mutation(s) of Dlx5/6, p63 and Bmp7 may be involved for the occurrence of particular hypospadias associated with SHFM. In fact, recent human genetic studies indicated the possible involvement of Bmp7 and Fgf(s) in the development of hypospadias.[41,42]

Epithelial differentiation during the UP formation of the embryonic external genitalia (GT)

One of the fundamental characters of the GT development is to develop endodermal epithelia along with GT formation, initially as CM and later forming the UP, subsequently the tubular urethra in the male GT.

Epithelial differentiation marked by a transition of immature epithelial markers, such as *K8* to a more differentiated stratification marker takes place during the UP to mature urethra formation.[4,11] The process of mature urethra formation includes multiple steps of bilateral mesenchyme growth/differentiation, initial midline fusion, epithelial remodeling and subsequent mesenchyme rearrangement.[1,4] It has been suggested that differential Keratin marker gene expressions correspond to several steps of the UP differentiation during urethra formation.[1,4] In fact, *K14* expression was detected in the ventral UP adjacent to the surface ectoderm at E12.5 stages onwards (data not shown). The ventral margin adjacent to the ventral midline shows differentiated epithelial state during the normal UP development[4] (Figure 1). *p63*, *Dlx5/6* and *Bmp7* are expressed in the GT during UP development (Figure 6). As shown in this study, *p63* and *Bmp7* are expressed in DUE with an overlapping manner during the UP development. Judged by the expression pattern and the similar symptoms, a possible developmental scenario may thus apply for p63 and Bmp7 in regulating the urethra formation. *Dlx5/6* D-KO UPs also retained the immature character during development with persisting *K8* expression. Furthermore, *Dlx5/6* D-KO and *Bmp7* KO embryonic GTs also showed the reduction of K14 gene expression (data not shown). Thus, such an abnormal epithelial status has been observed in *p63* KO, *Dlx5/6* D-KO and *Bmp7* KO GTs.

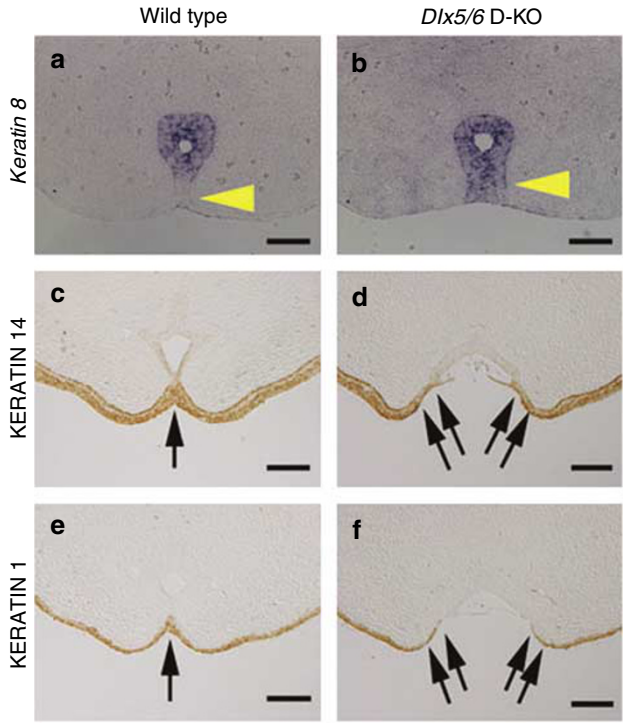
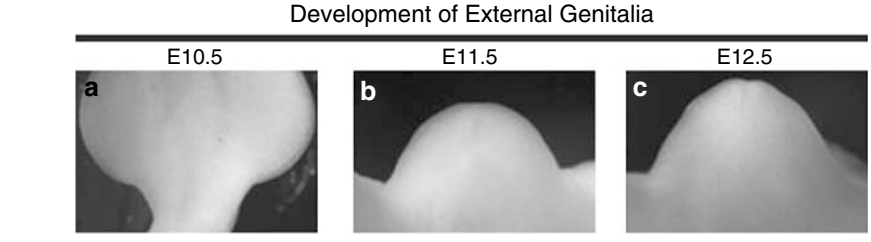


Figure 5 Abnormal epithelial differentiation of the *Dlx5/6* D-KO embryos. (**a** and **b**) *K8* expression is not detected in the ventral margin of the UP in wild-type GTs at E12.5 (**a**: yellow arrowhead). In contrast, the *Dlx5/6* D-KO UP shows the remaining *K8* expression in the corresponding region (**b**: yellow arrowhead). (**c**–**f**) *Dlx5/6* D-KO embryonic GTs show the reduction of K14 and K1 protein expression in contrast to the wild-type GTs at E14.5 (black arrows). Scale bars: 100 μm in (**a**–**f**).

How such abnormal epithelial differentiation was induced by the above genes remains unclear. Some reports have suggested a possible transcriptional regulation of several Keratin genes for epithelial cell proliferation and/or differentiation.[43,44] Some types of Keratins can regulate cell growth through several signaling pathways.[45] Whether an alteration of Keratin gene expression, such as the case of *K8*, thus consequently affects on urethra differentiation, requires further analyses. *Dlx5/6* was shown to be initially expressed in the CM and subsequently in DUE during the GT formation from E10.5 to E11.5 in this study. The lack of *Fgf8* expression, the DUE marker, in the double mutant GTs does not necessarily indicate the absence of DUE because of the possible compensation with other growth factors expressed there. During E10.5–E11.5, DUE expressed *Fgf8*, *Dlx5/6*, *p63* and *Bmp7*. This initial *Dlx5/6* expression suggests that DUE formation may contribute to the entire prospective UP, which will eventually give rise to the UP phenotypes. According to this hypothesis, DUE might thus eventually contribute to the entire P-D aspect of the UP formation, which needs to be studied by epithelial lineage studies in the future. Some previous reports described such early staged UP as a cloacal plate, implying that such an early staged GT is composed with uniform immature endodermal epithelia.[46] Irrespective of these interpretations, the UP defects in the *p63* KO, *Dlx5/6* D-KO and *Bmp7* KO GTs provide research materials for investigating the developmental mechanisms of vertebrate urethra formation and the onset of hypospadias. Similar UP defects of epithelial differentiation in *p63* KO, *Bmp7* KO and *Dlx5/6* D-KO GTs are striking considering the different nature of these genes. Although the frequency of SHFM with hypospadias represents not so frequent among the total hypospadias, an indication of transcription factors and



	E10.5 epithelia (CM)	E10.5 mesenchyme	E11.5 epithelia (UP)	E11.5 mesenchyme	E12.5 epithelia (UP)	E12.5 mesenchyme
p63	+	-	+	-	+	-
Dlx5/6	+	-*1	+	+	+*2	+*2
Bmp7	+	-	+	-	+	+

Figure 6 An illustration showing the kinetics of GT development with the list of gene expression pattern for the developing epithelia and mesenchyme of GTs. (**a**–**c**) The upper figures display the developmental morphologies of the mouse GT at E10.5, E11.5 and E12.5. The lower table represents the state of expression of developmental genes, p63, Dlx5/6 and Bmp7, in the epithelia and mesenchyme of GTs. +: expressed, −: not expressed, *1: faint expression in the mesenchyme, *2 restricted expression in the distal region.

Bmp7 for regulating urethra development will offer a unique candidate developmental cascade for further investigation.

Putative genetic cascades including Bmp7 during the GT development

One of the key distal epithelial regulators, Bmp7, was suggested as a downstream candidate gene of p63. Bmp7 has been suggested to regulate epithelial differentiation during embryonic development,[27,28] although its function during GT formation has remained unknown.

p63 has been implicated in many processes of epithelization during organogenesis. In the case of p63-related gene, p53, multiple downstream target genes for regulating cell growth/differentiation have been suggested.[47] In the case of p63, some candidate genes for the regulation of epithelialization or differentiation have been suggested.[13,14,48] Recently, the Thesleff group suggested that Bmp7 is located downstream of p63 during tooth formation providing evidence for its vital role during tooth formation.[29] The signaling epithelia for tooth formation, the enamel knot, expresses several growth factors, for example, *Bmp7*, *Bmp2*, *Shh*, *Fgf4* in addition to several transcription factors.[49] In the case of DUE, a similar but different set of growth factors are expressed, including, for example, *Fgf8*, *Bmp7* and *Fgf9*.[6,8,50] As epithelial (DUE)-mesenchymal interactions have been suggested in the distal GT region with several Bmps involved in such interactions,[8,10] it is possible that in addition to the identified role of Bmp7 for epithelial differentiation, its mesenchymal influences also existed. As for human developmental anomalies, whether Bmp7 is involved for or can modify the occurrence of SHFM remains unknown. *Bmp7* KO analysis on its null mutation reported subtle digit phenotypes, which are different from those of p63.[51,52] In sum, this study offers a unique developmental context with set of different transcription factors and growth factors in external genitalia formation. Because of the multiple phenotypic complexity and many candidate genes, detailed genetic analyses are very difficult by human genetic studies. The study should be further pursued to better understand the mechanisms of human heritable limbs/GT disorders.

Acknowledgements

Invaluable support from Liz Robertson is appreciated. We thank Drs Giorgio R Merlo, Alex Joyner, Hisayo Nishida, Mingjun Sun, Shigeaki Kato, Denis Duboule, Chi-Chung Hui, Gail Martin, John McLachlan, Anne M Moon, Sawako Fujikawa, Kenta Sumiyama, Yoshihiko Satoh and Yukiko Ogino for encouragement and helps. We express our appreciation to Shiho Kitagawa for her valuable assistance. This study was supported by a Grant-in-Aid for Scientific Research on Priority Areas; General promotion of Cancer research in Japan, by a Grant-in-Aid for Scientific Research on Priority Areas; Mechanisms of Sex Differentiation, by the Global COE Research Program and by a Grant for Child Health and Development (17-2) from the Ministry of Health, Labour and Welfare. GL is supported by the grant 'GENDACTYL' of the French Agence National pour la Recherche (ANR). OB is supported by the Telethon (Italy) grant GP0218Y01.

References

1 Yamada G, Satoh Y, Baskin LS, Cunha GR: Cellular and molecular mechanisms of development of the external genitalia. *Differentiation* 2003; **71**: 445–460.
2 Yamada G, Suzuki K, Haraguchi R *et al*: Molecular genetic cascades for external genitalia formation: an emerging organogenesis program. *Dev Dyn* 2006; **235**: 1738–1752.
3 Huang WW, Yin Y, Bi Q *et al*: Developmental diethylstilbestrol exposure alters genetic pathways of uterine cytodifferentiation. *Mol Endocrinol* 2005; **19**: 669–682.
4 Baskin LS, Erol A, Jegatheesan P, Li Y, Liu W, Cunha GR: Urethral seam formation and hypospadias. *Cell Tissue Res* 2001; **305**: 379–387.
5 Haraguchi R, Mo R, Hui C *et al*: Unique functions of Sonic hedgehog signaling during external genitalia development. *Development* 2001; **128**: 4241–4250.
6 Haraguchi R, Suzuki K, Murakami R *et al*: Molecular analysis of external genitalia formation: the role of fibroblast growth factor (Fgf) genes during genital tubercle formation. *Development* 2000; **127**: 2471–2479.
7 Perriton CL, Powles N, Chiang C, Maconochie MK, Cohn MJ: Sonic hedgehog signaling from the urethral epithelium controls external genital development. *Dev Biol* 2002; **247**: 26–46.
8 Suzuki K, Bachiller D, Chen YP *et al*: Regulation of outgrowth and apoptosis for the terminal appendage: external genitalia development by concerted actions of BMP signaling. *Development* 2003; **130**: 6209–6220.
9 Yamaguchi TP, Bradley A, McMahon AP, Jones S: A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. *Development* 1999; **126**: 1211–1223.
10 Morgan EA, Nguyen SB, Scott V, Stadler HS: Loss of Bmp7 and Fgf8 signaling in Hoxa13-mutant mice causes hypospadia. *Development* 2003; **130**: 3095–3109.
11 Kurzrock EA, Baskin LS, Cunha GR: Ontogeny of the male urethra: theory of endodermal differentiation. *Differentiation* 1999; **64**: 115–122.
12 Kurzrock EA, Baskin LS, Li Y, Cunha GR: Epithelial–mesenchymal interactions in development of the mouse fetal genital tubercle. *Cells Tissues Organs* 1999; **164**: 125–130.
13 Yang A, Schweitzer R, Sun D *et al*: p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* 1999; **398**: 714–718.
14 Mills AA, Zheng B, Wang XJ, Vogel H, Roop DR, Bradley A: p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* 1999; **398**: 708–713.
15 Basel D, Kilpatrick MW, Tsipouras P: The expanding panorama of split hand foot malformation. *Am J Med Genet A* 2006; **140**: 1359–1365.
16 Simeone A, Acampora D, Pannese M *et al*: Cloning and characterization of two members of the vertebrate Dlx gene family. *Proc Natl Acad Sci USA* 1994; **91**: 2250–2254.
17 Crackower MA, Scherer SW, Rommens JM *et al*: Characterization of the split hand/split foot malformation locus SHFM1 at 7q21.3–q22.1 and analysis of a candidate gene for its expression during limb development. *Hum Mol Genet* 1996; **5**: 571–579.
18 Merlo GR, Paleari L, Mantero S *et al*: Mouse model of split hand/foot malformation type I. *Genesis* 2002; **33**: 97–101.
19 Robledo RF, Rajan L, Li X, Lufkin T: The Dlx5 and Dlx6 homeobox genes are essential for craniofacial, axial, and appendicular skeletal development. *Genes Dev* 2002; **16**: 1089–1101.
20 Panganiban G: Distal-less function during *Drosophila* appendage and sense organ development. *Dev Dyn* 2000; **218**: 554–562.

21 Merlo GR, Zerega B, Paleari L, Trombino S, Mantero S, Levi G: Multiple functions of Dlx genes. *Int J Dev Biol* 2000; **44**: 619–626.
22 Zerucha T, Ekker M: Distal-less-related homeobox genes of vertebrates: evolution, function, and regulation. *Biochem Cell Biol* 2000; **78**: 593–601.
23 Ghanem N, Jarinova O, Amores A *et al*: Regulatory roles of conserved intergenic domains in vertebrate Dlx bigene clusters. *Genome Res* 2003; **13**: 533–543.
24 Stock DW, Ellies DL, Zhao Z, Ekker M, Ruddle FH, Weiss KM: The evolution of the vertebrate Dlx gene family. *Proc Natl Acad Sci USA* 1996; **93**: 10858–10863.
25 Park BK, Sperber SM, Choudhury A *et al*: Intergenic enhancers with distinct activities regulate Dlx gene expression in the mesenchyme of the branchial arches. *Dev Biol* 2004; **268**: 532–545.
26 Ianakiev P, Kilpatrick MW, Toudjarska I, Basel D, Beighton P, Tsipouras P: Split-hand/split-foot malformation is caused by mutations in the p63 gene on 3q27. *Am J Hum Genet* 2000; **67**: 59–66.
27 Godin RE, Takaesu NT, Robertson EJ, Dudley AT: Regulation of BMP7 expression during kidney development. *Development* 1998; **125**: 3473–3482.
28 Zeisberg M, Hanai J, Sugimoto H *et al*: BMP-7 counteracts TGF-beta1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. *Nat Med* 2003; **9**: 964–968.
29 Laurikkala J, Mikkola ML, James M, Tummers M, Mills AA, Thesleff I: p63 regulates multiple signalling pathways required for ectodermal organogenesis and differentiation. *Development* 2006; **133**: 1553–1563.
30 Beverdam A, Merlo GR, Paleari L *et al*: Jaw transformation with gain of symmetry after Dlx5/Dlx6 inactivation: mirror of the past? *Genesis* 2002; **34**: 221–227.
31 Temtamy SA, McKusick VA: The genetics of hand malformations. *Birth Defects Orig Artic Ser* 1978; **14** (i–xviii): 1–619.
32 Faiyaz ul Haque M, Uhlhaas S, Knapp M *et al*: Mapping of the gene for X-chromosomal split-hand/split-foot anomaly to Xq26–q26.1. *Hum Genet* 1993; **91**: 17–19.
33 Nunes ME, Schutt G, Kapur RP *et al*: A second autosomal split hand/split foot locus maps to chromosome 10q24–q25. *Hum Mol Genet* 1995; **4**: 2165–2170.
34 Del Campo M, Jones MC, Veraksa AN *et al*: Monodactylous limbs and abnormal genitalia are associated with hemizygosity for the human 2q31 region that includes the HOXD cluster. *Am J Hum Genet* 1999; **65**: 104–110.
35 O'Quinn JR, Hennekam RC, Jorde LB, Bamshad M: Syndromic ectrodactyly with severe limb, ectodermal, urogenital, and palatal defects maps to chromosome 19. *Am J Hum Genet* 1998; **62**: 130–135.
36 Giltay JC, Wittebol-Post D, van Bokhoven H, Kastrop PM, Lock MT: Split hand/split foot, iris/choroid coloboma, hypospadias and subfertility: a new developmental malformation syndrome? *Clin Dysmorphol* 2002; **11**: 231–235.
37 Garcia-Ortiz JE, Banda-Espinoza F, Zenteno JC, Galvan-Uriarte LM, Ruiz-Flores P, Garcia-Cruz D: Split hand malformation, hypospadias, microphthalmia, distinctive face and short stature in two brothers suggest a new syndrome. *Am J Med Genet A* 2005; **135**: 21–27.
38 Elliott AM, Evans JA, Chudley AE: Split hand foot malformation (SHFM). *Clin Genet* 2005; **68**: 501–505.
39 Baskin LS (ed.): Hypospadias and genital development. *Adv Exp Med Biol* 2004 Philadelphia: Kluwer Academic/Plenum Publication.
40 Baskin L: Hypospadias: a critical analysis of cosmetic outcomes using photography. *BJU Int* 2001; **87**: 534–539.
41 Beleza-Meireles A, Lundberg F, Lagerstedt K *et al*: FGFR2, FGF8, FGF10 and BMP7 as candidate genes for hypospadias. *Eur J Hum Genet* 2007; **15**: 405–410.
42 Chen T, Li Q, Xu J *et al*: Mutation screening of BMP4, BMP7, HOXA4 and HOXB6 genes in Chinese patients with hypospadias. *Eur J Hum Genet* 2007; **15**: 23–28.
43 Corcoran JP, Ferretti P: Keratin 8 and 18 expression in mesenchymal progenitor cells of regenerating limbs is associated with cell proliferation and differentiation. *Dev Dyn* 1997; **210**: 355–370.
44 Casanova ML, Bravo A, Ramirez A *et al*: Exocrine pancreatic disorders in transsgenic mice expressing human keratin 8. *J Clin Invest* 1999; **103**: 1587–1595.
45 Paramio JM, Segrelles C, Ruiz S, Jorcano JL: Inhibition of protein kinase B (PKB) and PKCzeta mediates keratin K10-induced cell cycle arrest. *Mol Cell Biol* 2001; **21**: 7449–7459.
46 Penington EC, Hutson JM: The urethral plate – does it grow into the genital tubercle or within it? *BJU Int* 2002; **89**: 733–739.
47 Wei CL, Wu Q, Vega VB: A global map of p53 transcription-factor binding sites in the human genome. *Cell* 2006; **124**: 207–219.
48 Koster MI, Kim S, Mills AA, DeMayo FJ, Roop DR: p63 is the molecular switch for initiation of an epithelial stratification program. *Genes Dev* 2004; **18**: 126–131.
49 Vaahtokari A, Aberg T, Jernvall J, Keranen S, Thesleff I: The enamel knot as a signaling center in the developing mouse tooth. *Mech Dev* 1996; **54**: 39–43.
50 Satoh Y, Haraguchi R, Wright TJ *et al*: Regulation of external genitalia development by concerted actions of FGF ligands and FGF receptors. *Anat Embryol (Berlin)* 2004; **208**: 479–486.
51 Luo G, Hofmann C, Bronckers AL, Sohocki M, Bradley A, Karsenty G: BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. *Genes Dev* 1995; **9**: 2808–2820.
52 Dudley AT, Lyons KM, Robertson EJ: A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. *Genes Dev* 1995; **9**: 2795–2807.