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ARTICLE

Genetic analysis of the role of *Alx4* in the coordination of lower body and external genitalia formation

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Although several syndromes include abnormalities of both the ventral body wall and external genitalia, the developmental bases of this correlation are largely unknown. Naturally occurring mutations in *Aristaless-like 4* (*Alx4, Strong's luxoid*: $Alx4^{Lst}$) have ventral body wall and pelvic girdle abnormalities. We sought to determine whether the development of the genital tubercle (GT) and its derivatives, the external genitalia, is affected by this mutation. We thus performed genetic and tissue labeling analyses in mutant mice. $Alx4^{Lst/Lst}$ mutants displayed hypoplasia of the dorsal GT and reduced expression of Fibronectin. We analyzed cell migration during GT formation by tissue labeling experiments and discovered that the cells located in the proximal segment of the umbilical cord (infra-umbilical mesenchyme) migrate toward the dorsal part of the GT. The $Alx4^{Lst/Lst}$ mutants also displayed augmented expression of Hh signal-related genes. Hence, we analyzed a series of combinatorial mutants for *Alx4*, *Sonic hedgehog* (*Shh*) and *GLI-Kruppel family member 3* (*Gli3*). These phenotype–genotype analyses suggested a genetic interaction between *Alx4* and Hh signaling during GT formation. Moreover, Hh gain-of-function mutants phenocopied some of these phenotypes. These observations reveal novel information regarding the pathogenic mechanisms of syndromic lower ventral body malformations, which are largely unknown.
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INTRODUCTION

Organogenesis is a developmentally coordinated process such that the adjacent tissues and structures influence one another's morphogenesis. For instance, bladder exstrophy (OMIM: %600057) is associated not only with defects in the bladder wall but also in the external genitalia and the pelvic girdle.[1–4] The ventral body wall defect called omphalocele is also associated with cardiac and anorectal malformations in more than 50% of cases.[5,6] Although methodologies for genetic analysis have been improved recently, the pathogenic mechanisms of such syndromic malformations are unclear.[7–9]

The genital tubercle (GT) is the anlage of the external genitalia in both males and females. The GT is composed of a urethral plate/tube derived from the cloacal membrane together with the mesenchyme and the outer ectoderm.[10] Congenital abnormalities frequently result from abnormal GT formation and its subsequent development. A common defect is hypospadias, in which the urethra opens aberrantly into the ventral GT.[11] Recent studies have identified the causative genes and signaling pathways related to hypospadias-like pathogenesis, including Hedgehog (Hh) and Wnt.[12–19] On the other hand, epispadias is a congenital abnormality reflecting aberrant development of the dorsal part of the GT. Patients with epispadias often present with other abnormalities such as bladder exstrophy.[2,3] The incidence of dorsal GT abnormalities including epispadias (2.4 in 100 000) is rare relative to hypospadias (1 per 300 live births).[2,4] Hence, the pathogenic mechanisms underlying dorsal GT hypoplasia are poorly understood and require investigation.

The *Aristaless-like homeobox 4* (*Alx4*) is an essential gene for skull and appendage development in humans and mouse.[20–23] Several heterozygous and homozygous mutations in the human *ALX4* gene have been identified and the human phenotypes vary with the type of mutations.[22–24] Common defects observed with *ALX4* mutations in both heterozygotes and homozygotes are parietal foramina, which are oval defects of the parietal bones. Affected individuals also display alopecia, coronal craniosynostosis, hypertelorism, severely depressed nasal bridge and ridge, bifid nasal tip, hypogonadism/cryptorchidism, callosal body agenesis and/or mental retardation.[25] Several mouse *Alx4* mutants have also been reported.[26] Representative phenotypes include preaxial polydactyly, hemimelia of the tibia, craniofacial abnormalities (reduction of the parietal and frontal bones), eyelid abnormalities, ventral body wall defects, cryptorchidism and alopecia.[20,21,26]

One of the *Alx4* mouse mutants, $Alx4^{Lst}$ (*Strong's luxoid: Lst*^{l}), is a naturally occurring 16-bp deletion in the homeobox-encoding domain.[26,27] Such deletion disrupts the DNA recognition helix and results in a frame-shift of the downstream protein-coding sequence.[26] We previously utilized combinatorial mutants of $Alx4^{Lst}$, $Gli3^{Xt}$ and

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Shh to show that omphalocele pathogenesis likely relates to disruption of the ventral body wall and pelvic girdle formation.[28] Here we investigate the correlation of these phenotypes with the broad spectrum of lower body abnormalities, including GT morphogenesis. We analyzed the function of *Alx4* during GT formation with a series of genetically modified mouse models and tissue labeling experiments. We found that the *Alx4*^{Lst} compound mutants display dorsal GT hypoplasia and some of the above defects could be remedied by modulating Hh signaling. In addition, tissue labeling and extirpation experiments revealed that mesenchymal cells located in the proximal umbilical cord normally migrate to the dorsal GT. These results suggest an essential interaction between *Alx4* function and Hh signaling for coordinated development of lower ventral body structures.

MATERIALS AND METHODS

Mice

The mutant mice used herein were *Shh*, *Gli3*^{Xt} (*Xt*^{J}), *Alx4*^{Lst} (*Lst*^{J}), *CAGGS-CreER*^{TM} and *Rosa26-SmoM2*.[26,27,29–32] The genotypes of each strain were determined as reported previously. To obtain *Alx4*^{Lst}*; Gli3*^{Xt}*; Shh* combinatorial mutants, single, double or triple heterozygous male and female mice were crossed. Noon of the day when the vaginal plug was identified was considered as embryonic day 0.5 (E0.5). All experimental procedures and protocols were approved by the Committees on Animal Research at Wakayama Medical University and at Kumamoto University.

Histological analyses

Mouse embryos were fixed overnight in 4% paraformaldehyde (Sigma, St Louis, MO, USA) with phosphate-buffered saline, dehydrated through methanol, embedded in paraffin, and 8 μm serial sections were prepared. Hematoxylin and eosin staining was performed using standard procedures.[14,17] Immunohistochemical analysis was performed by standard procedures using anti-fibronectin antibody (1:500, Sigma). Signal amplification was performed using VECTASTAIN ABC Rabbit IgG Kit (Vector Laboratories, Burlingame, CA, USA) and the immunocomplexes were detected with DAB staining.

Statistical analysis

For the statistical analyses of the length between the umbilical cord and the GT, the length was measured with a slide gauge. Data were analyzed using Student's *t*-test (two tailed). $P<0.001$ was considered to be statistically significant. Values are given as means ± SD.

In situ hybridization

Whole-mount and section *in situ* hybridizations for gene expression analyses were performed as previously described.[28] The antisense riboprobe templates have been described previously: *Alx4* (kindly provided by Dr Toshihiko Shiroishi and Dr Frits Meijlink), transcription factor AP-2 alpha (*Ap2α*) (kindly provided by Dr Chi-chung Hui), *Mab-21* (male abnormal 21: *C. elegans*) like 2 (*Mab21l2*),[33] paired-like homeodomain transcription factor 1 (*Pitx1*) (kindly provided by Dr YiPing Chen), fibroblast growth factor 8 (*Fgf8*) (kindly provided by Dr Brigid LM Hogan), Sonic hedgehog (*Shh*) (kindly provided by Dr Chisa Shukunami) and Patched homolog 1 (*Ptc1*) (kindly provided by Dr Jun Motoyama).

Hh signaling gain-of-function analysis

Rosa26-SmoM2 (*R26-SmoM2*) homozygous female mice were crossed with male mice bearing the tamoxifen-inducible *CAGGS-CreER*^{TM} transgene.[31,32,34] Pregnant *R26-SmoM2* females were treated with tamoxifen (2 mg per 40 g maternal body weight) orally using a gavage needle. No overt teratological effects of tamoxifen were observed in the control embryos under these conditions.[13–15]

DiI tissue labeling

The harvested embryos at E12.0 were embedded in saline with 3% agarose and sectioned sagittally using a vibratome. The DiI solution (0.01% DiI dissolved in dimethylsulfoxide) was injected into the proximal umbilical mesenchyme with a glass needle.

Explanted tissue cultures

DiI-labeled and dissected tissue samples were cultured for 36 or 48 h on ISOPORE membrane filters (Millipore, Bedford, MA, USA) in BGJb medium (Gibco BRL, Carlsbad, CA, USA) with 10 mM HEPES (pH 7.4) and 0.1 mg/ml L-ascorbic acid at 37 °C in air and 5% CO_2.

RESULTS

Alx4$^{Lst/Lst}$ mutants have hypoplasia of the dorsal GT

To gain insight into the role of *Alx4* during body wall and GT formation, we examined the expression of *Alx4* in the body wall and urogenital organs including GT in E10.5–13.5 wild-type embryos. *Alx4* expression was observed around the proximal umbilical cord region at E10.5, before GT protrusion (Figures 1a and a′). At E11.5, *Alx4* expression was prominent at the dorsal GT mesenchyme and the ventral body wall region (Figures 1b and b′). Expression in the proximal umbilical mesenchyme persisted until E12.5 and then gradually decreased later (Figures 1c′ and d′). Expression in the dorsal GT mesenchyme was observed at E12.5 and E13.5 (Figures 1c–d′).

Urogenital phenotypes of *Alx4*$^{Lst/Lst}$ mutants were apparent by E11.5 (Figures 1e and j). The proximal umbilical mesenchyme and dorsal GT were significantly hypoplastic in *Alx4*$^{Lst/Lst}$ mutants at E11.5 and E12.5 (Figures 1j and k; blue arrowheads). This hypoplastic region corresponds to an *Alx4* expression domain (Figures 1b′ and c′). In the late fetal stages, *Alx4*$^{Lst/Lst}$ mutants displayed pelvic girdle abnormalities and ventral body wall defects such as omphalocele, thin body wall and bladder wall hypoplasia (Figure 1l; blue arrow, data not shown). The dorsal GT was hypoplastic in the mutants at E18.5 (Figures 1l–n; red arrowheads). *Alx4*$^{Lst/Lst}$ mutants also had reduced size of glans penis and dorsal preputial tissues (Figures 1l–n). These results indicate that *Alx4* function is essential for the normal formation of the caudal regions of the embryo.

Expression of several differentiation markers is altered in *Alx4*$^{Lst/Lst}$ mutants

Ap2α function is known to be essential for the formation of the ventral body wall and GT.[35–37] The *Ap2α*-positive domain in the dorsal GT mesenchyme was smaller in *Alx4*$^{Lst/Lst}$ mutants compared with controls at E12.0 (Figures 2a and b; red arrowheads). *Pitx1* gene is also expressed in the dorsal GT and proximal umbilical mesenchyme (Figure 2c). Such expression was retained at a high level in the dorsal GT and reduced in the proximal umbilical mesenchyme of *Alx4*$^{Lst/Lst}$ mutants (Figure 2d; red arrowheads). Expression of *Mab21l2* gene and fibronectin protein (detected using immunohistochemistry) was markedly decreased throughout the GT in *Alx4*$^{Lst/Lst}$ mutants (Figures 2e–h; red arrowheads). *Mab21l2* gene is expressed in the proximal umbilical mesenchyme and the dorsal GT at E12.5 (Figure 2e). Congenital body wall defects due to *Mab21l2* mutation have been reported.[33]

Previous reports have suggested that *Alx4* gene function is related to Hh signaling, such as in the developing limb.[20,27,28,38,39] Hence, we analyzed the expression of genes in the Hh pathway. The expression of *Shh*, one of the Hh ligands, was increased in the mutant cloacal epithelium (Figure 2j; red arrow). *Ptc1* encodes the receptor for Hh ligands, and is a downstream target of Hh signaling;[40,41] its expression

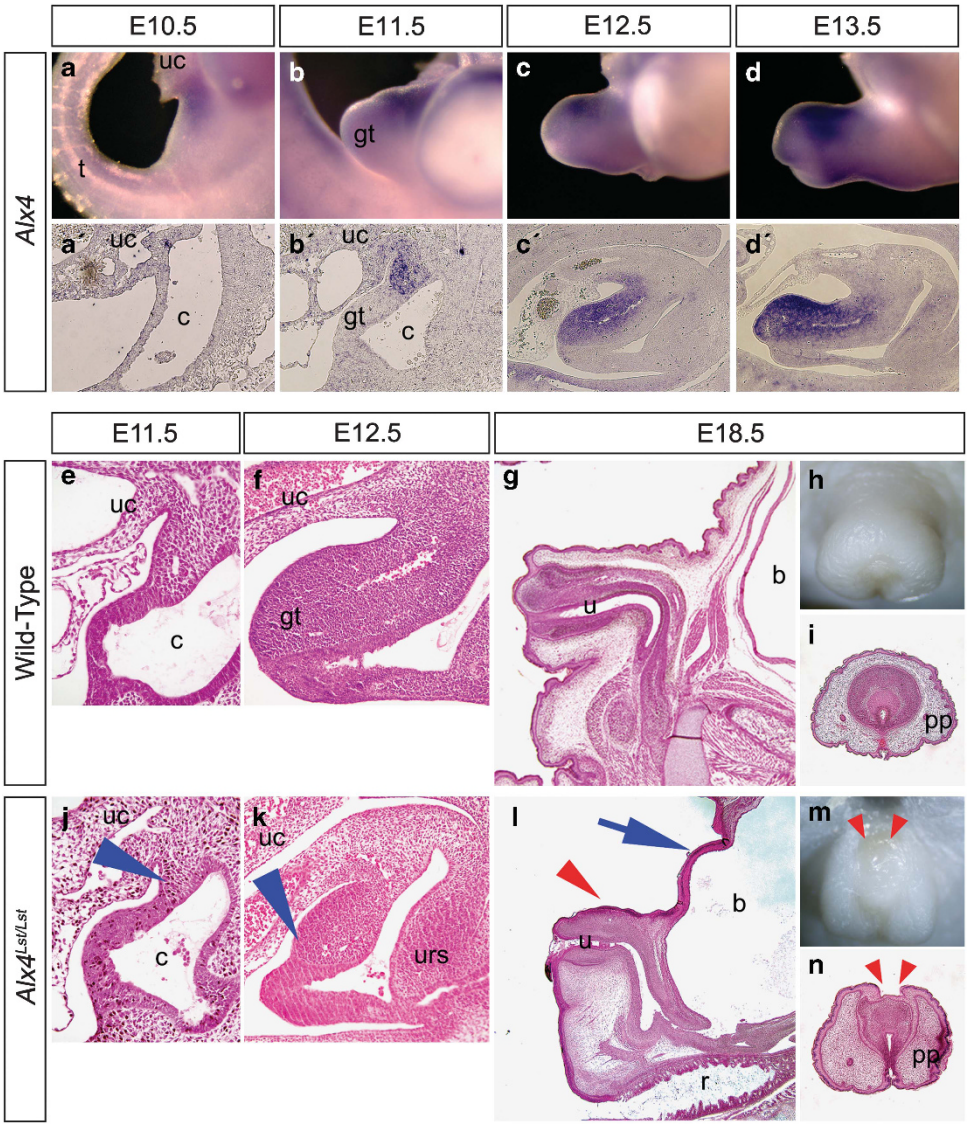

Figure 1 Expression of *Alx4* and urogenital abnormalities of *Alx4*$^{Lst/Lst}$ mutants. Lateral view of *Alx4* gene expression in whole-mount embryos at E10.5-E13.5 (**a**–**d**). *Alx4* expression in sagittal sections at E10.5-E13.5 (**a**′–**d**′). Sagittal sections of urogenital organs of wild-type embryos (**e**–**g**) and *Alx4*$^{Lst/Lst}$ mutants (**j**–**l**) at E11.5, E12.5 and E18.5. Blue arrowheads indicate dorsal hypoplasia of the GT in *Alx4*$^{Lst/Lst}$ mutants. Sagittal sections (**g**, **l**), frontal view (**h**, **m**) and coronal sections (**i**, **n**) of the GT in E18.5 embryos. Red arrowheads in **l**–**n** indicate hypoplastic dorsal GT in *Alx4*$^{Lst/Lst}$ mutants at E18.5. Blue arrow in **l** indicates hypoplastic ventral body wall of *Alx4*$^{Lst/Lst}$ mutants. b: bladder, c: cloaca, gt: genital tubercle, pp: prepuce, r: rectum, t: tail, u: urethra, uc: umbilical cord, urs: urorectal septum.

was also increased in the cloacal mesenchyme of the mutants (Figure 2l; red arrows).

Migration of proximal umbilical mesenchymal cells is impaired by *Alx4* mutation

We observed a significant reduction in the distance between the umbilical cord and GT in *Alx4*$^{Lst/Lst}$ mutants at E18.5 (Figure 2m; wild-type *versus* *Alx4*$^{Lst/Lst}$ mutants: 1.75 ± 0.42, $n = 10$ *versus* 0.54 ± 0.09, $n = 5$; $P < 0.001$). The distance between the fetal umbilical cord and the GT in early gestation has been suggested to be useful for early pre-natal diagnosis of congenital bladder exstrophy.[42] As Fibronectin (Fn1) is an extracellular matrix component essential for cell adhesion and migration,[43] and its expression was decreased in *Alx4*$^{Lst/Lst}$ mutants (Figure 2h; red arrowheads), the decreased distance between the umbilical cord and the GT prompted us to examine cell migration during the GT formation. We first performed tissue extirpation experiments to determine the significance of the cells in the proximal umbilical mesenchyme. The lower body tissues from E12.5 wild-type embryos with or without proximal umbilical mesenchyme were cultured in the BGJb medium (Figures 3a and c). After 48 h in culture, we examined the gross morphology of the control tissue *versus* that lacking umbilical cord (Figures 3b and d). We observed dorsal GT hypoplasia in explants cultured without proximal umbilical mesenchyme (Figure 3d; red arrow). We next performed tissue labeling experiments. Sagittally sliced tissues were labeled with DiI at E12.0 and incubated for 36 h. We observed the DiI-labeled cells distributed from the proximal umbilical mesenchyme to the dorsal part of GT (Figures 3e–f′). When we performed this

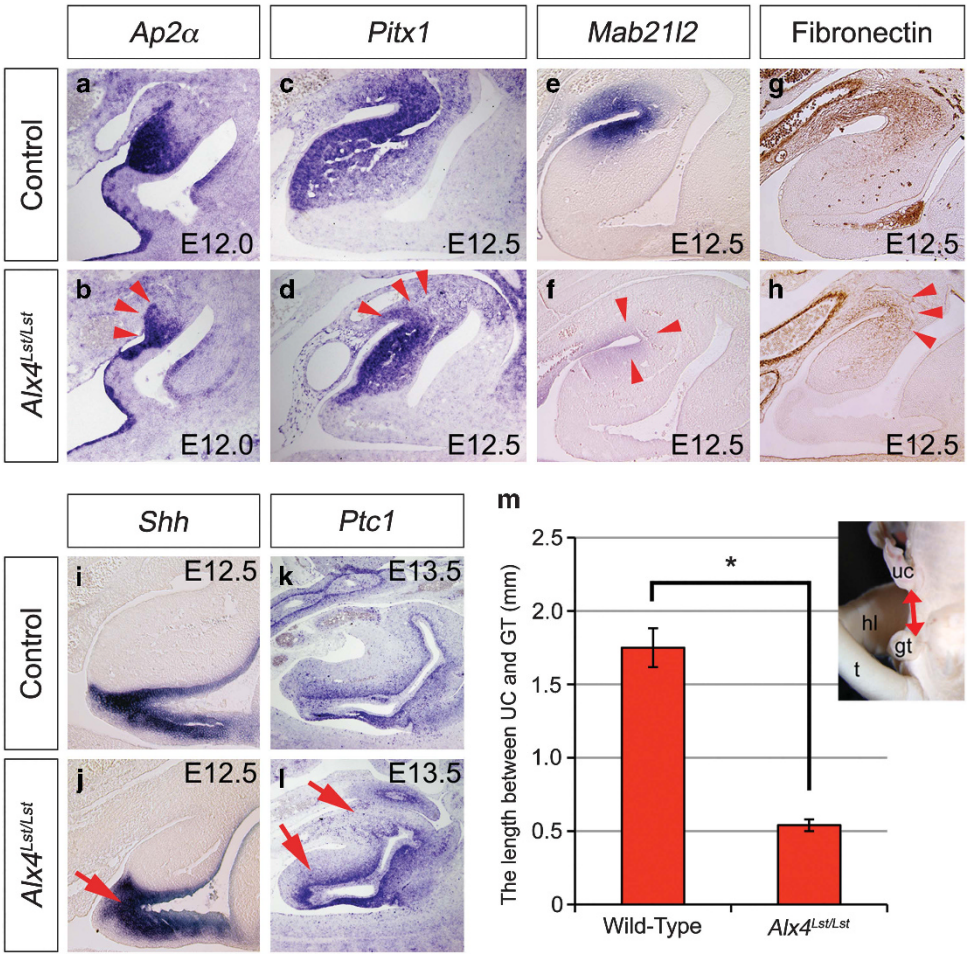

Figure 2 Expression of developmental markers in *Alx4*$^{Lst/Lst}$ mutants. Sagittal sections of the control and *Alx4*$^{Lst/Lst}$ mutants (**a–l**). Expression of *Ap2a* (**a, b**), *Pitx1* (**c, d**) and *Mab21l2* (**e**, **f**) genes was examined at E12.0 or E12.5. Immunohistochemistry to detect Fibronectin protein was performed in E12.5 sagittal sections (**g, h**). Red arrowheads indicate reduced expression of markers (**b**, **d**, **f**, **h**). *Shh* and *Ptc1* expression in sagittal sections of the control and *Alx4*$^{Lst/Lst}$ mutants at E12.5 and E13.5 (**i–l**). Red arrows indicate augmented expression of *Shh* and *Ptc1* in *Alx4*$^{Lst/Lst}$ mutants. The length between the umbilical cord and the dorsal GT was measured at E18.5 (**m**; inset). The significant difference in the length is indicated by an asterisk. Results are presented as means ± SD. *$P<0.001$. gt: genital tubercle, hl: hindlimb, t: tail, uc: umbilical cord.

labeling experiment with *Alx4*$^{Lst/Lst}$ mutants, we found that labeled cells in mutants migrated a shorter distance than those from control embryos (Figures 3g and h). These results suggest that cells in the proximal umbilical mesenchyme can migrate toward the GT mesenchyme and that this process is hampered by the *Alx4* mutation.

Genetic analysis of *Alx4* and Hh signaling genes during GT formation

The current results suggest that *Alx4* regulates urogenital organ development. We next analyzed candidate genes that may interact with *Alx4* in the developing urogenital system. Previous reports suggested a genetic interaction between *Alx4* and Hh signaling during early limb development.[38,39] We thus analyzed the genetic interaction between *Alx4* and Hh signaling in the developing urogenital system. *Fgf8* expression is a marker for the distal GT.[15,44] Conventional and conditional mutants for *Shh* gene are known to display GT hypoplasia and reduced expression of *Fgf8*, suggesting a possibility of distal GT hypoplasia.[15,17] Hence, we analyzed the expression of *Fgf8* in the GT of *Shh; Alx4*^{Lst} combinatorial mutants. In wild-type embryos, Fgf8 expression was detected in the distal GT (Supplementary Figures S1a and a′) and its expression was slightly augmented in the ventral GT of *Alx4*$^{Lst/Lst}$ mutants (Supplementary Figures S1b and b′). In contrast, *Shh*$^{-/-}$ mutants with GT agenesis did not display such expression (Supplementary Figures S1c and c′). We next examined *Fgf8* expression in double mutants of *Alx4* and *Shh*. *Fgf8* expression was restored with slight protrusion of the GT in the mutants (Supplementary Figures S1d and d′; red arrows). These genetic analyses suggest that *Alx4* can negatively regulate Hh signaling during GT formation.

Defective GT formation by aberrant Hh signaling

We then assessed the genetic interaction between *Alx4* and *Gli3*, which mainly works as a negative regulator of Hh signaling, during GT formation. The introduction of *Alx4*^{Lst} mutation into a *Gli3*$^{Xt/Xt}$ mutant background led to severe hypoplasia of the dorsal GT (Figures 4a–c′; black arrows). The *Gli3*$^{Xt/Xt}$ single mutants displayed slight hypoplasia of dorsal GT (data not shown). An additional introduction of *Alx4*^{Lst} mutation to the *Gli3*$^{Xt/Xt}$ mutant background (*Alx4*$^{Lst/+}$*; Gli3*$^{Xt/Xt}$ mutants) resulted in hypoplasia of the dorsal GT (Figure 4b; black arrow). Furthermore, double homozygous mutants

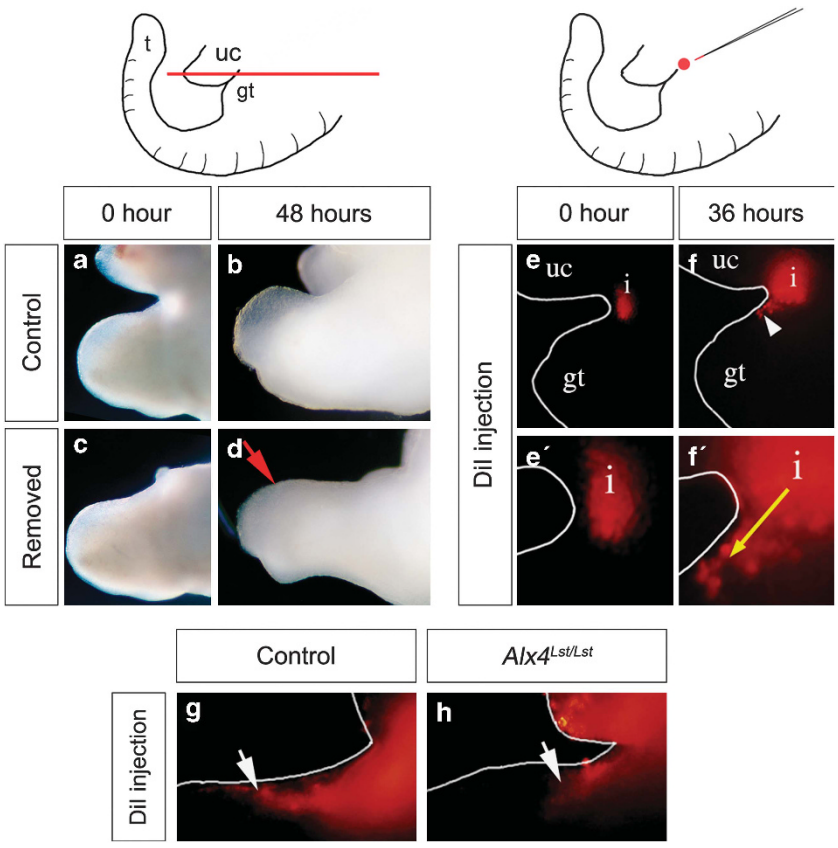

Figure 3 Tissue extirpation and cell migration of proximal umbilical mesenchyme. Surgical removal of the proximal part of umbilical cord at E12.5 (0 h) (**a**, **c**) resulted in hypoplasia of the dorsal GT (**b**, **d**; red arrow). DiI was injected into the embryonic tissue slices at E12.0 (0 h) (**e**, **e′**) and the slices were cultured for 36 h (**f**, **f′**). **e′** and **f′** are magnified views of **e** and **f**. Yellow arrow indicates the direction of migration of DiI-labeled cells. DiI-injected tissues of *Alx4*$^{Lst/Lst}$ mutants displayed reduced migration (**g**, **h**; white arrows). gt: genital tubercle, i: DiI, t: tail, uc: umbilical cord.

(*Alx4*$^{Lst/Lst}$; *Gli3*$^{Xt/Xt}$) displayed more severe hypoplasia of both dorsal and ventral GT structures (Figure 4c; black arrows). These mutants also displayed hypoplastic body wall and a shortened distance between the umbilical cord and GT at E15.5 (Figures 4a′–c′; blue arrows). The expression of several marker genes such as *Pitx1* and *Mab21l2* was reduced in the upper cloacal mesenchyme in *Alx4*$^{Lst/Lst}$; *Gli3*$^{Xt/Xt}$ mutants (Supplementary Figures S2a–d; red arrowheads).

Alx4$^{Lst/Lst}$; *Gli3*$^{Xt/+}$ mutants also displayed dorsal GT hypoplasia with ventral body wall abnormalities (Figures 4d–f; red arrows and blue arrowhead). Notably, decreasing *Shh* gene dosage partially rescued the morphology of the GT and body wall in *Alx4*$^{Lst/Lst}$; *Gli3*$^{Xt/+}$; *Shh*$^{+/-}$ mutants (Figures 4g–i). These data are all consistent with increased Shh signaling contributing to the complex phenotypes of *Alx4* mutants.

We performed complementary experiments by using *R26-SmoM2* allele, which possesses a constitutively active form of Smoothened (*SmoM2*) and a floxed stop cassette under the ubiquitous *Rosa26* promoter.[32,34] By crossing *R26-SmoM2* female mice and *CAGGS-CreER*^{TM} transgenic male mice, an augmentation of Hh signaling is achieved in embryos after induction with tamoxifen.[28] Tamoxifen treatment at E10.5 resulted in hypoplastic prepuce and uncovered the glans penis of the mutant GT at E17.5 (Figure 4k; red arrowhead). These results further support our hypothesis that excess Hh signaling could contribute to dorsal GT hypoplasia.

DISCUSSION

Although the causative genes and mutations for inherited urogenital diseases have been identified,[45] the functions of these genes in complex syndromes remain elusive. In particular, cellular mechanisms for tissue coordination during development of the urogenital organs are largely unknown, even though discoordination likely contributes to malformation of these structures. We previously reported several mutants with body wall defects associated with abdominal muscle and pelvic girdle abnormalities.[28] Here we show that *Alx4* mutants have reduced expression of *Ap2α*, *Mab21l2* and *Pitx1* genes, all of which are essential for body wall and/or pelvic girdle formation.[33,35,46] In the case of *Alx4*^{Lst}; *Gli3*^{Xt} combinatorial mutants, the severities of GT phenotypes correlate to those of the body wall and pelvic girdle. From these observations, we suggest that the GT, the ventral body wall and the pelvic girdle develop coordinately. In addition, we also showed that cell migration is crucial for proper GT formation and is a likely cellular mechanism for coordinating the development of neighboring urogenital tissues. The current study suggests part of the pathogenic mechanisms for the broad-spectrum symptoms of some clinical patients.

Bladder exstrophy (OMIM: %600057) is a complex birth defect often associated with abnormalities of the external genitalia, rectus muscles and separation of the pubic bones.[4] In addition, the umbilical cord is aberrantly located immediately above the bladder.[1] Such features resemble the phenotypes of *Alx4*$^{Lst/Lst}$ mouse mutants.

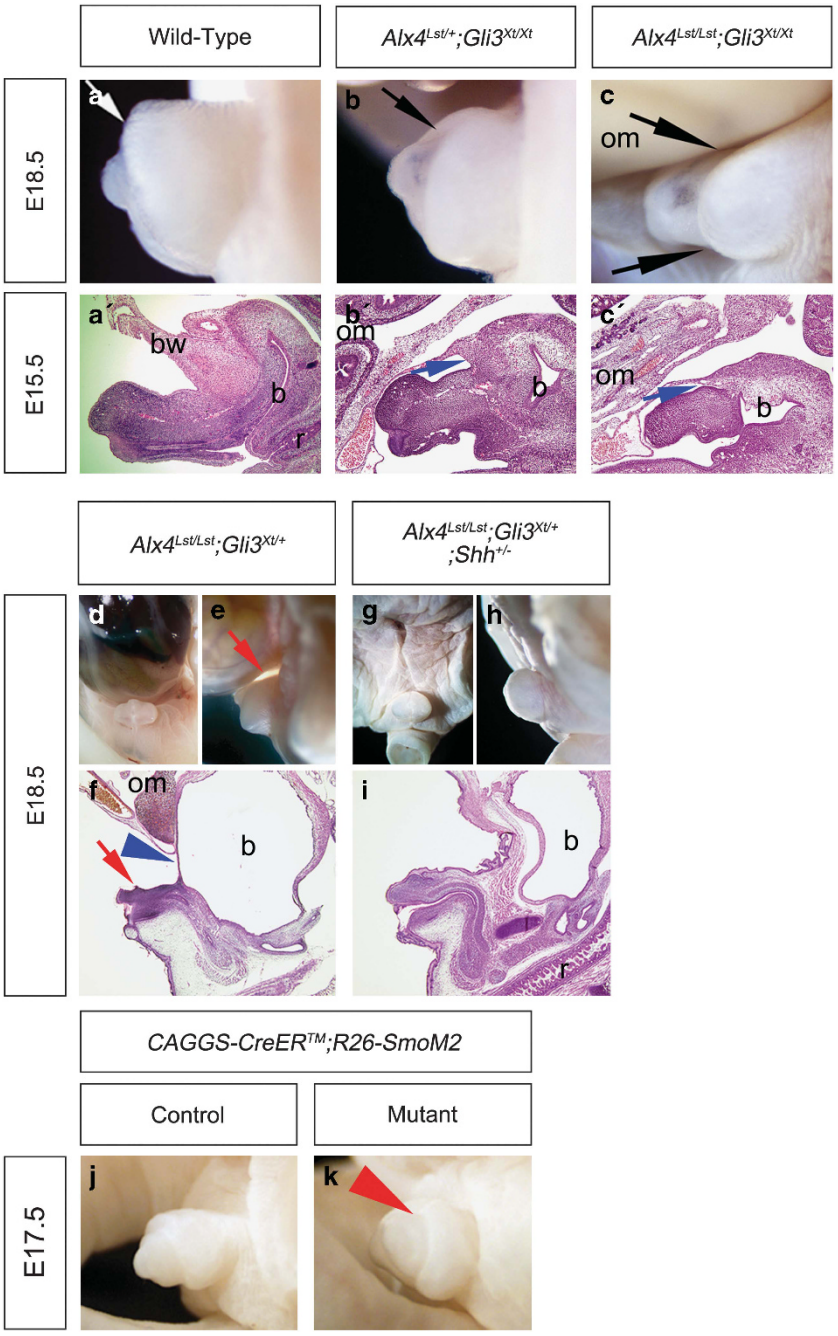

Figure 4 Genetic interaction between *Alx4* and the Hedgehog signaling pathway during lower body formation. Lateral view and sagittal sections of wild-type, $Alx4^{Lst/+}$; $Gli3^{Xt/Xt}$ and $Alx4^{Lst/Lst}$; $Gli3^{Xt/Xt}$ embryos (**a**–**c**′). Lateral view of E18.5 GT with $Alx4^{Lst}$ mutations to the $Gli3^{Xt/Xt}$ genetic background (**b**, **c**). White arrow indicates normal GT (**a**). Black arrows indicate hypoplasia of the GT (**b, c**). Sagittal sections of wild-type, $Alx4^{Lst/+}$; $Gli3^{Xt/Xt}$ and $Alx4^{Lst/Lst}$; $Gli3^{Xt/Xt}$ embryos at E15.5 (**a**′–**c**′). Blue arrows in panels **b**′ and **d**′ indicate hypoplasia of the ventral body wall. Frontal (**d, g**) and lateral (**e**, **h**) view of the GT in $Alx4^{Lst/Lst}$; $Gli3^{Xt/+}$ and $Alx4^{Lst/Lst}$; $Gli3^{Xt/+}$; $Shh^{+/-}$ mutants. $Alx4^{Lst/Lst}$; $Gli3^{Xt/+}$ mutants display omphalocele and hypoplasia of the dorsal GT at E18.5 (**d, e**). These phenotypes are rescued in $Alx4^{Lst/Lst}$; $Gli3^{Xt/+}$; $Shh^{+/-}$ mutants (**g, h**). The sagittal sections of $Alx4^{Lst/Lst}$; $Gli3^{Xt/+}$ and $Alx4^{Lst/Lst}$; $Gli3^{Xt/+}$; $Shh^{+/-}$ mutants (**f**, **i**). Red arrows in panels **e** and **f** indicate hypoplasia of the dorsal GT. Blue arrowhead indicates hypoplastic ventral body wall in $Alx4^{Lst/Lst}$; $Gli3^{Xt/+}$ mutants. Lateral view of the GT of Hh gain-of-function mutants at E17.5 (**j, k**). Red arrowhead indicates hypoplasia of prepuce (**k**). b: bladder, bw: body wall, om: omphalocele, r: rectum.

Additional phenotypes including craniofacial abnormalities have been reported in both human patients and $Alx4^{Lst/Lst}$ mouse mutants. However, human patients and mouse mutants often do not display identical phenotypes. Even in the case of human patients, there is significant variability in phenotypes resulting from different *ALX4* mutations, such as nonsyndromic craniosynostosis by V7F and K211E mutations and syndromic craniofacial abnormalities including hypertelorism and cryptorchidism (undescended testis) with homozygous R265X mutation.[25,47] A case report with deletion of chromosome 11p12 extending to bands p11.2 and p13 (*ALX4* is

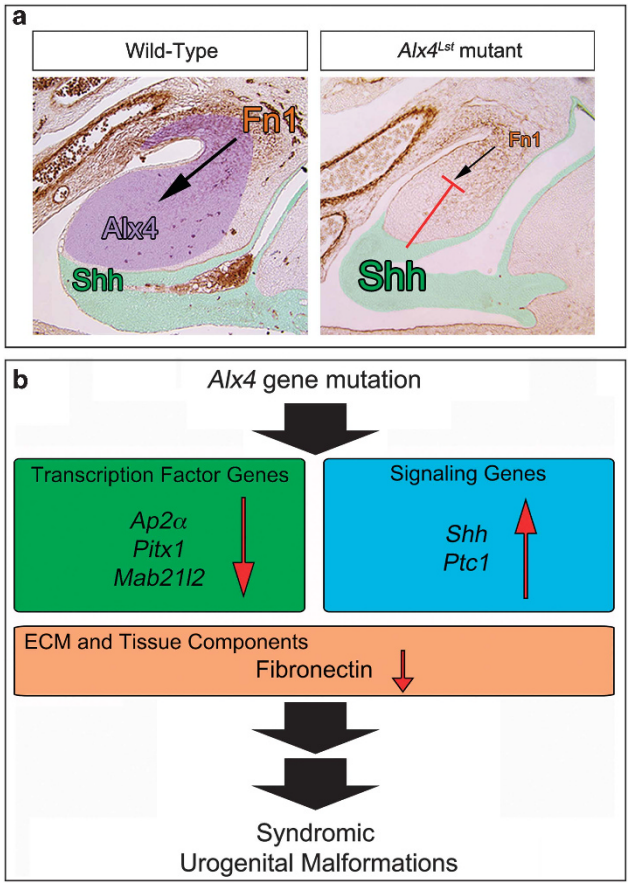

Figure 5 The summary schemes (**a**, **b**). (**a**) Brown signal indicates Fibronectin (Fn1) expression. The *Alx4*-positive region is marked in purple. *Shh* expression is marked in green. Cells in the proximal umbilical mesenchyme migrate toward the GT region in wild-type embryos (black arrow). The introduction of mutation in *Alx4* gene affects Hh signaling with reduced Fibronectin (Fn1) expression, leading to defective cell migration (right panel). (**b**) The *Alx4* mutation resulted in the reduced expression or expression area of several transcription factor genes such as *Ap2α*, *Pitx1* and *Mab21l2*. On the other hand, the expression of *Shh* and *Ptc1* was augmented. In one of the extracellular matrix and tissue components, fibronectin was also downregulated. As a result, it is suggested that *Alx4* mutants displayed syndromic urogenital malformation.

located on 11p11.2) had micropenis phenotype in addition to mild facial dysmorphism.[48] Therefore, additional, as yet unidentified, mutations such as those seen in $Alx4^{Lst}$ (Lst^{J}: a 16-bp deletion within the paired-type homeodomain, which causes a frame shift potentially producing a truncated protein at amino acid position 332) may cause urogenital syndromic malformations in humans. Additional genetic events, such as copy number variation or epigenetic modifications of *ALX4*, may also cause phenotypes in humans. Recently, genome-wide copy number variation studies have been performed for anorectal malformations and have identified some affected genes;[49] however, the importance of these findings is not yet clear. On the other hand, *ALX4* is frequently methylated in adenocarcinomas of the gastrointestinal tract.[50] Hence, such human studies may become more essential for human *ALX4* analyses.

The current analyses speculate that the loss of proximal umbilical cells and/or the disturbance of their migration toward the dorsal GT may contribute to syndromic malformations of structures derived from these tissues by utilizing *Alx4* and Hh signaling (Figure 5). Such pathogenic mechanisms are likely applicable to other genes that affect cell migration and cell number in the proximal umbilical mesenchyme. In response to the growing number of infertility patients, assisted reproductive technology such as *in vitro* fertilization is becoming increasingly common. The exstrophy–epispadias complex is known to occur more frequently in children conceived by *in vitro* fertilization.[51] We believe the current study will contribute to understanding the basis for such defects. Further clinical analyses of patients together with model animal studies will reveal the pathogenic mechanisms for human syndromic malformations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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