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Kumamoto University
Molecular analysis of external genitalia formation: the role of fibroblast growth factor (Fgf) genes during genital tubercle formation

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SUMMARY

The molecular mechanisms underlying the development of the external genitalia in mammals have been very little examined. Recent gene knockout studies have suggested that the developmental processes of its anlage, the genital tubercle (GT), have much in common with those of limb buds. The Fgf genes have been postulated as regulating several downstream genes during organogenesis. Fgf8 was expressed in the distal urethral plate epithelium of the genital tubercle (GT) together with other markers such as the Msx1, Fgf10, Hoxd13 and Bmp4 expressed in the mesenchyme. To analyze the role of the FGF system during GT formation, an in vitro organ culture system was utilized. It is suggested that the distal urethral plate epithelium of GT, the Fgf8-expressing region, regulates the outgrowth of GT. Ectopic application of FGF8 beads to the murine GT induced mesenchymal gene expression, and also promoted the outgrowth of the GT. Experiments utilizing anti-FGF neutralizing antibody suggested a growth-promoting role for FGF protein(s) in GT outgrowth. In contrast, despite its vital role during limb-bud formation, Fgf10 appears not to be primarily essential for initial outgrowth of GT, as extrapolated from Fgf10−/− GTs. However, the abnormal external genitalia development of Fgf10−/− perinatal mice suggested the importance of Fgf10 in the development of the glans penis and the glans clitoridis. These results suggest that the FGF system is a key element in orchestrating GT development.

Key words: FGF8, FGF10, Msx1, Hox, Bmp4, Genital tubercle, Clitoris, Penis, Limb, AER, Epithelial-mesenchymal interaction, Gene knockout mouse

INTRODUCTION

Recent molecular cloning of many of the genes controlling development, including growth factors and homeobox genes, has led to the formulation of basic plans of organogenesis (Kessel and Gruss, 1990; Chisaka et al., 1992; Gehring et al., 1994; Johnson and Tabin, 1997; Hogan, 1996, 1999; Gehring, 1999). During fetal and neonatal development of mammals, the last structures to develop along the embryonic anteroposterior (AP) axis are the external genitalia, organs displaying characteristic proximodistal-oriented outgrowth adjacent to the cloacal membrane (Fig. 1). The mammalian external genitalia are highly developed structures to permit efficient internal fertilization.

The external genital anlage, the genital tubercle (GT), differentiates into a penis in males and a clitoris in females (Murakami and Mizuno, 1986; Hildebrand, 1995). Because of the lack of a suitable experimental system, the mechanisms of GT formation have been very little studied by molecular developmental analysis. Pioneering study has suggested that GT development may have some similarities to limb development, with both structures exhibiting organ outgrowth (Dolle et al., 1991; Yamaguchi et al., 1999). Vertebrate limb development depends on the establishment and maintenance of the apical ectodermal ridge (AER), a specialized ectoderm region at the distal tip of the limb bud (Duboule, 1993; Tickle and Eichele, 1994; Johnson and Tabin, 1997). It has been speculated that the GT epithelium and mesenchyme develop through presumptive epithelial-mesenchymal interactions, although no discrete structures resembling an AER have been described, nor have any candidate molecules for those “AER functions” been suggested in studies of external genitalia formation (Murakami and Mizuno, 1986; Dolle et al., 1991).

Epithelial-mesenchymal interactions play an essential role in regulating a wide variety of developmental processes (Le Douarin and Jotereau, 1975; Hall 1981; Slavkin et al., 1984; Thesleff et al., 1991, 1995; Tickle and Eichele 1994; Johnson and Tabin, 1997; Hogan, 1999). Signaling between the
epithelium and the mesenchyme governs many aspects of organogenesis, from the initiation of organ development to differentiation (MacKenzie et al., 1992; Dassule and McMahon, 1998; Tucker et al., 1999). The developing limb has long served as a good model system for studying such mechanisms during organ development (Tabin, 1991; 1995; Duboule, 1993, 1994). The limb bud is initiated through the continued proliferation of cells of the lateral plate mesoderm. Signals emanating from the rapidly proliferating mesodermal cells induce the limb-bud ectoderm to form the AER. Once induced, the AER becomes essential for sustained outgrowth and the patterning of a limb by interacting with the underlying mesenchyme through molecules, including fibroblast growth factors (FGFs) (Niswander and Martin, 1992; Niswander et al., 1993, 1994; Laufer et al., 1994; Tickle and Eichele 1994; Niswander, 1999; Pizette and Niswander, 1999).

FGFs are essential signaling molecules for embryogenesis (Cohn et al., 1995; Wall and Hogan, 1995; Yamasaki et al., 1996; Belluscì et al., 1997; Martin, 1998; Ornitz, 2000). One of the Fgf family genes, Fgf8, is expressed and functions in a variety of developmental events, including gastrulation and the development of limbs, the central nervous system (CNS), the mandible and teeth (Ohuchi et al., 1994; Crossley and Martin, 1995; Crossley et al., 1996a,b; Johnson and Tabin, 1997; Lee et al., 1997; Neubüser et al., 1997; Bei and Maas, 1998). Fgf8-deficient mice show a variety of malformations, lacking all embryonic mesoderm-derived structures, including the heart and somites (Lewandoski et al., 1997; Meyers et al., 1998). Previous studies have shown that Fgf8 is important for the growth and patterning of embryonic limbs (Crossley et al., 1996b; Ohuchi et al., 1997), and an elegant recent conditional gene knockout study indicated its importance in development of the jaw (Trumpp et al., 1999). It has been suggested that Fgf10 functions in brain, lung and limb development based on its spatiotemporal expression (Yamasaki et al., 1996; Ohuchi et al., 1997), and, in support, Fgf10 knockout mice displayed drastic outgrowth defects in the limbs and died at birth due to the lack of lung development (Min et al., 1998; Sekine et al., 1999). Roles of the FGF system during the outgrowth of GT and in the subsequent development of the penis and the clitoris are examined in this paper.

MATERIALS AND METHODS

Mouse genital tubercle organ culture

ICR mice (6 week old; Japan SLC Inc) were utilized. All experiments with animals were done in accordance with the experimental animal rights guidelines of Kumamoto University. Embryos were aseptically dissected and genital tubercles were microsurgically dissected. In some cultures, the distal urethral plate epithelium of the GT was removed by a tungsten needle to eliminate the Fgf8-expressing region.

Dissected genital tubercles were oriented with the dorsal surface upward on a 5% gelatin-coated Millipore type HTTP filter in tissue culture dishes. Cultures were fed with BGM Medium (Gibco BRL) containing 0.1 mg/ml L-ascorbic acid and 10 mM Hepes (pH 7.4). Genital tubercles were maintained at the interface between the gas (5% CO2) and liquid phases of the culture, and the level of the medium was adjusted so that the tissue was immersed but not covered by the medium. After 1-4 days in culture, the genital tubercles were processed for histological analysis. For antibody inoculation, anti-FGF8 antibodies (MAB323 or AF-423-NA, R&D systems Inc, CA, USA) or control Ig class-matched Ab (PM-01020D, anti-CD90 antibody, Pharmingen) were added to the culture medium at a concentration of 50 μg/ml. Both anti-FGF8 antibodies gave same results.

Preparation of FGF8 protein beads

Recombinant mouse FGF8b protein and human FGF10 protein (423-F8 and 345-FG, R&D systems Inc, CA, USA) were used at a concentration of 1.0 mg/ml in phosphate-buffered saline (PBS). Heparin acrylic beads (Sigma H-5263) were washed with PBS and subsequently soaked overnight with the above proteins at 4°C. Control beads were treated with PBS containing 0.1% bovine albumin (Sigma, USA).

In situ hybridization for gene expression

Whole-mount in situ hybridization was performed with digoxigenin-labeled probes by standard procedures (Wilkinson, 1992). The following probes were used for in situ hybridization: Fgf8 (kindly provided by B. L. Hogan), Fgf10 (kindly provided by H. Ohuchi and N. Isho), Fgrf2 (Riken Inst.), Hoxd13 (Dolle et al., 1991), Bmp4 (Jones et al., 1991), Shh (kindly provided by C. Shukunami) and Msx1 (Satokata and Maas, 1994). Histological sectioning was performed as described previously (Belo et al., 1998).

Histological analysis

Newborn tissues were fixed in Bouin’s fixative or paraformaldehyde. They were dehydrated in graded ethanol, embedded in paraffin and sectioned. The sections were stained with Hematoxylin and Eosin.

Fgf10-deficient mice

Fgf10−/− newborns were identified as described previously and external genitalia were analyzed using standard histological procedures (Sekine et al., 1999).

RESULTS

Expression of Fgf8, Fgf10, Fgrf2 and other epithelial or mesenchymal genes during murine genital tubercle development

Before the onset of GT outgrowth, Fgf8 and Fgf10 were both expressed in the epithelium of the outermost part of the urogenital sinus at 10.5 d.p.c. (Fig. 2A,B,D,E). Following initial expression, Fgf8 was expressed at the distal region of urethral plate epithelium of the GT in embryos from 11.5 d.p.c. (Fig. 2F) and, subsequently, Fgf8 mRNA levels decreased gradually up to 14.5 d.p.c. (data not shown). Yamaguchi et al. (1999) has also partly reported expression of Fgf8 in GT.

In contrast, Fgf10 was expressed in the mesenchyme abutting the distal region of the urethral plate epithelium during the outgrowth and development of the GT.
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The outgrowth phase of GT (Fig. 2C). It was also expressed at a low level in the broad subectodermal mesenchyme (Fig. 2C). Hence, both Fgf8 expression in the distal urethral plate epithelium and mesenchymal Fgf10 expression continued through the outgrowth period of the GT from 11.5 to 14.5 d.p.c.

It is known that multiple Fgf genes are expressed in an overlapping manner during organ formation. Fgf4 expression was not evident during GT development (data not shown). FGF ligand activities are mediated by FGF receptors, which contain tyrosine kinase domains (reviewed in Johnson and Williams, 1993; Arman et al., 1998; Partanen et al., 1998). Fgfr2 was found to be expressed in the GT epithelium (shown by a black arrow in Fig. 2G) and mesenchyme (shown by white arrows).

During limb development, the epithelial-mesenchymal interaction is considered essential in the regulation of the expression of mesenchymal genes. Therefore, expression of several makers, such as the Hoxd13, Msx1 and Bmp4 genes, was examined. Intriguingly, they were expressed in the mesenchyme of the outgrowing GT from 10.5 to 14.5 d.p.c. (Fig. 2H-J). Expression of Bmp2 was not detected (data not shown). The other key signaling gene for limb patterning, sonic hedgehog (Shh), was found to be expressed in the distal region of the urethral plate epithelium where Fgf8 was also expressed (K). Urogenital expression of Shh and Bmp4 has been previously reported (Bitgood and McMahon, 1995). The expression pattern of these genes led us to investigate whether similar gene expression regulatory mechanisms occur during GT formation.

Induction of Fgf10, Msx1, Bmp4 and Hoxd13 gene expression by FGF8 protein; GT outgrowth is promoted by the distal urethral plate epithelium and FGF8 protein

To analyze GT development, a GT organ culture system was established. Genital tubercles were microsurgically removed from embryos and were oriented with the dorsal surface upward on gelatin-coated membrane filters in tissue culture dishes. Cultures were performed without serum in a modified BGJb medium to examine the effects of growth factor activities (see Materials and Methods). Genital tubercles were maintained at the interface between the gas and liquid phases of the culture so that the tissue was immersed but not covered with the medium.

GTs at several embryonic stages (from 11.5 d.p.c. up to 13.5 d.p.c.) could be cultured to a stage approximately equivalent to embryonic 14 d.p.c. Fig. 3 demonstrates the outgrowth
kinetics of the cultured GT in vitro, as compared with corresponding GTs in vivo (12.5-14.5 d.p.c.). Analysis of gene expression patterns after culture also showed GT development to be equivalent to that observed in vivo (an example of Fgf8 expression is shown in Fig. 3E,I). Thus, this organ culture system was helpful in analyzing murine GT formation.

During GT development, the role of the distal region of the urethral plate epithelium, the Fgf8-expressing region, in the regulation of the expression of several genes was first examined by cultivating GTs after microsurgically removing the region. Mesenchymal (endogenous) Fgf10 expression was markedly reduced 24 hours after the removal (Fig. 4B). In support of the above results, when FGF8 beads were transplanted to the GT mesenchyme, expression of the mesenchymal genes, Fgf10, Msx1, Bmp4 and Hoxd13, were augmented (Fig. 4C-F; compare with the endogenous signal by control beads in the opposite side of the same specimen).

In loss-of-function experiments, administering anti-FGF8 neutralizing antibody reduced mesenchymal (endogenous) Bmp4 expression 24 hours after addition (Fig. 4G,H). It is not known why the expression of other mesenchymal genes, Fgf10 and Msx1, was not inhibited under the same conditions (data not shown). Augmentation of mesenchymal gene expression (Fgf10, Msx1, Hoxd13 and Bmp4) was observed 24 hours after, but not 12 hours after, application of FGF8 beads (Fig. 4I; Bmp4 expression is the same for FGF8 and control beads). Thus, it appears that induction may be due to several sequential responses as elicited by FGF. An elegant recent experiment, using another FGF, suggested that these proteins may diffuse from beads 4 hours after being implanted (Iseki et al., 1999). More studies are required to analyze the regulation of these genes in GT mesenchyme.

To check the competence of mesenchymal responsiveness, several stages of GT explants were tested for gene expression in response to FGF8 bead transplantation. The inducibility decreased significantly in mesenchymes at 12.5 d.p.c. and 13.5 d.p.c. (data not shown). We next examined the growth-promoting role of the corresponding region and of FGF proteins in GT organ culture. The GT from 11.5 d.p.c. embryos

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Fig. 4. The distal region of urethral plate epithelium of GT, the Fgf8-expressing region, could regulate mesenchymal gene expression during GT development. Endogenous mesenchymal Fgf10 expression was significantly reduced 24 hours after removing the distal tip of the urethral plate epithelium (B) in contrast to the control (A). Augmentation of Fgf10 (C), Msx1 (D), Bmp4 (E) and Hoxd13 (F) expression in the cultured murine GTs by FGF8 beads. The GT explants from 11.5 d.p.c. embryos were cultured for 24 hours after applying the FGF8 protein beads and control beads in the same specimen (f, FGF8 beads; c, control BSA beads). To detect augmentation of gene expression, color staining reaction for in situ expression analysis was performed for 6 hours (E,F), or for 14 hours (C,D). In C,F, induction of gene expression by the beads was relatively strong, resulting in the detection of weak endogenous signals. Anti-FGF8 neutralizing antibody reduced mesenchymal (endogenous) Bmp4 expression 24 hours after addition (Fig. 4G,H; 16 hours in situ color staining). Bmp4 expression was not augmented 12 hours after FGF8 beads addition (I). Bars: (A-I) 0.5 mm.

Fig. 5. GT outgrowth is dependent on the presence of the distal region of the urethral plate epithelium. Absence of Fgf8 expression was confirmed after removing the urethral plate epithelium (A) in contrast to the control (B). The absence of the Fgf8-expressing region is indicated by an arrowhead (A). After removing the tip of the urethral epithelium (the Fgf8-expressing region), GTs from 11.5 d.p.c. embryos were cultured for 48 hours with exogenously applied FGF8 beads (D) or with control BSA beads (C). The GT outgrowth was restored by the exogenous FGF8 bead application but was not with control beads. Anti-FGF8 neutralizing antibody addition (F) inhibited GT outgrowth in contrast to the controls (E). (G) Summary of the growth inhibition or promotion rate by anti-FGF8 antibody administration or by FGF8 or FGF10 beads transplantation. The length of the outgrowth in between the position of prepuce and the distal tip of the GT was measured (length in control taken as 100%). Bars: (A-F) 0.5 mm.
were cultured for 48 hours after removing the distal urethral plate epithelium (the Fgf8-expressing region). In order to confirm the extent of the removal, absence of Fgf8 expression was checked (Fig. 5A,B). GT outgrowth was found to be dependent on the presence of the Fgf8-expressing region (Fig. 5C). In contrast, the GT with exogenously applied FGF8 beads had outgrowth restored (Fig. 5D). Experiments utilizing anti-FGF8 neutralizing antibody confirmed the growth-promoting role of FGF in GT outgrowth (Fig. 5E,F; see Discussion). The growth-promoting activities of FGF10 beads were found to be not as marked as those of FGF8 beads (Fig. 5G).

**Fgf10 gene mutation did not affect initial GT outgrowth**

Since Fgf10−/− embryos display drastic outgrowth defects in limbs (shown by an arrow in Fig. 6A; Min et al., 1998; Sekine et al., 1999), we next examined the external genitalia of Fgf10 knockout mice.

Mutant embryos did not show marked GT outgrowth defects, as judged by the size of each GT and the normal Fgf8 expression patterns at early stages (compare Fig. 6B and E (at 10.5 d.p.c.), Fig. 6C and F (at 11.5 d.p.c.)). This suggested that early expression of Fgf10 may not be essential during GT development from 10.5 to 11.5 d.p.c. Because Fgf10 knockout abolished Fgf8 gene expression in the limb bud, cross-regulatory gene expression mechanisms have been suggested previously (Min et al., 1998; Sekine et al., 1999).

During the outgrowth phase of the GT from 11.5 d.p.c. to 14.5 d.p.c., the mutant mice GTs were comparable to the wild-type GTs in proximal-distal outgrowth, however, significant growth retardation was observed from 12.5 d.p.c. (Fig. 7C,D). Following this retardation, the development of the urethral plate was also consequently affected (shown by Fgf8 expression pattern at 13.5 d.p.c. in Fig. 6D,G). These results suggested that Fgf10 mutation leads to GT growth retardation, although the phenotype is much less prominent than that observed in the limbs.

**Abnormal glans penis and glans clitoridis formation in newborn Fgf10 knockout mice**

The penis and the clitoris of the Fgf10 knockout newborn mouse was histologically examined. Characteristic morphological defects were found in the ventral (lower) side of the glans penis and the clitoridis, especially in
the urethral epithelium and prepuce (Fig. 7F,H). In normal type female newborn mouse, morphogenesis of the urethra and prepuce in the glans clitoridis is largely similar to that of penis, though the incorporation of the tubular urethral epithelium is incomplete (Fig. 7G). In mutants, tubular urethra was not formed at the glans penis and the clitoridis, and fusion of the prepuce at the ventral (lower) midline of the glans region was completely suppressed (Fig. 7F,H). It is suggested that the urethral plate epithelium remains as an epithelium on the ventral surface of the glans (Fig. 7F,H). Rudiments of corpus cavernosum glands were found in both sexes of Fgf10 mutants.

Following mesenchymal differentiation in the genital tubercle, Fgf10 was expressed in the mesenchyme adjacent to the midline urethral plate (groove) at 13.5 d.p.c., when there was no gross sexual dimorphism (Fig. 7B). These results suggested that Fgf10 might play an important role in the morphogenesis of the most distal structure of the glans penis, and of the glans clitoridis, becoming involved in the developmental process of prepuce fusion and urethral tube formation (see Discussion).

**DISCUSSION**

Organogenesis has been assumed to include several fundamental processes: firstly, the initiation of organ formation by inducing organ outgrowth, and, secondly, subsequent tissue differentiation through various mechanisms e.g. epithelial-mesenchymal interactions (Hogan, 1999). The external genitalia show marked morphological variations among vertebrates but little research has been undertaken into their development.

*Hox* gene expression in developing GT (Dolle et al., 1991) and *Hoxd13* and *Shh* expression in urogenital sinus (Oefelein et al., 1996; Podlacsek et al., 1999) have been reported. The posteriormost *Hoxd* and *Hoxa* genes are required for proper growth and patterning of GT (Kondo et al., 1997; Warot et al., 1997). Gene regulatory mechanisms of *Hox* gene expression in limbs and other tissues, including GT, suggest the presence of shared aspects of gene regulation (Kondo et al., 1998). In addition, there have been reports of human malformations of limbs and external genitalia associated with *Hox* gene mutations (Goff and Tabin, 1996; Kondo et al., 1997; Mortlock and Innis, 1997). Another line of recent molecular analysis has demonstrated that *Wnt5a* can regulate the outgrowth of the embryonic axis and facial, genital, ear and limb primordia; indicating that *Wnt5a* might be located downstream of such transcriptional regulatory genes (Yamaguchi et al., 1999).

**Expression and function of the FGF system during GT formation**

*Fgf8* was expressed in urogenital sinus and its expression gradually confined to the distal region of the urethral plate epithelium. *Fgf10* was expressed in outgrowing GT mesenchyme while urethral expression of *Fgf8* remained.

A null mutation of *Fgfr2* results in peri-implantation lethality at 4.5-5.5 d.p.c. (Arman et al., 1998, 1999), while embryos with homozygous hypomorphic alleles die around 10.5 d.p.c. with no limb buds (Xu et al., 1998). Recent elegant *Fgfr2* knockout experiments also suggest its importance during organogenesis (De Molerloose et al., 2000). Analysis of such receptor functions during GT formation awaits further analysis.

An in vitro system was used to culture GT from 11.5-13.5 d.p.c. mouse embryos up to the stage closely corresponding to 14.5 d.p.c. in vivo. The success of the culture seems to depend largely on keeping organs at the interface of the medium. Currently it is not feasible to cultivate GTs at later stages, which presumably requires additional improvements, e.g., higher doses of oxygen supply.

Other experimental methods could potentially be applicable to study GT development; however, the whole embryo culture system can only be utilized for embryos up to about 10.5 d.p.c. (Tam, 1989), and, in the case of exo utero techniques, it is technically difficult to manipulate murine GTs through the yolk sac. Chick embryos have served as excellent tools for experimental manipulations, especially in the area of limb development (Cohn and Tickle, 1996). However, chicks, which copulate by pressing their cloacas together, might exhibit moderately diverged GT morphogenesis compared with mammals (Bakst, 1986; Hildebrand, 1995).

Utilizing our serum-free organ culture system, it is suggested that the distal region of the genital urethral plate epithelium, *Fgf8*-expressing region, has a role in controlling mesenchymal gene expression and outgrowth of the GT anlage, presumably acting as a signaling center for GT development. Ectopic application of FGF8 beads induced expression of mesenchymal markers such as *Fgf10*, *Mx1*, *Bmp4* and *Hoxd13*, and induced GT outgrowth. Experiments with neutralizing antibodies also suggested a role for FGF8 or related FGF(s) in GT outgrowth.

It was previously shown that the anti-FGF8 antibody used in this study has a specific neutralizing activity for FGF8-mediated cell proliferation (Dorkin et al., 1999). This specificity was also shown in embryonic tissues (Jung et al., 1999). Moreover, a different anti-FGF8 antibody showed similar results (see Materials and Methods). However, it is possible that other related molecule(s), e.g., other FGF(s), will show similar activities in this system.

Transplanted FGF10 beads displayed growth-promoting activities, but less prominent compared with those of FGF8 beads. In this assay, FGF10 beads were applied to GT after removing the *Fgf8*-expressing region, leading to downregulation of endogenous *Fgf10* in the treated mesenchyme. One might speculate that FGF8 could induce growth-promoting factor(s) other than FGF10, but it remains to be analyzed further.

*Fgf4* expression was not evident during GT development (data not shown) although it is expressed in posterior AER and is postulated to function in the control of limb mesenchyme proliferation. This may, therefore, represent a different mode of gene expression profile during GT formation compared with that of limbs. Several other *Fgf* genes are expressed in the urethral plate epithelium or in GT mesenchyme (R. H. and G. Y., unpublished results). Combinations of such *Fgf* gene expressions may constitute a redundant network and play various roles during GT development. Such redundant mode of FGF system was also shown by the absence of limb phenotypes in *Fgf4* conditional mutants (Moon et al., 2000).

To our knowledge, neither such *Fgf8*-expressing regions nor candidate molecules for GT growth promotion have been previously identified. Furthermore, the mesenchymal *Fgf10*
gene was shown to be important during GT formation based on the analysis of the gene knockout phenotypes (see below). Thus, the FGF system appears to be one of the key signals involved in controlling outgrowth and in regulating various developmental processes of mammalian GT.

The inductive effects of epithelia on the development of various tissues through epithelial-mesenchymal interactions have been reported previously (Cunha et al., 1983; Roberts et al., 1995). Such interactions have been also reported in developing urogenital systems by groups such as the Cunha laboratory (Cunha and Chung, 1981; Hayward et al., 1998; Kurzrock et al., 1999b). Fgf genes have frequently been described as part of gene regulatory loops (Thomson and Cunha, 1999; Lebeche et al., 1999). It has also been suggested that several surface epithelia of genital tubercles are involved in epithelial-mesenchymal interactions in relation to the androgen system (Cunha and Lung, 1978; Murakami and Mizuno, 1986; Murakami, 1987).

During limb-bud development, Fgf8 transcripts have been detected in the prelimg field ectoderm prior to the formation of AER (Tickle and Eichele, 1994; Johnson and Tabin, 1997; Ohuchi et al., 1997). It is known that beads releasing different FGFs are able to induce the development of additional limbs when applied to the flank of chick embryos (Cohn et al., 1995; Crossley et al., 1996b; Ohuchi et al., 1997). It should be noted that, in GT development, Fgf8 expression resides in the urethral plate epithelium, which is endodermal in origin. Extensive recent experiments confirmed the distal end of the urethra as endodermal in origin (Kurzrock et al., 1999a). One might still envisage that part of the initial GT outgrowth before urethral tube formation might be achieved by applying FGF proteins. However, it would be necessary to exploit another experimental system that would allow the application of protein beads to murine embryos at earlier stages, because our organ culture can only be efficiently performed during the outgrowth phase of GTs.

In this paper, alterations of GT mesenchymal competence on application of FGF8 beads are suggested (data not shown). This responsiveness correlates well with the endogenous Fgf8 gene expression profile, with strong expression at 11.5 d.p.c. and a gradual decrease later.

It has been suggested that the BMP system could exert negative activities against growth-promoting signals emanating from the AER in limbs (Niswander, 1999). The effect of BMP4 beads application on the regulation of GT outgrowth was not as prominent under similar culture conditions (data not shown). More detailed analysis would be necessary as negative activities may be balanced by positive (growth-promoting) signals (Niswander, 1999).

**Fgf10 knockout mice did not show drastic outgrowth defects in the GT**

We have shown that Fgf10 may not be essential for the early phase of GT development, but Fgf10 knockout drastically affects morphogenesis of the glans penis and the glans clitoridis, the most distal structures of GTs.

It is of interest that Fgf8 gene expression remained normal in Fgf10−/− GT at 10.5 and 11.5 d.p.c., suggesting that Fgf10 is not required for the induction of Fgf8 expression in GTs. One might speculate that the gene regulatory loop between Fgf8 in AER and the mesenchymal Fgf10 might only apply to limb formation. This difference might also be due to gene compensatory mechanisms by other functionally and/or structurally related genes during GT development as generally shown in other recent gene knockout studies (Rudnicki et al., 1993; Kondo et al., 1997; Belo et al., 1998; Sun et al., 1999).

Redundant gene networks may also be indicated by the absence of obvious phenotypes during GT formation of Msx1−/− mice (unpublished results). The results from Msx1 and Msx2 double knockout mice, may be of future interest as the Msx2 gene is also expressed during GT development (R. H. et al., unpublished results).

Fgf8 expression in later stage mutant GTs was altered, possibly as a result of the several morphological changes of the lower (ventral) part of the urethral plate.

**Abnormal glans penis and glans clitoridis formation in newborn Fgf10 knockout mice**

Following initial outgrowth, distal portions of GTs differentiate into penes or clitorides with the prepucce glands, urethrae, prepuces and the corpus cavernosum glands. In normal development of the penis, urethral plate becomes separated from the surface epithelium of the glans penis in proximodistal sequence and forms an epithelial tube that runs through the glans penis (Grenister, 1954; Murakami, 1987). The prepucce grows to cover the glans penis, being fused at the ventral (lower) midline of the GT (Fig. 7A). Morphogenesis of the urethra and prepuce in the glans clitoridis is largely similar to that of penis. Extensive anatomical evaluations and related references about the mode of urethral tube formation are found in recent papers (Kurzrock et al., 1999a,b).

Histological analysis of newborn Fgf10 mutant external genitalia revealed striking defects, including defects in urethral tube formation of the glans regions. Mesenchymal-derived elements in the glans region, such as the rudiment of the corpus cavernosum glands and the rudiment of os penis/clitoridis, were found to be formed as mesenchymal condensations in the glans penis/clitoridis of the knockout mice.

It was shown that Fgf10 is expressed in mesenchyme adjacent to the midline at later stages of GT development, when the differentiation of urethral tube formation occurs. These phenotypes, together with the above mesenchymal expression of Fgf10, may suggest an essential role of Fgf10 in the morphogenesis of the distalmost structures of GTs, especially those in the lower (ventral) side of GTs formation. Mutant male newborns did not show drastic phenotypes in the testis (data not shown).

Multiple morphogenic events would be involved during formation of the urethral plate and the subsequent urethral tube formation. Previous histological studies have suggested a dynamic expression profile of cell-matrix proteins, e.g. the tenasin gene, in these processes (Murakami et al., 1990). Analysis of these candidate molecules is in progress (R. H. and G. Y., unpublished results). Further molecular analysis is required to clarify whether Fgf10 might regulate such molecules or not.

In humans, one of the frequent hereditary genital abnormalities, hypospadias, displays an abnormal positioning of the urethral opening (Sutherland et al., 1996). Multiple causative genetic backgrounds have been suggested as potential candidate genes for hypospadias, including androgen
systems (Anderson and Clark, 1990; Nordenskjold et al., 1999). Because Fgf10 knockout mice display multiple agenesis of vital organs such as the lung, it might be implied that Fgf10 gene mutation in humans would result in more severe phenotypes than those in hypospadias.

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