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Genitourinary Functions of Hoxa13 and Hoxd13

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In the United States, Japan, United Kingdom, and Sweden, birth defects affecting the growth and development of the genitourinary (GU) regions are becoming increasingly prevalent, with incidences ranging as high as 1 in 125 live births. To understand the basis for these malformations, scientists have begun to examine the function of developmental genes in GU tissues. At the forefront of these investigations are studies examining the role of the 5′ HOX proteins during the formation of the GU region. In this report we discuss what is known about HOXA13 and HOXD13 function during GU development, highlighting some of the cellular and molecular mechanisms controlled by these proteins during the GU formation. Finally, the translational benefits of identifying HOX target genes are discussed; first to explain the prevalence of some GU defects as well as a mechanism to facilitate their prevention in the birth population.

Key words: external genitalia, hand foot genital syndrome, Hox, hypospadias, synpolydactyly.

In humans and mice, reproduction proceeds by internal fertilization, a process facilitated by specialized structures collectively termed the external genitalia. Developmentally, these structures form from the genital tubercle (GT), a ventro-caudal structure comprised of cells from the intermediate mesoderm bisected by an endodermally derived epithelium (*1–3*) (Fig. 1). At approximately 50 days of human gestation (embryonic day 14–15 in mice) the genitalia exhibit secondary sexual characteristics, a process initially believed to be predominantly controlled by the presence (male) or absence (female) of androgen signaling (*2*, *4–6*).

Additionally, several growth factors have also been implicated in the regulation of genitourinary (GU) development. In particular, fibroblast growth factors 8 and 10 (FGF-8, FGF-10), sonic hedgehog (SHH), and bone morphogenetic proteins 2, 4, and 7 (BMP2, BMP4, BMP7) appear to control many of the proliferative and apoptotic processes required for GT patterning (*7–12*). The source of many of these growth factors is the centralized GT epithelium, a region first reported by Murakami and Mizuno (*5*) as having an inductive effect on the genital tubercle mesenchyme. Subsequent studies confirm that the epithelium separating the two shelves of genital tubercle mesenchyme functions as a *bona fide* signaling center supplying both proliferative and polarizing signals to the developing external genitalia (*13*, *14*) (Fig. 1).

Interestingly, while the GT-specific functions of these growth factors are beginning to be understood, surprisingly little is known about how these genes are transcriptionally regulated during the development of the external genitalia. To address this issue, this review will highlight some recent investigations of transcription factor function in the developing GT, focusing on the role of Hoxa13 and Hoxd13 in GT formation.

Hox genes and their associated GU syndromes

Hox genes encode a highly conserved family of transcription factors. In humans and mice, the Hox genes are distributed into four linkage groups (HOXA, B, C, D) comprising 39 genes located on human chromosomes 7, 17, 12, and 2 and mouse chromosomes 6, 11, 15, and 2 (Fig. 2). To date only Hoxa13 and Hoxd13 have been definitively linked to syndromes affecting genitourinary development (*15–19*).

Mutations in Hoxa13 cause Hand-Foot-Genital Syndrome (HFGS) an autosomal dominant disorder that profoundly affects the development of limb and genitourinary (GU) structures, including the external genitalia, uterus, bladder, ureter, cervix, and rectum (*15*, *20–23*). In the external genitalia, the loss of Hoxa13 function also causes hypospadias, a common genitourinary (GU) malformation affecting the formation and closure of the meatus (coronal hypospadias), ventral urethra (penile hypospadias), scrotum (scrotal hypospadias) and perineum (perineal hypospadias) (*24–27*) (Fig. 3). A second syndrome, Guttmacher Syndrome (GS) is also associated with mutations in Hoxa13 (*18*, *28*). Here mutations in the Hoxa13 promoter and coding region presumably affect the regulation of Hoxa13 expression and DNA-binding properties, resulting in limb phenotypes remarkably different from HFGS. Individuals affected by GS also exhibit hypospadias, suggesting that the Hoxa13 has separate limb and GU functions. More importantly, the mutations in Hoxa13 causing GS also suggests an absolute requirement for wild-type Hoxa13 protein expression and function in the developing external genitalia.

Using gene targeting, models of HFGS have also been produced in mice which exhibit many of the GU pheno-

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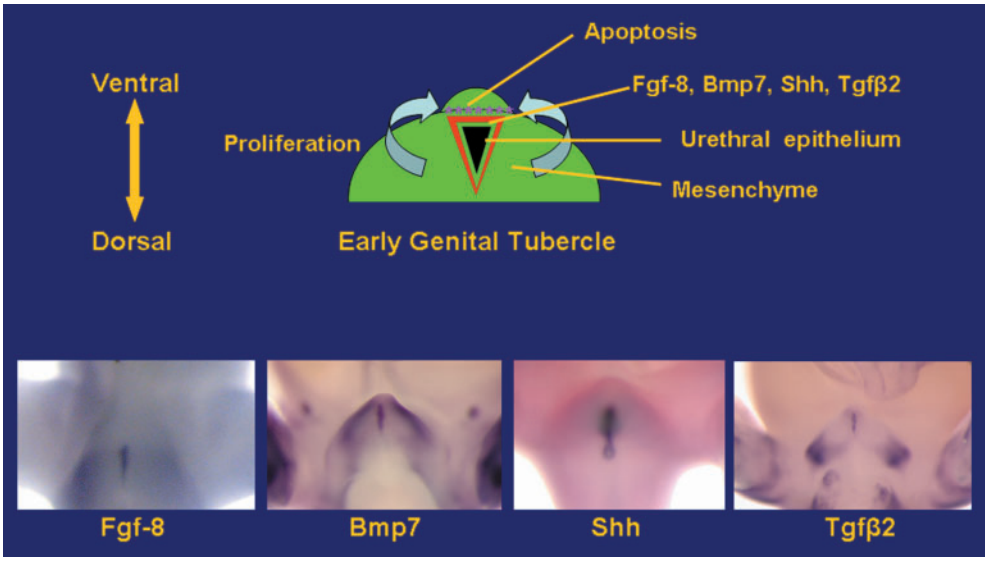


Fig. 1. **Cell processes and gene expression controlling early genital tubercle growth and development.** Arrows represent the sites of high cell proliferation in the genital tubercle mesenchyme. Asterisks represent sites of high apoptosis. Lower panels depict the expression of various growth factors in the urethral epithelium signaling center.

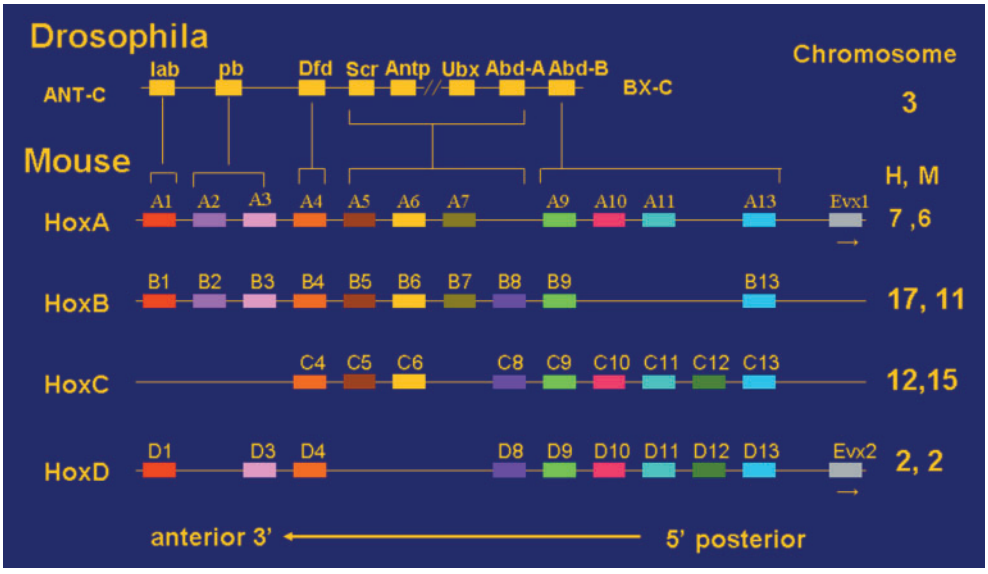
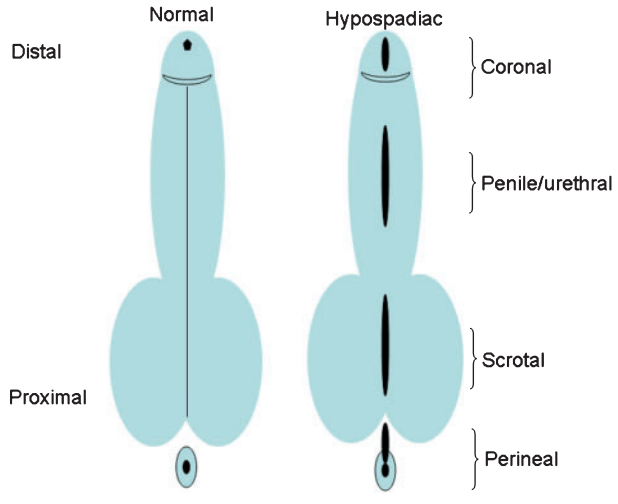


Fig. 2. **Chromosomal arrangement of the human and mouse HoxA-D gene clusters.** Paralogous genes within the A–D cluster are denoted by the same color.



Proximal-Distal Classification of Hypospadias in the External Genitalia

types seen in HFGS-affected individuals including malformations of the rectum, Müllerian ducts, ureters, bladder, and hypospadias (*7*, *29–31*).

Mutations in Hoxd13 cause Synpolydactyly (SPD) a disorder that is also autosomal dominant and affects many of the same tissues as HFGS including skeletal elements of the distal limb and the external genitalia (hypospadias) (*16*, *17*). A comparison of the HFGS and SPD phenotypes suggest that Hoxa13 and Hoxd13 may have redundant and independent target genes. In the limb, individuals affected by HFGS exhibit reductions in digit size (hypodactyly digit I), whereas individuals affected by SPD often exhibit supernumerary digits and enlarged skeletal elements (*16*, *17*, *29*, *30*, *32*). In the absence of both Hoxa13 and Hoxd13, malformations of the limbs

Fig. 3. **Classification of hypospadias in the male external genitalia.** Figure is reproduced with permission (*39*).

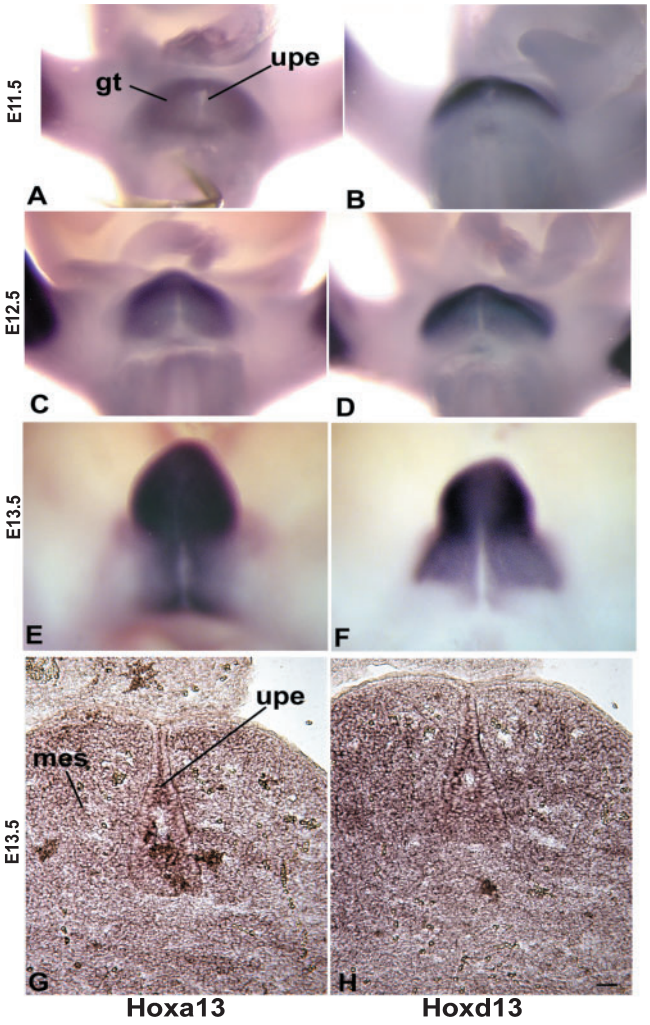


Fig. 4. **Expression of Hoxa13 and Hoxd13 in the developing genital tubercle.** (A–F) Whole mount *in situ* hybridization showing the co-localization of Hoxa13 and Hoxd13 transcripts embryonic days 11.5–13.5 gt = genital tubercle mesenchyme, upe = urethral plate epithelium. (G) and (H) *In situ* hybridization of consecutive sections through an E 13.5 genital tubercle. Note the similarly in distribution of Hoxa13 and Hoxd13 transcripts throughout the mesenchyme (mes) and in the urethral plate epithelium (upe). Bar is 50 μm.

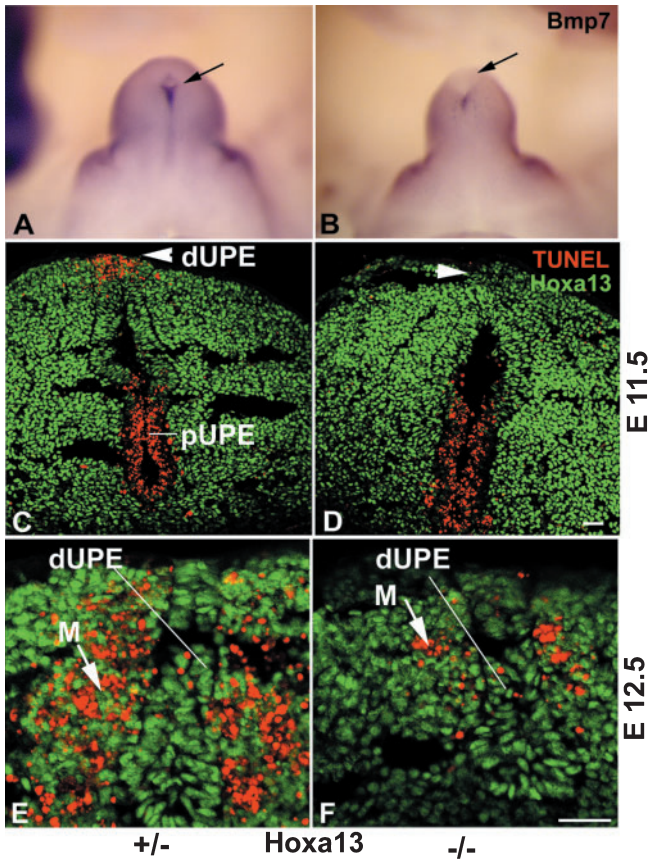


Fig. 5. **Male embryos lacking Hoxa13 exhibit decreased programmed cell death (PCD) in the developing genital tubercle.** (A) Bmp7 expression distal urethral epithelium and developing meatus of Hoxa13 heterozygous control embryos. (B) Bmp7 expression is reduced in the distal urethral epithelium and meatus of age-matched Hoxa13 homozygous mutant embryos. Arrows denote site of Bmp7 expression in the developing meatus. (C) At E 11.5, PCD is restricted to the urethral plate epithelium (UPE) (arrowhead) as well as the more proximal sinus epithelium (PSE). (D) PCD is reduced in the UPE (arrowhead) but not in the proximal sinus epithelium of Hoxa13^{GFP} homozygous mutants. (C) By E 12.5, PCD shifts from the UPE to the mesenchyme (M) flanking the UPE (arrow). (D) Homozygous mutants also exhibit a shift in PCD to the flanking mesenchyme, although the number of TUNEL positive cells in the mutant mesenchyme is consistently reduced when compared to heterozygous controls. Bars are 50 μm. Figure is reproduced with permission (*7*).

and GU regions appear to diverge from comparable compound mutations in mice, suggesting that Hoxa13 and Hoxd13 perform unique roles during the specification of homologous structures (*30*, *33*). In most cases, individuals affected by SPD do not exhibit GU malformations unless their mutations cause large expansions of a polyalanine tract, suggesting that the polyalanine expansion confers a novel function on the mutant Hoxd13 protein (*16*, *17*). Moreover, the efficacy of this gain of function is directly proportional to the size of the alanine tract expansion, which at its greatest length, perturbs both the limb and GU-developmental pathways (*16*, *17*). Further investigations will be required to determine mechanisms underlying the hypospadias associated with the Hoxd13 polyalanine tract expansions. Of particular emphasis should be investigations aimed at determining whether the mutant Hoxd13 protein functions as a specific inhibitor of the Hoxd13 transcriptional hierarchy or as general antagonist of the GT developmental program. Furthermore, it is also possible that the mutant Hoxd13 protein acquires the capacity to affect Hoxa13's developmental functions, mimicking the phenotypes associated with HFGS.

Expression of Hoxa13 and Hoxd13 during GU development

As a general rule, Hox genes are expressed in nested domains along the A-P axis of the embryo. Typically the genes located in the 3′ regions of the Hox A-D clusters are expressed in the anterior regions of the embryo, whereas the more 5′ Hox genes are expressed in increasingly posterior regions (*13*) (Fig. 2). In the GT, Hoxa13 and Hoxd13

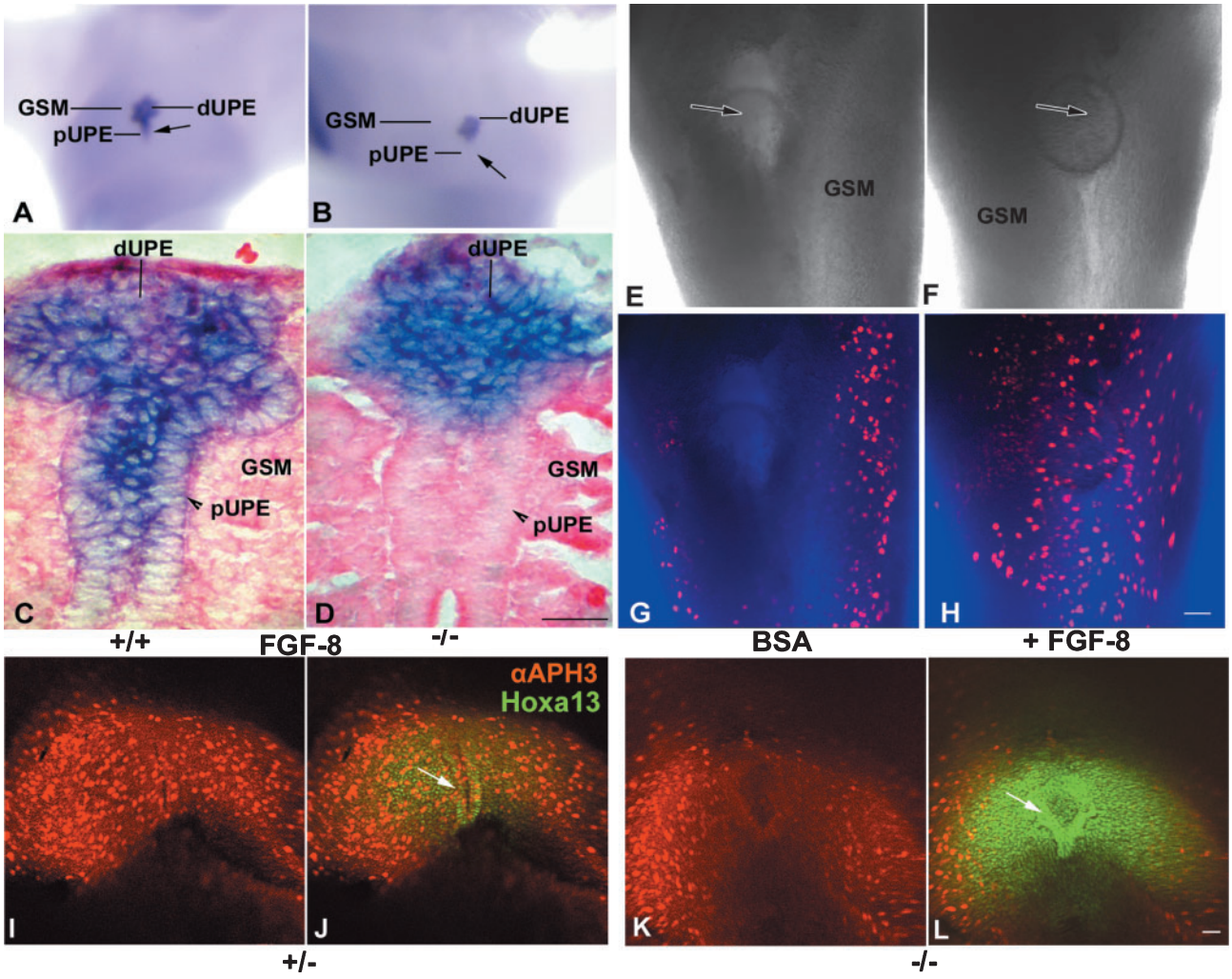


Fig. 6. **Rescue of proliferative defects in the GT of Hoxa13 mutants by ectopic FGF-8.** (A) Expression of Fgf-8 in the genital tubercle of E 11.5 wild-type male embryos. Note that Fgf-8 expression is seen in both dUPE and pUPE (black arrow) of E11.5 wild-type male embryos. Genital Shelf Mesenchyme (GSM) (B) Fgf-8 expression is absent in the pUPE (black arrow)of E 11.5 Hoxa13^{GFP} homozygous mutants. (C) Section analysis of Fgf-8 expression in the urethral plate epithelium (UPE) of E 11.5 wild type male embryos. Note that Fgf-8 expression is present in both the proximal (black arrowhead) and distal UPE. (D) Fgf-8 expression is restricted to the dUPE in E 11.5 homozygous mutants. Note the complete loss of Fgf-8 expression in the pUPE (black arrowhead). (E and G) Implantation of heparin beads (black arrow) treated with BSA into the UPE of E 11.5 homozygous mutants had no effect on the reduced proliferation seen in the GSM. (F and H) Implantation of beads treated with 0.1 mg/ml FGF-8b (black arrow) stimulated proliferation of the GSM in age-matched homozygous mutant embryos. (I) and (J) Represent typical levels of cell proliferation in the E 11.5 GT of normally developing heterozygous male embryos. Arrow denotes normal thickening of the UPE. (K) and (L) Cell proliferation in the GT of a E 11.5 Hoxa13^{GFP} homozygous male mutant. Arrow denotes the earliest detection of the abnormally thickened UPE. Note how Fgf-8 applications alter cell proliferation in the mutant GT to resemble proliferation levels exhibited by heterozygous littermates (compare H and I) whereas mutant embryos treated with BSA maintain reduced levels of cell proliferation (compare G and K). Proliferation was determined the level of positive αAPH3 staining (red signal). Hoxa13 expression in the GT is denoted by the green cells. Bars are 50 μm. This figure is reproduced with permission (*7*).

are the most highly expressed Hox genes. Analysis of Hoxa13 and Hoxd13 expression in the GT reveals a remarkably similar distribution of expression including co-localization in the GT mesenchyme and in the epithelial signaling center (Fig. 4). The degree of co-localization between Hoxa13 and Hoxd13 in the GT suggests that Hoxa13 and Hoxd13 may function in a redundant manner in the GT. Indeed, the analysis of GU defects in Hoxa13/Hoxd13 compound mouse mutants supports this conclusion as the severity of malformations increases in additive fashion culminating in complete agenesis of the GT in Hoxa13/Hoxd13 double homozygous mutants (*31*). In humans, the combined loss of HOXA13 and HOXD13 also affects the developing GT causing reductions in the labia majora as well as frequent urinary tract infections (*33*). Interestingly, a comparison of limb defects present in this same individual with the comparable mouse mutants revealed significant differences in the sites of malformation as well as their severity (*30*, *33*). This discrepancy raises an important consideration when comparing mutant phenotypes. In particular the affect a particular mutation has on the structure of the Hoxa13 or

Hoxd13 proteins will ultimately dictate the function of the mutant molecule. Thus phenotypes produced by the loss of an important protein domain such as the DNA-binding domain may not necessarily coincide with mutations causing polyalanine tract expansions which could alter the sites of protein–protein interaction or proper protein folding creating a separate phenotype based on the acquisition of a novel protein function.

Cellular and molecular consequences of loss of Hoxa13 function

The loss of Hoxa13 function impacts both cell proliferation and programmed cell death during GT development. In the early GT, the sites of programmed cell death (PCD) dynamically move from the urethral plate epithelium to the genital shelf mesenchyme (Fig. 5) (*7*). In absence of Hoxa13 function, PCD is dramatically reduced, particularly in the medial regions of the genital shelf mesenchyme, a site that requires tissue remodeling to facilitate fusion. Concomitant with this reduction in PCD is the loss of Bmp7 expression in the GT (*7*) (Fig. 5). It is not known whether Bmp7 is a direct target of Hoxa13 in the developing GT, however, in the limb, Hoxa13 has been shown to directly bind an enhancer element upstream of Bmp7 (*34*). In the absence of Hoxa13 function, PCD in the limb is reduced and can be restored with BMP7 supplementation, suggesting a similar regulatory link may be present in the developing GT (*34*).

Cell proliferation is also reduced in the GT of Hoxa13 mutant mice (*7*). Analysis of proliferative factors affected by the loss of Hoxa13 function revealed that Fgf-8 expression in the proximal urethral plate epithelium was severely reduced, and that supplementation of the mutant GT with FGF-8 restored proliferation (Fig. 6). At this point, it is not known whether Hoxa13 directly regulates Fgf-8 expression in the GT. The regulation of Fgf-8 by Hoxa13 in the GT would present an interesting functional divergence, as other structures such as the developing limb bud typically segregate the expression domains of these genes to the underlying mesenchyme (Hoxa13) and overlying apical ectodermal ridge (Fgf-8) (*29*, *35*).

Concluding thoughts and a challenge to the research community—Defining GU transcriptional hierarchies

To date, only a few transcription factors have been investigated for a role in GU development. By defining how GU-specific genes are regulated, insights will be gained that explain the susceptibility of this region to malformation. At the forefront of these investigations should be analyses directed towards defining the DNA sequences bound by the GU-transcription factors as well as accessory proteins that mediate their specific transcriptional regulation. Only by understanding the biochemical functions of GU-specific proteins can we truly understand why defects such as hypospadias and vesicouretral reflux are common in the human birth population (*24*, *36–38*). Finally, the current frequency and diversity of GU defects also suggests that epigenetic influences may be affecting the development of these tissues as the rate of these defects outpaces single gene mutation rates. To address this issue we must also examine how epigenetic factors can affect the protein–DNA and protein–protein interactions required for normal GU development, providing both an explanation and possibly the means to prevent congenital GU malformations.

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