Published in final edited form as:
Dev Dyn. 2012 February ; 241(2): 365–375. doi:10.1002/dvdy.23710.

Dynamic Expression of Tbx2 Subfamily Genes in Development of the Mouse Reproductive System

Nataki C. Douglas[1,*], **Kathleen Heng**[1], **Mark V. Sauer**[1], and **Virginia E. Papaioannou**[2]

[1]Department of Obstetrics and Gynecology, Columbia University Medical Center, New York, New York

[2]Department of Genetics and Development, Columbia University Medical Center, New York, New York

Abstract

Background—*Tbx2, Tbx3, Tbx4*, and *Tbx5*, members of the Tbx2 subfamily of T-box transcription factor genes, are important for many aspects of embryonic development and mutations in some human TBX2 subfamily genes cause developmental syndromes. In addition, *TBX2* and *TBX3* are overexpressed in a variety of cancers, including reproductive system cancers. This study characterizes the expression of Tbx2 subfamily genes during development of the reproductive system.

Results—We show that these genes are expressed in both the internal and external reproductive systems. *Tbx2* is expressed in gonads and genital ducts, the Wolffian and Müllerian ducts, while *Tbx3* is only expressed in genital ducts. *Tbx4* is expressed in embryonic and postnatal germ cells. All four genes are expressed in mesenchyme in external genitalia, with *Tbx3* and *Tbx5* expression in the epithelium as well.

Conclusion—This study lays the foundation for investigation of functional requirements for Tbx2 subfamily genes in development of the mammalian reproductive system.

Keywords

Tbx2; *Tbx3*; *Tbx4*; *Tbx5*; T-box; testis, ovary; Wolffian; Müllerian; genital tubercle

INTRODUCTION

The Tbx2 subfamily of the T-box transcription factor genes plays a critical role in the determination of cell fate decisions during organogenesis and in the development and differentiation of many organ systems (Naiche et al., 2005; Wardle and Papaioannou, 2008; Conlon and Yutzey, 2010). The highly conserved T-box DNA binding motif is common to all members of the T-box gene family. The Tbx2 subfamily consists of *Tbx2, Tbx3, Tbx4*, and *Tbx5*. *Tbx2* and *Tbx3* derive from a common ancestral gene, as do *Tbx4* and *Tbx5* (Holland et al., 1994; Agulnik et al., 1996; Ruvinsky and Silver, 1997). Mutations in these genes are associated with developmental defects in both mice and humans.

Targeted mutagenesis in mice to produce homozygous disruption of *Tbx2* (Harrelson et al., 2004), *Tbx3* (Davenport et al., 2003; Mesbah et al., 2008), *Tbx4* (Naiche and Papaioannou,

[*]Correspondence to: Nataki C. Douglas, MD, PhD, Department of Obstetrics and Gynecology, PH 16-64, Columbia University Medical Center, 622 W. 168th Street, New York, NY 10032. nd2058@columbia.edu.

2003), or *Tbx5* (Bruneau et al., 2001) results in embryonic lethality. *Tbx2* and *Tbx5* null embryos die during midgestation due to cardiovascular defects (Bruneau et al., 2001; Harrelson et al., 2004; Suzuki et al., 2004). Heterozygous *Tbx3* mutant mice are fertile, but all adult females have a split clitoris and some do not possess a vaginal opening (Davenport et al., 2003). Homozygous null *Tbx3* mutants die by embryonic day (E) 16.5 with yolk sac, limb, mammary gland, and heart abnormalities (Davenport et al., 2003). In homozygous null *Tbx4* mutants, lack of chorio-allantoic fusion, which prevents formation of the umbilical vessels, results in death by E10.5 (Naiche and Papaioannou, 2003).

While there are no documented human developmental syndromes associated with mutations in *TBX2*, mutations causing heterozygous loss of *TBX3, TBX4*, or *TBX5* in humans result in ulnar-mammary (Bamshad et al., 1995, 1997), small patella (Bongers et al., 2004), or Holt-Oram (Basson et al., 1997; Li et al., 1997) syndromes, respectively. The phenotype of humans affected with ulnar-mammary syndrome (UMS) is similar to, although less severe than, that observed in homozygous null *Tbx3* mutant embryos (Davenport et al., 2003). Humans with UMS have abnormal development of the ulnar aspect of the hand and/or forearm, breasts, teeth, and external genitalia. UMS may also result in delayed onset of puberty in males (Schinzel, 1987; Schinzel et al., 1987; Franceschini et al., 1992; Davenport et al., 2003). The similarities between mice and humans underlie the importance of the Tbx2 subfamily of T-box genes in mammalian development and strongly suggest conservation of function among different species.

In addition to critical roles in development, two members of the TBX2 subfamily, *TBX2* and *TBX3*, are overexpressed in human cancers, including those of the reproductive system. Increased *TBX2* and *TBX3* expression was detected in ovarian, uterine, cervical, and breast cancers (Jacobs et al., 2000; Sinclair et al., 2002; Adem et al., 2004; Lomnytska et al., 2006; Lyng et al., 2006; Liu et al., 2010a,b). Although TBX2 protein was detectable in only 2% of normal uterine endometrial or cervical tissue samples, more than 50% of endometrioid endometrial adenocarcinoma and squamous cell cervical carcinoma samples expressed TBX2 (Liu et al., 2010a,b). Increased expression of TBX3 was detected in plasma protein extracts from ovarian and breast cancer patients (Lomnytska et al., 2006), and TBX3 was detected in gene expression microarrays of metastatic squamous cell cervical carcinoma (Lyng et al., 2006).

The central role of Tbx2 subfamily genes in mammalian development coupled with the overexpression of TBX2 and TBX3 in tumors of the reproductive system strongly suggests that T-box genes may also be essential for development of the reproductive system and sexual differentiation. It was noted that *Tbx2* and *Tbx3* are expressed in the murine genital ridge and that all four Tbx2 subfamily genes are expressed in the genital papilla (Chapman et al., 1996); however detailed expression of Tbx2 subfamily genes during development of the mammalian reproductive system has not been previously reported.

In this study, we examine the spatio-temporal expression patterns of Tbx2 subfamily genes in the internal and external reproductive systems throughout organogenesis and postnatal, pre-pubertal differentiation using in situ hybridization (ISH), immunofluorescence (IF), and comparison with cell-type specific markers. Our results show that *Tbx2, Tbx3*, and *Tbx4* are expressed in the internal reproductive system. *Tbx5* expression was not detected in the internal reproductive system by ISH or RTPCR (data not shown) at any stage. *Tbx2* is expressed in the interstitium of the testis and *Tbx4* is expressed in germ cells in the testis and ovary. *Tbx2* and *Tbx3* are expressed in the genital ducts, the Wolffian (mesonephric) ducts and Müllerian (paramesonephric) ducts, and their derivatives from E9.5 to postnatal day (P) 16, with overlapping, yet distinct expression patterns. In addition, we show that all

four members of the Tbx2 subfamily are expressed in the genital tubercle (GT) mesenchyme during both androgen-independent and androgen-dependent developmental phases.

RESULTS AND DISCUSSION

Expression of Tbx2 Subfamily Genes in the Urogenital Ridge

The urogenital ridges, comprised of bipotential gonads and mesonephroi, arise from the intermediate mesoderm by E9.5. Primordial germ cells, which arise in the epiblast between E7 and E7.5, migrate to and enter the gonads. By E10.5, the bipotential gonads, with germ cells and somatic cells, are detected as thickenings on the ventromedial surface of the mesonephroi (Kaufman and Bard, 1999; Staack et al., 2003).

Tbx2, Tbx3, Tbx4, but not *Tbx5* (data not shown) are expressed in the urogenital ridge. *Tbx2* and *Tbx3* expression was first detected in the epithelium of the developing Wolffian ducts at E9.5 (Fig. 1), which are marked by *Pax2* expression (Torres et al., 1995). At E11.5, *Tbx2* is expressed in both Wolffian ducts and cranial mesonephric tubules (Fig. 2B, G), which later become the efferent ductules between the testis and epididymis (Sainio et al., 1997). *Tbx3* is expressed in the Wolffian duct epithelium and mesonephric mesenchyme (Fig. 2C, H), but not in the mesonephric tubules (data not shown). *Pax2* expression in Wolffian ducts, cranial and caudal mesonephric tubules (Sainio et al., 1997) is shown for comparison (Fig. 2A, F). Whereas neither *Tbx2* nor *Tbx3* is expressed in the bipotential gonad (Fig. 2G, H), ventral views of urogenital ridges and transverse sections at E11.5 reveal *Tbx4* expression in gonads (Fig. 2D, I) in a pattern similar to that of *Oct4*, which is expressed in germ cells (Urven et al., 1993) (Fig. 2E, J). The unique expression patterns of *Tbx2, Tbx3*, and *Tbx4* in the urogenital ridges suggest that these Tbx2 subfamily genes have non-redundant roles in the development of the internal reproductive system.

Expression of Tbx2 Subfamily Genes in the Internal Reproductive System After Sexual Differentiation

Gonads—Bipotential gonads differentiate into testes or ovaries. Expression of *Sry*, the Y-linked testis-determining gene, in somatic cells of XY gonads at E10.5 results in *Sox9*-mediated differentiation of these bipotential cells into Sertoli cells, which then organize the testes into two main compartments, the epithelial testis cords and the mesenchymal interstitium between the testis cords. Testis cords consist of Sertoli cells and germ cells enclosed by peritubular myoid cells. The interstitium between the testis cords contains androgen-producing Leydig cells, vascular endothelial cells, fibroblasts, and hematopoietic cells (McLaren, 2000; Wilhelm et al., 2007; Sekido and Lovell-Badge, 2009). In contrast to what is seen in testes, embryonic ovaries, which also contain germ, somatic, and vascular endothelial cells, are morphologically similar to bipotential gonads. Thus, by E13.5, male and female gonads are morphologically distinct.

Tbx2 expression in the interstitium around testis cords (Fig. 3A–C) was observed at E13.5. Double immunofluoresence (IF) with antibodies to Tbx2 and Pecam, which labels germs cells and vascular endothelial cells (Wakayama et al., 2003), shows Tbx2 protein expressed throughout the interstitium (Fig. 3G–I). *Tbx2* expression was also observed in ovaries at E13.5 (Fig. 3D–F). Neither *Tbx3* (Fig. 4) nor *Tbx5* (data not shown) is expressed in testes or ovaries at E13.5. *Tbx4* expression was observed in both XY and XX gonads (Fig. 5). *Tbx4* is expressed within testis cords (Fig. 5A, B) and throughout the ovaries (Fig. 5D, E), similar to *Oct4* (Fig. 5C, F), which is expressed in germ cells. To determine if *Tbx4* is expressed in embryonic germ cells, we treated pregnant female mice with busulfan and analyzed *Tbx4* and *Oct4* expression in the gonads of embryos from busulfan-treated mice. Busulfan is an alkylating agent that has been used to deplete germ cells and the study of gene expression in

gonads from mice treated with busulfan has been previously described (Ross et al., 2007). A reduction in *Oct4* expression in testis cords of E13.5 XY gonads (Fig. 5H, J) confirms depletion of germ cells after busulfan administration. *Tbx4* expression was not detected in XY and was greatly reduced in XX gonads after germ cell depletion (Fig. 5G, I, data not shown for XX). These data are consistent with *Tbx4* expression in XY and XX germ cells at E13.5. Expression of *Tbx2* and *Tbx4* does not overlap in testes. Thus, our results suggest unique roles for *Tbx2* and *Tbx4* in sexual differentiation of the gonads.

Genital ducts—The Wolffian and Müllerian ducts, anlagen of XY and XX genital ducts, respectively, are unipotential structures that develop in both male and female embryos. Whereas Wolffian ducts are present by E10.5, Müllerian ducts arise between E11.5 and E13.5 (Kobayashi and Behringer, 2003; Staack et al., 2003; Hannema and Hughes, 2007). After sexual differentiation of the gonads, Wolffian ducts regress in females and Müllerian ducts regress in males by E16.5 (Kobayashi et al., 2004).

Both *Tbx2* and *Tbx3* are expressed in the Wolffian duct epithelium and Müllerian duct mesenchyme in XY embryos at E13.5 (Figs. 3C, C′, 4B, B′). In addition, *Tbx3*, but not *Tbx2* expression was observed in the Wolffian duct mesenchyme (Figs. 3C, C′, B, B′). *Tbx2* and *Tbx3* are expressed in the Wolffian duct epithelium in XX embryos at E13.5 (Figs. 3F, F′, 4D, D′), and, as seen in males, *Tbx3* is expressed in mesenchyme around both Wolffian and Müllerian ducts (Fig. 4D, D′). *Tbx2* and *Tbx3* are also expressed in the coelomic epithelium (Fig. 3F′, arrow; data not shown for *Tbx3*). The coelomic epithelium proliferates and undergoes an epithelial-to-mesenchymal transition to give rise to mesenchymal cells that contribute to connective tissue in the internal reproductive tracts (Moore et al., 1998; Guioli et al., 2007). Thus, *Tbx2*- and *Tbx3*-positive cells in the Müllerian duct mesenchyme may reflect their origin from coelomic epithelium.

In summary, our results show that *Tbx2* and *Tbx3* are expressed in the genital ducts from E9.5 to E13.5 in both unique and overlapping expression domains (Table 1) and sexually dimorphic *Tbx2* expression is observed in the Müllerian ducts. The patterns of *Tbx2* and *Tbx3* expression in the genital ducts suggest a role for *Tbx2* -, *Tbx3*-mediated cellular interactions in the sex-specific differentiation of the Wolffian and Müllerian ducts. For example, *Tbx2* and *Tbx3* may interact to induce Wolffian duct differentiation and/or Müllerian duct regression in male embryos. Additionally, *Tbx2* and *Tbx3* may interact to mediate Wolffian duct regression in female embryos.

Expression of Tbx2 Subfamily Genes in Postnatal, Pre-Pubertal Male and Female Internal Reproductive Systems

Elongation and coiling of the testis cords to form mature seminiferous tubules occurs before birth. Wolffian duct differentiation into the epididymides, vas deferentia, and seminal vesicles starts at E15.5 and is completed within the first 2 weeks of postnatal life (Archambeault et al., 2009). In contrast, primordial follicles, the first stage of ovarian folliculogenesis, form between P1 and P3 (Wilhelm et al., 2007), and Müllerian ducts differentiate into the oviducts, uterus, cervix, and upper vagina during the first 2 weeks of postnatal life.

Of the four Tbx2 subfamily genes, only *Tbx2* and *Tbx3* are expressed in postnatal, pre-pubertal males (Fig. 6). *Tbx2* is expressed in the testicular interstitium at P0 (Fig. 6A), but not at P14 (Fig. 6E). *Tbx3* is not expressed in postnatal testes (Fig. 6C, G), similar to E13.5. Both *Tbx2* and *Tbx3* are expressed in the epithelium of the epididymis (Fig. 6B, D, F, H white arrows) and seminal vesicles (Fig. 6J, L). *Tbx3*, but not *Tbx2*, is expressed in the vas deferens (Fig. 6K, I). Neither *Tbx2* nor *Tbx3* expression was observed in mesenchymal cells around epididymal coils (Fig. 6F, H, black arrows), although *Tbx3* was expressed in the

embryonic Wolffian duct mesenchyme (Figs. 2 and 4). Expression of *Tbx2* and *Tbx3* during Wolffian duct formation and differentiation suggests a role for these two genes in sperm maturation, transport, and/or storage.

In postnatal, pre-pubertal females at P16, non-overlapping expression of *Tbx2, Tbx3*, and *Tbx4* was observed (Fig. 7). *Tbx2* is expressed in epithelial cells in oviducts (Fig. 7A, D). *Tbx3* was not detected in ovaries or oviducts, but was observed in glandular and luminal uterine epithelium and uterine stroma (Fig. 7B, E), which contain cell types, such as fibroblasts, of mesenchymal origin. *Tbx4* is the only member of the Tbx2 subfamily expressed in postnatal, pre-pubertal ovaries. *Tbx4* is expressed in oocytes of primary and secondary ovarian follicles (data shown for secondary follicles) (Fig. 7C, F). Taken together, our data support unique, non-overlapping expression of *Tbx2* and *Tbx4* in sexually differentiated gonads; *Tbx2* is expressed in testicular and ovarian somatic cells at E13.5, while *Tbx4* is expressed in embryonic male and female germ cells and postnatal oocytes.

Expression of Tbx2 Subfamily Genes in the External Genitalia

The GT, a bipotential embryonic structure that is morphologically identical in males and females prior to E16.5, gives rise to the penis and foreskin, scrotum, clitoris, and labia. Androgen-independent outgrowth of the GT, ventral to the cloacal membrane, starts after E10.5 (Perriton et al., 2002; Yamada et al., 2003, 2006). However, differentiation of the GT mesenchyme, which results in corpora cavernosum with functional erectile tissue and chondrogenesis and osteogenesis in the os penis (Murakami and Mizuno, 1986; Murakami, 1987), is androgen dependent.

All four Tbx2 subfamily genes are expressed in the GT at E10.5 and E13.5 and in the sexually dimorphic GT at E18.5 (Fig. 8). At E10.5 (Fig. 8B–E), these T-box genes are expressed in mesenchyme lateral to the cloacal epithelium, which expresses *Shh* (Perriton et al., 2002) (Fig. 8A). At E13.5, whole mounts and sections reveal overlapping, yet unique expression domains of *Tbx2, Tbx3, Tbx4*, and *Tbx5* in the GT mesenchyme (Fig. 8G–J, L–O). Expression of *Shh* in the urethral plate epithelium (UPE) is shown for comparison (Fig. 8F, K). *Tbx3* is the only member of the Tbx2 subfamily expressed in the UPE at E10.5 (Fig. 8C) and E13.5 (Fig. 8H, M).

At E18.5, *Tbx2, Tbx3, Tbx4*, and *Tbx5* are expressed in mesencyhme that gives rise to the glans penis, glans clitoris, and foreskin (derived from the prepuce) (Fig. 8P–W). In addition, *Tbx3* is expressed in the UPE of males (Fig. 8Q) and females (Fig. 8U) and *Tbx5* is expressed in the male UPE (Fig. 8S). Signals from the epithelium control external genital development. Thus, *Tbx3* and *Tbx5* may interact with epithelial genes like *Shh* (Perriton et al., 2002) or mesenchymal genes like *Fgf10* (Haraguchi et al., 2000), which are integral to normal external genitalia development.

The pattern of Tbx2 subfamily gene expression in the embryonic and postnatal, pre-pubertal internal reproductive system is summarized in Table 1. Tbx2 subfamily gene expression was observed from initiation of organ development to sexual differentiation in both the internal and external reproductive systems. Thus, our study lays the foundation for investigation of functional requirements for *Tbx2, Tbx3, Tbx4*, and *Tbx5* in the development, differentiation, and post-pubertal function of the mammalian reproductive system.

EXPERIMENTAL PROCEDURES

Animals, In Situ Hybridization, and Immunofluorescence

Wild type adult ICR mice (Taconic, Germantown, NY) were bred to generate embryos from E9.5 to E13.5 and pups at P0, P14, and P16. All mice were housed in a temperature-

controlled facility with a 12-hr light/12-hr dark cycle. Noon on the day a mating plug was observed was designated E0.5. The sex of each embryo or pup was determined by staining the amnion with 2% orcein in 60% acetic acid to detect sex chromatin (Papaioannou and Behringer, 2005), by PCR of yolk sac lysates for the *Sry* gene, or by morphological differences in the gonads at later stages. Embryos, urogenital ridges, and gonads were dissected in cold phosphate buffered saline (PBS), fixed in 4% paraformaldehyde, washed in PBS containing Tween-20, dehydrated in a methanol series, and stored at −20°C until use. For section ISH and IF, whole embryos and internal reproductive organs from postnatal mice were isolated and fixed in 4% paraformaldehyde. Following fixation, samples were infiltrated with sucrose, embedded in Tissue-Tek® O.C.T.™ Compound (Sakura Fine Technical Co, Ltd, Tokyo, Japan), snap-frozen on dry ice in ethanol, and stored at −80°C. Animal protocols were approved by the Institutional Animal Care and Use Committee of Columbia University.

Whole mount and section ISH were performed using standard protocols (Wilkinson and Nieto, 1993; Grieshammer et al., 2004). Antisense and sense digoxigenin–labeled RNA probes were transcribed from linearized plasmids in the presence of digoxigenin-labeled dUTP (Roche, Nutley, NJ). For section ISH, frozen sections at 20 μm were made for E9.5 and E18.5 embryos, 16 μm for E10.5 embryos, and 10 μm for E11.5 and E13.5 embryos and postnatal organs. Sections were counter-stained with Nuclear Fast Red unless otherwise indicated.

ISH was performed on a minimum of 5 male and 5 female samples. ISH results for XY and XX embryos (E9.5–E11.5) and GT (E10.5–E13.5) were identical. Data are shown for XY embryos. IF was performed on 10-μm frozen sections at E13.5 using standard protocols (Wilhelm et al., 2005). The following antibodies were used: rat anti-Pecam (BD Pharmingen, San Diego, CA), mouse monoclonal anti-Tbx2 (C. R. Goding, unpublished data), Alexa fluor488 donkey anti-rat (Molecular Probes Inc., Eugene, OR), Cy3-conjugated goat anti-mouse (Jackson ImmunoResearch, West Grove, PA). Sections were stained with 4′, 6-diamidino-2-phenylindole (Sigma-Aldrich, St. Louis, MO) for nuclear visualization.

Images of whole-mount samples were taken under bright field on a Nikon SMZ1500 microscope (Nikon, Japan). Section ISH and IF staining was examined with a Nikon MICROPHOT-FXA microscope (Nikon, Japan) and images were captured using NIS-Elements D3.10 software.

Depletion of Embryonic Germ Cells

Intraperitoneal (IP) administration of busulfan (Sigma-Aldrich) was utilized to deplete embryonic gonads of germ cells. Pregnant female mice were injected with 150 μl of busulfan solution (20 mg/ml in 50% DMSO) or 50% DMSO (control) at E11.5. Gonads were removed at E13.5 and processed for ISH with probes for *Tbx4* and *Oct4* as described above.

Acknowledgments

We thank Dr. Colin R. Goding (Oxford, UK) for his generous gift of anti-Tbx2 antibody. This work was supported by the National Institutes of Health grant HD033082 (to V.E.P.) and the Reproductive Scientist Development Program grant NIH K12 HD000849 (to N.C.D.) and Robert Wood Johnson Foundation grant (N.C.D.).

Grant sponsor: National Institutes of Health; Grant sponsor: Robert Wood Johnson Foundation.

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Key findings

- This is the first study to characterize the expression of multiple T-box genes, the four closely related members of the Tbx2 subfamily, in both the internal and external reproductive systems of males and females.
- We show that *Tbx2* and *Tbx3* have both unique and overlapping expression domains in the internal reproductive system during initial stages of gonad and genital duct formation, after establishment of sexual dimorphism and at postnatal, pre-pubertal stages.
- β-*Tbx4* is expressed in embryonic male and female germ cells and postnatal oocytes.
- All four Tbx2 subfamily genes, *Tbx2, Tbx3, Tbx4*, and *Tbx5*, are expressed in mesenchyme in external genitalia, with *Tbx3* and *Tbx5* expression in the epithelium as well.

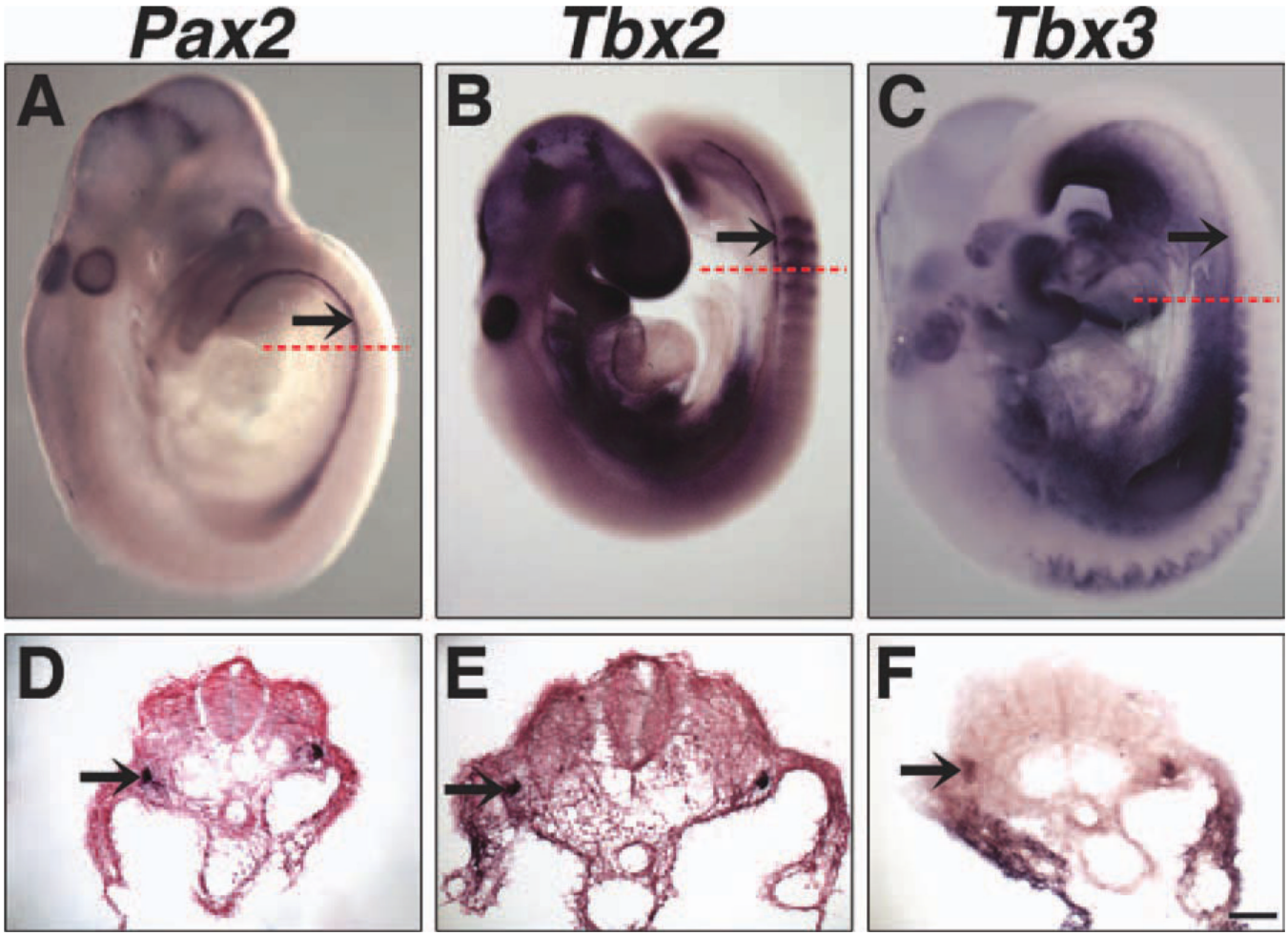

Fig. 1.

Expression of *Tbx2* and *Tbx3* in Wolffian ducts at E9.5. Dissected whole embryos and transverse sections were hybridized with antisense *Pax2* (**A, D**), *Tbx2* (**B, E**), and *Tbx3* (**C, F**) riboprobes. *Pax2* expression highlights the Wolffian duct. Both *Tbx2* and *Tbx3* are expressed in the Wolffian duct. Arrows identify Wolffian ducts and dashed red lines indicate the level of the corresponding transverse sections. Nuclear Fast Red counter-stain was not used in F. Scale bar in F = 50 μm (D–F).

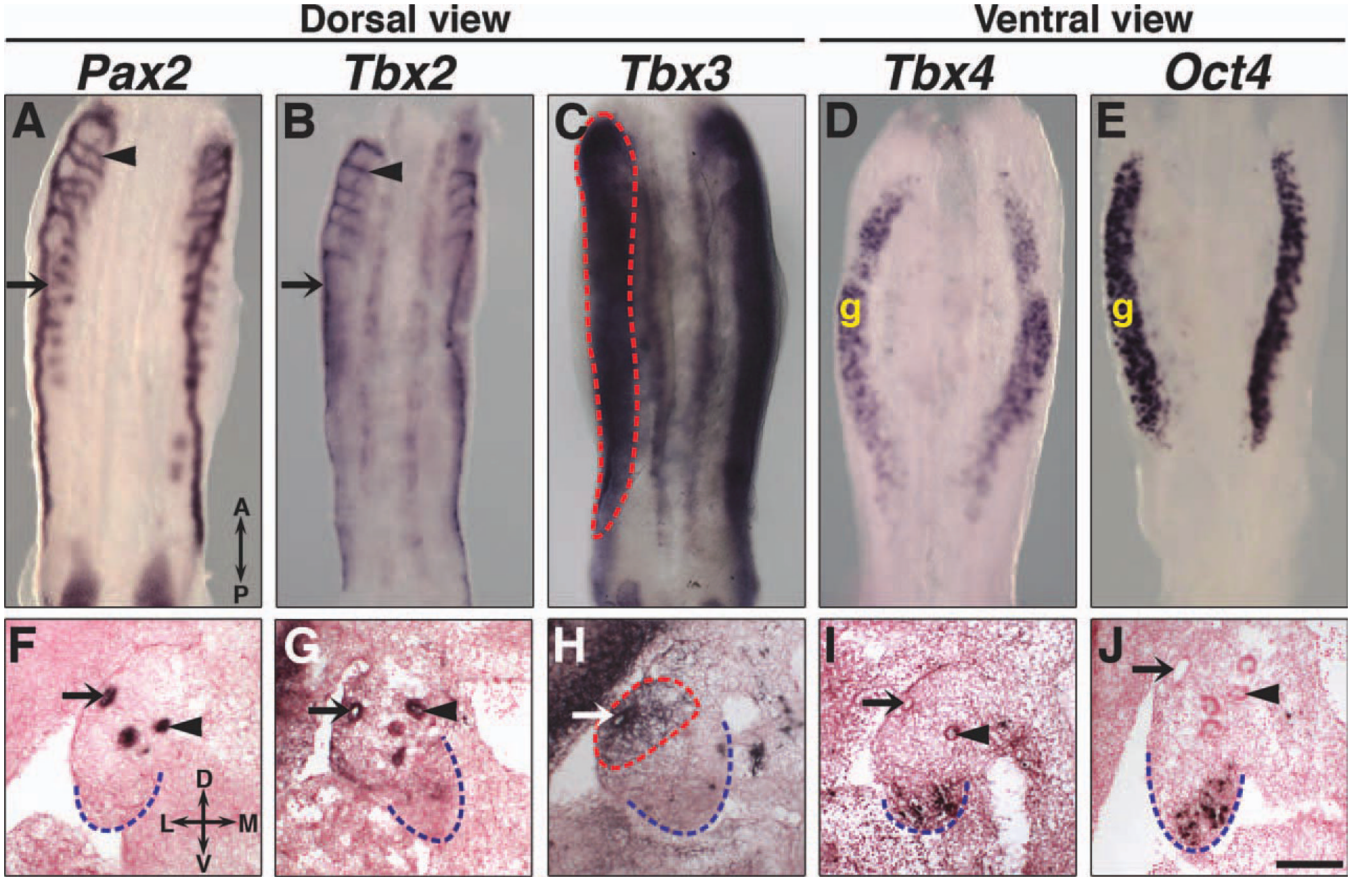

Fig. 2.

Tbx2, Tbx3, and *Tbx4* expression in urogenital ridges at E11.5. Whole-mount ISH on dissected urogenital ridges, dorsal views (A–C) and ventral views (D, E), and section ISH on transverse sections are shown. *Tbx2* is expressed in the epithelium of the Wolffian duct and mesonephric tubules (**B, G**). *Tbx3* is expressed in the Wolffian duct epithelium and mesonephric mesenchyme, highlighted by dashed red lines (**C, H**). *Tbx4* is expressed in gonads but not the Wolffian ducts or mesonephroi (**D, I**). *Pax2* expression in the epithelium of the Wolffian duct and mesonephric tubules (**A, F**) and *Oct4* expression in germ cells (**E, J**) are shown for comparison. Arrows identify Wolffian ducts and arrowheads identify mesonephric tubules. Dashed blue lines outline gonads in transverse sections. A, anterior; P, posterior; D, dorsal; L, lateral; M, medial; V, ventral; g, gonad. Scale bar in J = 100 μm (F–J).

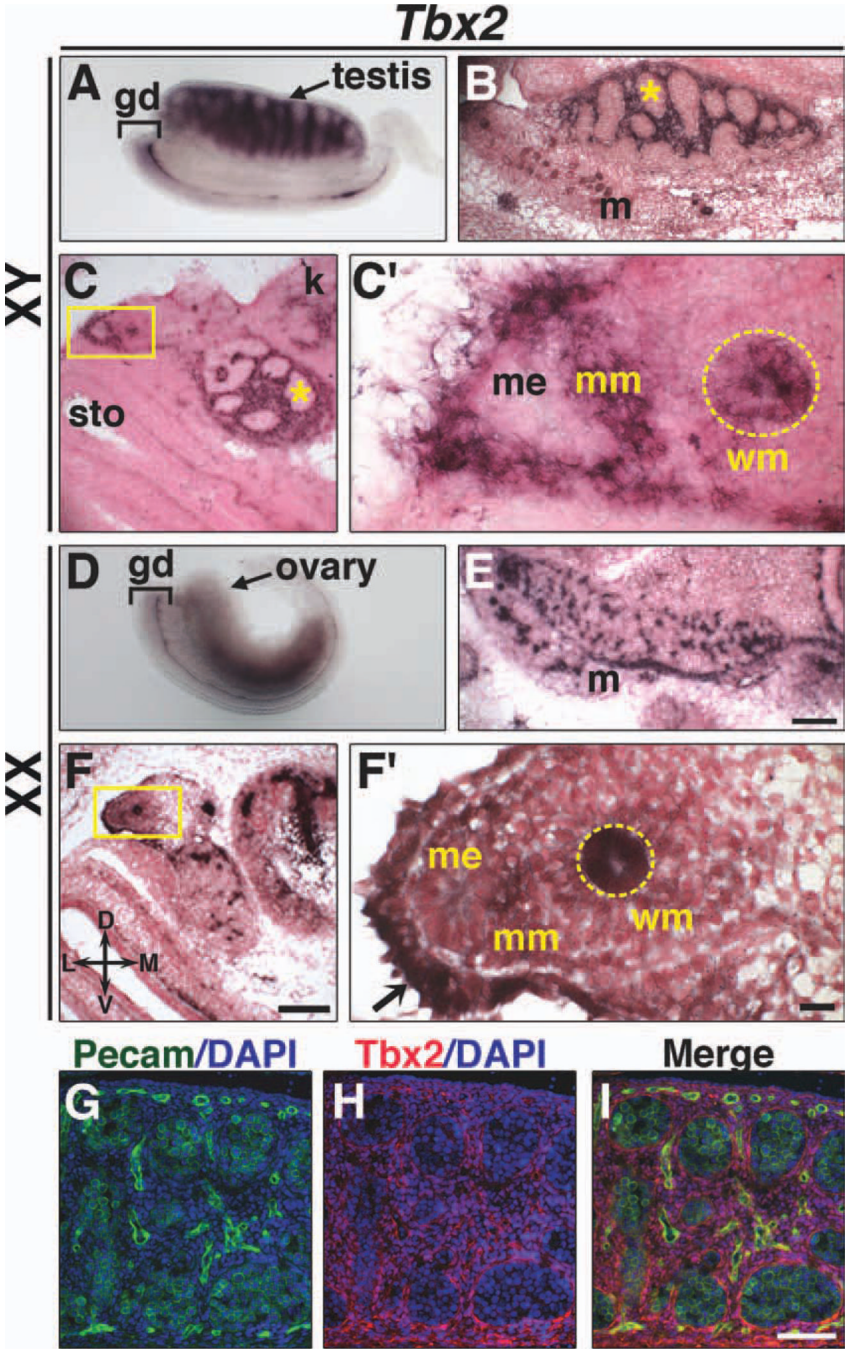

Fig. 3.

Expression of *Tbx2* in isolated internal reproductive systems at E13.5. Whole mount ISH (**A, D**), section ISH on sagittal (**B, E**) and transverse (**C, F**) sections of isolated gonads, and IF (**G–I**) are shown. In XY embryos, *Tbx2* is expressed in the interstitium around testis cords (asterisk) (A–C), Müllerian duct mesenchyme (**C′**), and Wolffian duct epithelium (C′, dashed yellow circle). In XX embryos, *Tbx2* expression is observed throughout ovaries (D–F). *Tbx2* is expressed in Wolffian duct epithelium (**F′**, dashed yellow circle) and coelomic epithelium (F′, arrow). *Tbx2* is not expressed in Müllerian duct mesenchyme in females or Müllerian duct epithelia of either sex. Double IF (G–I) staining of sagittal sections shows

Tbx2 expression in the interstitium around testis cords and not in Pecam-positive germ cells. Boxed areas in C and F are shown at higher magnification in C′ and F′. D, dorsal; L, lateral; M, medial; V, ventral; gd, genital ducts; k, kidney; m, mesonephros; me, Müllerian duct epithelium; mm, Müllerian duct mesenchyme; sto, stomach; wm; Wolffian duct mesenchyme. Scale bars in E and F = 100 μm (B, C, E, F); scale bar in F′ = 10 μm (C′, F′); scale bar in I = 50 μm (G–I).

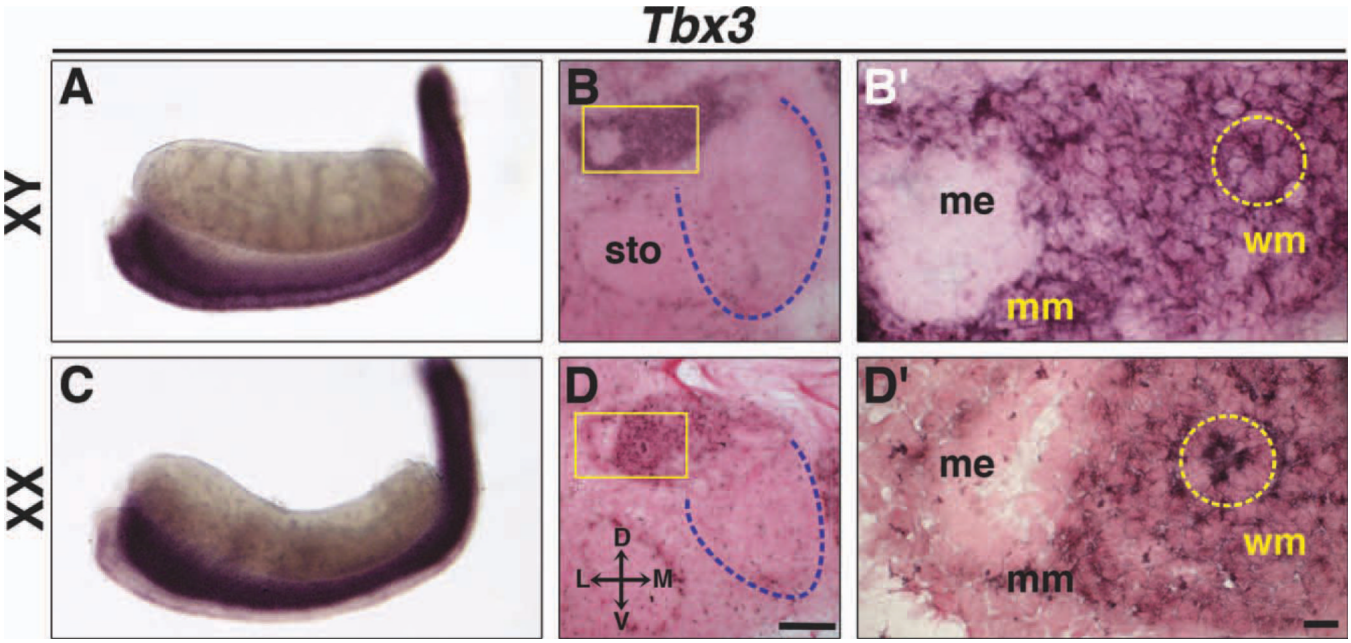

Fig. 4.

Expression of *Tbx3* in Wolffian and Müllerian ducts at E13.5. Whole-mount ISH and section ISH on transverse sections of XY (**A, B, B′**) and XX (**C, D, D′**) embryos. In XY and XX embryos, *Tbx3* is expressed in the Wolffian duct epithelium (dashed yellow circles) and mesenchyme around Wolffian and Müllerian ducts, but not in testes (A, B) or ovaries (C, D). Boxed areas in B and D are shown at higher magnification in B′ and D′. Dashed blue lines outline gonads in transverse sections. D, dorsal; L, lateral; M, medial; V, ventral; me, Müllerian duct epithelium; mm, Müllerian duct mesenchyme; sto, stomach; wm; Wolffian duct mesenchyme. Scale bar in D = 100 μm (B, D); scale bar in D′ = 10 μm (B′, D′).

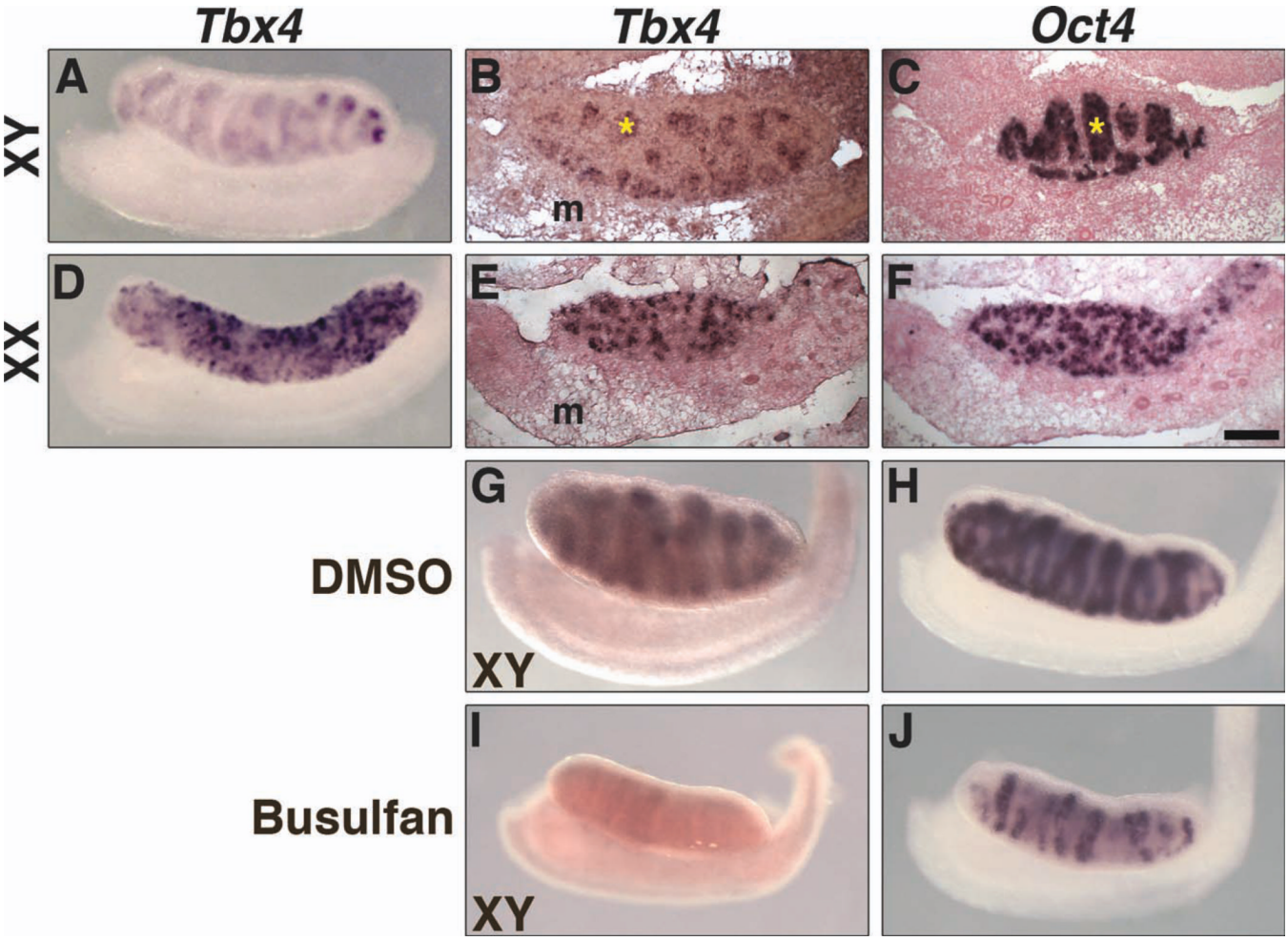

Fig. 5.

Expression of *Tbx4* in gonads at E13.5. Whole-mount and section ISH on sagittal sections are shown. In XY embryos, *Tbx4* is expressed in testis cords (asterisk) (**A, B**). In XX embryos, *Tbx4* is expressed throughout ovaries (**D, E**). *Oct4* expression in XY and XX germ cells (**C, F**) is shown for comparison. *Tbx4* and *Oct4* are expressed in control XY gonads (**G, H**). After administration of busulfan to deplete germ cells, expression of both *Tbx4* and *Oct4* is reduced in XY gonads (**I, J**). *Tbx4* is not expressed in genital ducts of either sex. m, mesonephros. Scale bar in F = 100 μm (B, C, E, F).

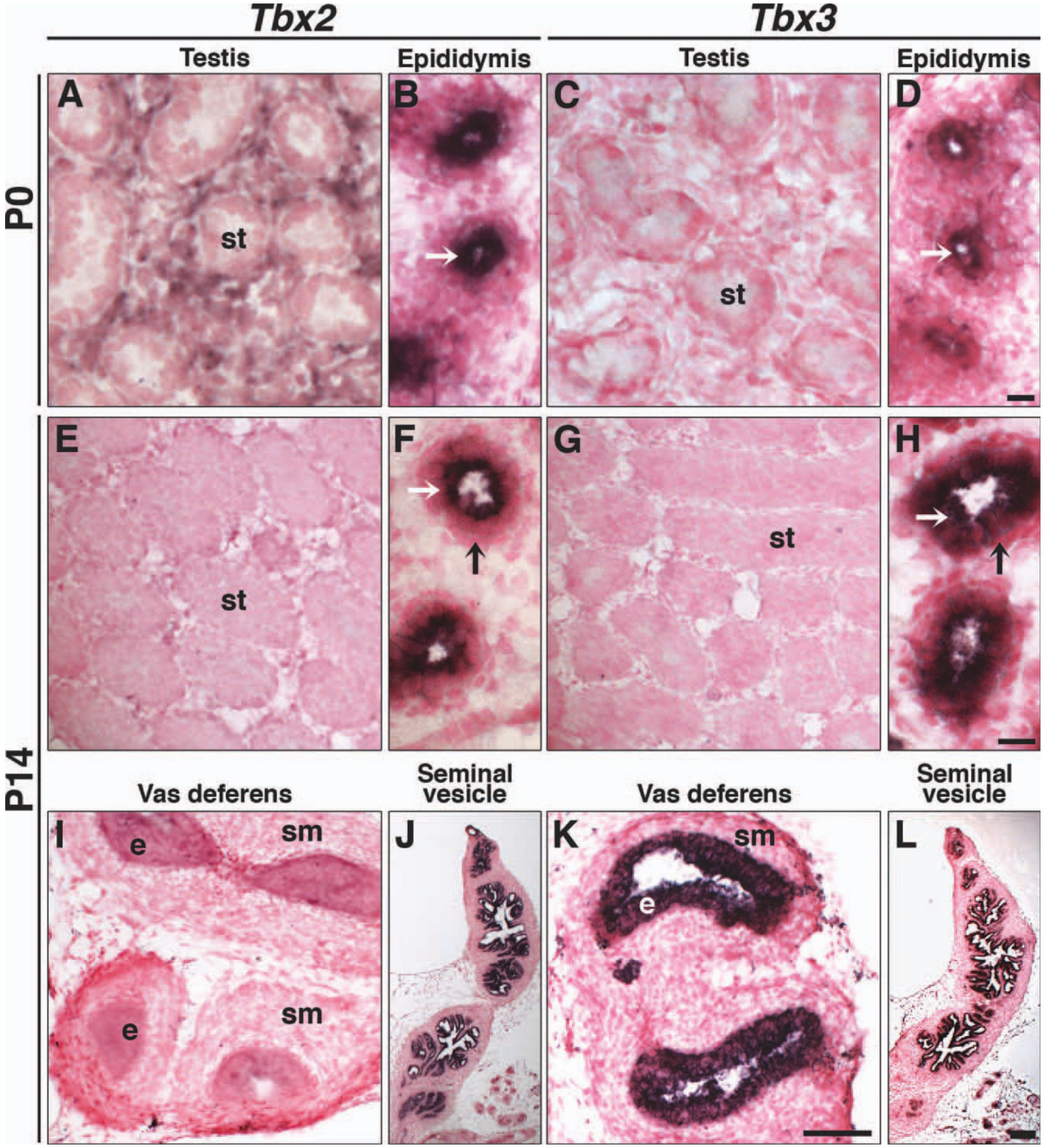

Fig. 6.

Tbx2 and *Tbx3* expression in postnatal testes and differentiated Wolffian ducts. ISH on sagittal sections of testes, epididymides, vas deferentia, and seminal vesicles. At P0, *Tbx2* is expressed in interstitial cells that surround seminiferous tubules (**A**) and epithelial cells of the epididymis (**B**, white arrow). *Tbx3* is not observed in testes (**C**), but is expressed in epithelial cells of the epididymis (**D**, white arrow). At P14, neither *Tbx2* nor *Tbx3* is expressed in testes (**E, G**). Both are expressed in epithelial cells (white arrows) of epididymal tubules and not mesenchymal cells (black arrows) surrounding epididymal tubules (**F, H**). *Tbx3*, but not *Tbx2*, is expressed in epithelial cells of the vas deferens (**I, K**)

and both are expressed in seminal vesicles (**J, L**). e, epithelium; st, seminiferous tubules; sm, smooth muscle. White arrows indicate epithelial cells of epididymal tubules. Black arrows indicate mesenchymal cells surrounding epididymal tubules. Scale bar in D = 10 μm (A–D); scale bar in H = 10 μm (F, H); scale bar in K = 50 μm (E, G, I, K); scale bar in L = 100 μm (J, L).

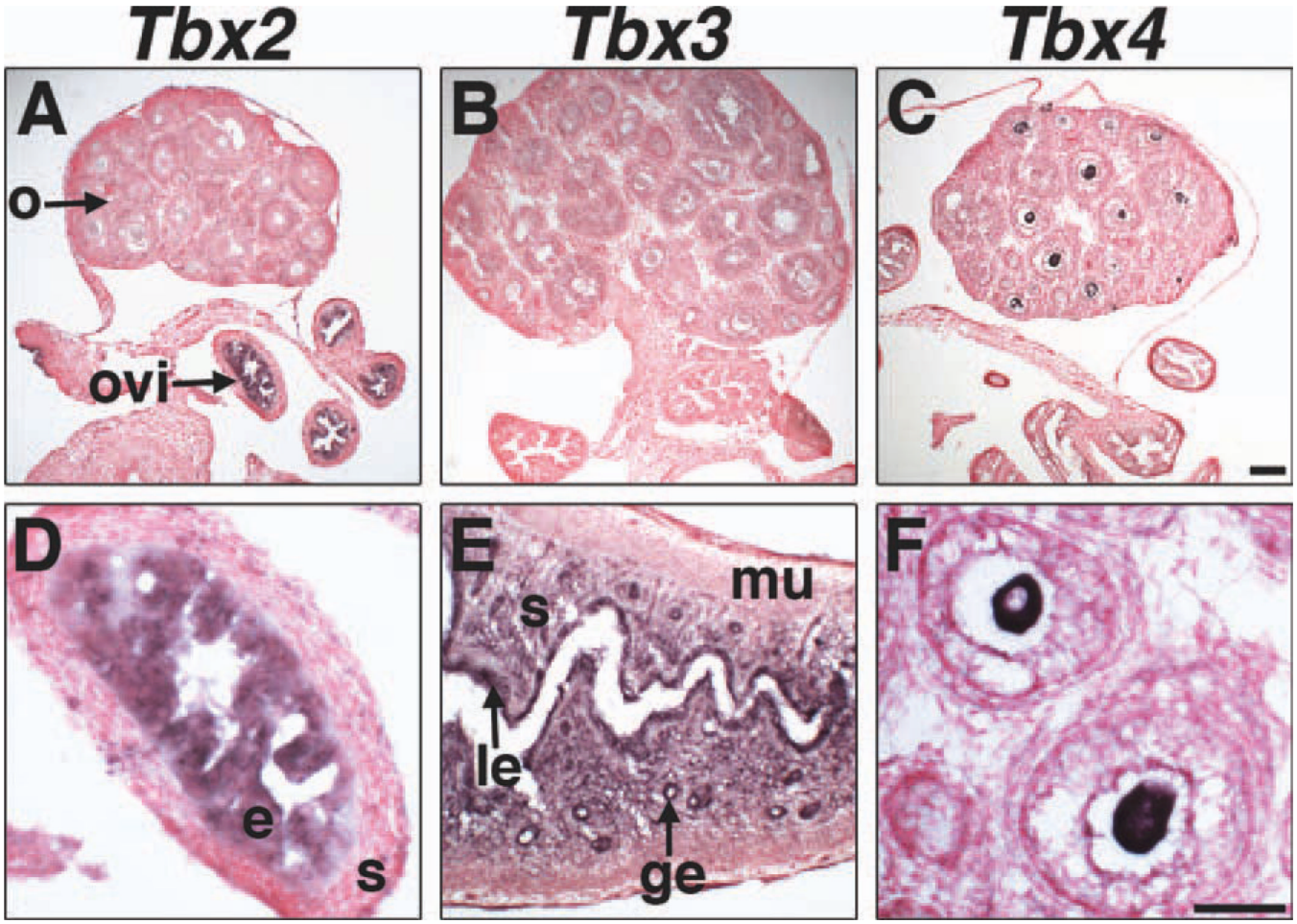

Fig. 7.

Tbx2, Tbx3, and *Tbx4* expression in the postnatal female internal reproductive system by section ISH on sagittal sections at P16. *Tbx2* is expressed in the epithelium, but not the stroma of the oviduct, and not in ovaries (**A, D**). *Tbx3* is expressed in the uterine luminal epithelium, glandular epithelium, and stroma (**E**), but not in ovaries or oviducts (**B**). *Tbx4* is expressed in oocytes, but not oviducts or in uterus (**C, F**). F shows expression of *Tbx4* in oocytes of two secondary follicles. ge, glandular epithelium; e, epithelium; le, luminal epithelium; m, muscle; o, ovary; ovi, oviduct; s, stroma. Scale bar in C = 100 μm (A–C); scale bar in F = 50 μm (D–F).

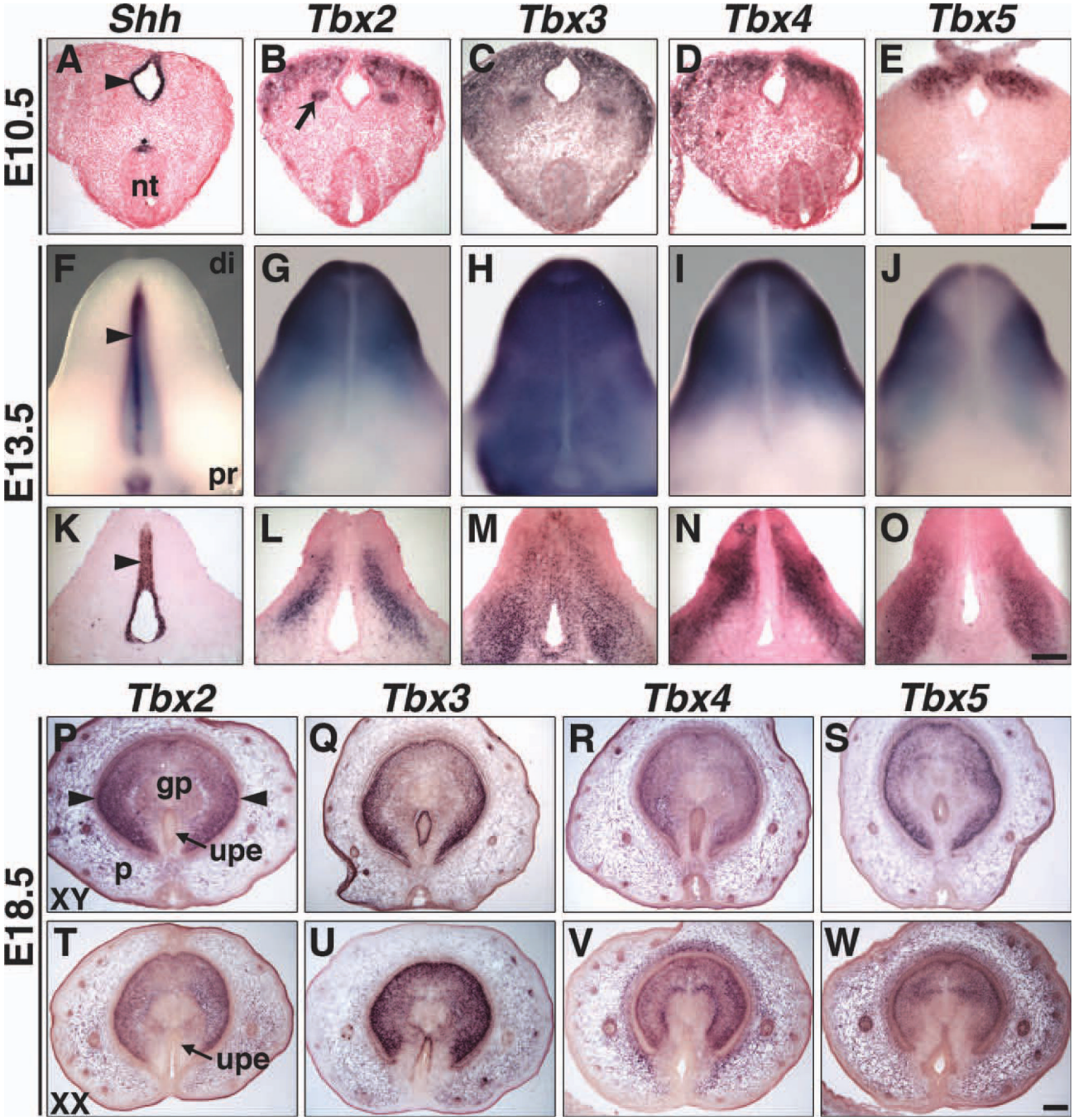

Fig. 8.

Expression of *Tbx2, Tbx3, Tbx4*, and *Tbx5* in genital tubercles at E10.5, E13.5, and E18.5 by ISH. E10.5 coronal sections (**A–E**), E13.5 ventral views of whole mounts (**F–J**) and longitudinal sections through GT (**K–O**), and E18.5 XY and XX transverse sections through GT (**P–W**) are shown. *Shh* expression in the epithelium of the cloaca (A, arrowhead) and UPE (F, K, arrowheads) is shown for comparison. *Tbx2* is expressed in the GT mesenchyme, but not epithelium (B, G, L). The arrow in B highlights *Tbx2* expression in the Wolffian duct. *Tbx3* is expressed in the epithelium and mesenchyme of the GT (C, H, M). *Tbx4* (D, I, N) and *Tbx5* (E, J, O) are both expressed in the GT mesenchyme, but their

patterns of expression differ. Neither *Tbx4* nor *Tbx5* are expressed in the epithelium at E10.5 and E13.5. At E18.5, the dorsal surface of the GT is at the top of each section (P–W). *Tbx2, Tbx3, Tbx4*, and *Tbx5* are expressed in mesenchyme, including the glans penis and prepuce, but not the glans lamellae (arrowheads) of XY (P–S) and XX (T–W) GT. Neither *Tbx2* (P, T) nor *Tbx4* (R, V) is expressed in urethral plate epithelial cells of XY or XX embryos. *Tbx3* is expressed in the UPE of both XY (Q) and XX (U) GT. *Tbx5* is expressed in XY (S), but not XX (W) UPE. di, distal; gp, glans penis; nt, neural tube; p, prepuce; pr, proximal; upe, urethral plate epithelium. Scale bars in E, O, and W = 100 μm (A–E, K–O, and P–W). Nuclear Fast Red counter-stain was not used at E18.5 (P–W).

TABLE 1

Summary of Tbx2 Subfamily Gene Expression in the Embryonic and Postnatal, Pre-Pubertal Internal Reproductive System[a]

			E13.5				
					P0	P14	P16
Gene	**E9.5**	**E11.5**	**XY**	**XX**	**XY**	**XY**	**XX**
Tbx2	we	we	Testis interstitium[b], we, mm	Ovary, we	Testis interstitium[b], epididymis	Epididymis, seminal vesicle	Oviduct
Tbx3	we	we, wm	we, wm, mm	we, wm, mm	Epididymis	Epididymis, vas deferens, seminal vesicle	Uterus
Tbx4	Absent	Gonad	gc in testis cords	gc in ovary	Absent	Absent	Oocytes

[a] gc, germ cells; we, Wolffian duct epithelium; wm, Wolffian duct mesenchyme; mm, Müllerian duct mesenchyme.

[b] Mesenchymal compartment.

[c] Epithelial compartment.