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Irx3 is differentially up-regulated in female gonads during sex determination

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Abstract

Irx3 is a member of the Iroquois homeobox gene family that encodes a protein known for its essential role in spinal cord development. Transcript screening of male and female gonads during the critical period of sex determination (E12–13.5) revealed a sexually dimorphic expression pattern for *Irx3* with female gonads exhibiting a sixfold increase in expression over time. Whole mount in situ hybridization confirmed the sexually dimorphic nature of *Irx3* expression and immunohistochemical analysis of gonads at E13.5 determined that IRX3 and GATA4 proteins co-localized to somatic cells of XX gonads. The *Irx3* signal persisted in germ cell-depleted XX gonads resulting from Busulfan treatment suggesting that its expression was independent of germ cell regulation. Quantitative real-time PCR analysis over an extended time course determined that *Irx3* message was low initially and then increased in XX gonads until E13.5, remained elevated until birth, diminished shortly after birth, and remained low in the adult ovary. In contrast, *Irx3* message was 50% lower in male compared to female gonads at the initial time point, and continued to decrease over time. Further analysis of adult ovaries suggested that IRX3 expression is not present in any subpopulations of cells of the differentiated ovary. Together, these results suggest that the *Irx3* signal is restricted to the somatic cell component of XX gonads and is present at a discreet period of ovarian development that ends abruptly at birth. This timing coincides with the transition of female primordial germ cells from mitotic proliferation to meiotic division, and the organization of germ cell cysts prior to primordial follicle development at birth.

Keywords: *Irx3*; *Iroquois*; Ovary; Gonad; Gonad development; Somatic cell

The chain of events surrounding the differentiation of a bipotential gonad into an ovary or testis occurs within a very narrow time frame during fetal development. The mechanisms underlying these processes are only beginning to be understood. We have discovered that expression of a factor belonging to the Iroquois homeobox family, Irx3, dramatically increases in female, but not in male gonads during this critical period of development. Compelling evidence has been presented in organisms ranging from plants to vertebrates that implicate Iroquois expression in critical developmental processes such as patterning and axis formation (Peters et al., 2000; Briscoe et al., 2000; Kudoh and Dawid, 2001). That this family of genes and their functions transcend multiple biological kingdoms confers significant credence to their importance. During development in lower vertebrates, the ortholog of *Irx3* acts as an organizer in establishing dorsal–ventral axis formation and influences cellular expansion and differentiation (Kudoh and Dawid, 2001). In mammalian models, *Irx3* expression influences dorsal-ventral patterning in spinal cord development and ultimately coordinates with other homeobox genes to determine specific neuronal fates in this tissue (Briscoe et al., 2000; Takahashi and Osumi, 2002).

Characterization of the *Iroquois* family member genes during mouse development has revealed cluster-specific and regionalized patterns in ectoderm, mesoderm and endoderm derived tissues as revealed by in situ hybridization. Only the IrxB cluster was expressed in gonadal tissue with a strong signal for *Irx3*, weak detection of *Irx5*, and no expression identified for *Irx6* (Houweling et al., 2001; Mummenhoff

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et al., 2001). Importantly, the sex of the tissues surveyed was not noted in these studies.

1. Results and discussion

1.1. Irx3 has a female-specific expression profile in developing gonads during sex determination

Gene expression profiles obtained by Affymetrix GeneChip® analysis determined that *Irx3* was slightly increased compared to the median signal intensity at embryonic day 12 (E12, Normalized intensity signal value, NISV 1.3), and then increased over sixfold (NISV 8.2) by E13.5 in female gonads. In contrast, *Irx3* expression in the male gonad (NISV 0.7) was approximately half the value of female gonads and signal detection diminished over time (Fig. 1A). Whole mount in situ hybridization (*WISH*) of urogenital ridges (gonad plus mesonephros) using a probe specific for *Irx3* first detected a signal in XX gonads by E12.5 that was considerably stronger at E13.5 (dark purple staining). In contrast, no *Irx3* signal was detected in XY gonads at any time (Fig. 1B). Together, these data verified the GeneChip® findings and illustrated the female-specific expression pattern of *Irx3*.

1.2. IRX3 is localized to the somatic cells in the female gonad

Double-label immunohistochemistry (IHC) was used to determine cell type-specific expression of IRX3 in embryonic gonads at E13.5. Co-localization was facilitated by simultaneously detecting GATA4, a marker for somatic cells of the developing gonad (Viger et al., 1998). GATA4 (green, FITC labeled secondary antibody) was detected in the same cells as IRX3 (red, Rhodamine labeled secondary antibody) only in female gonads (Fig. 2). No signal was detected for either protein in mesonephros tissue (arrow, XX gonad). In male gonads, no IRX3 was detected and GATA4 expression was more intense in newly differentiated Sertoli cells inside the testis cords, but also present in sporadic cells outside of the testis cords consistent with previous characterization of GATA4 expression at this developmental age (Fig. 2, XY gonad; Viger et al., 1998).

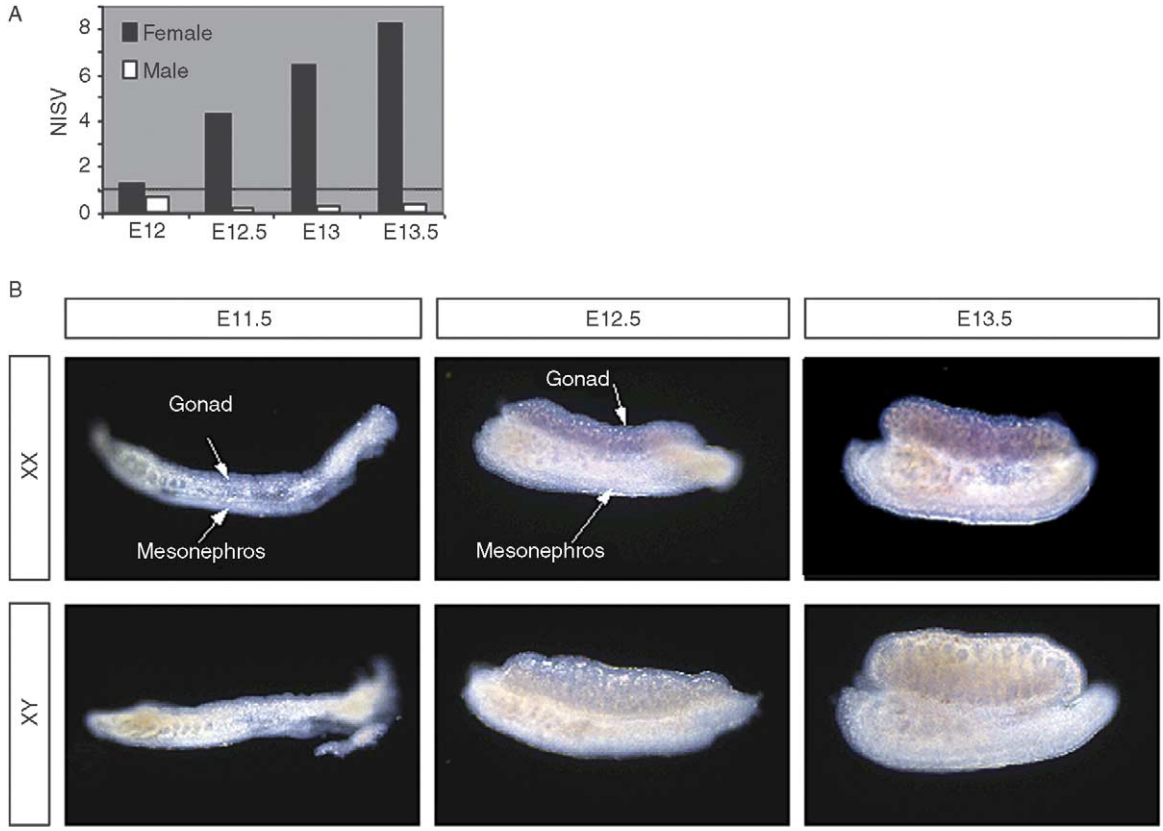

Fig. 1. *Irx3* has a female-specific expression profile in developing gonads. (A) Normalized signal intensity values (NISV) from Affymetrix GeneChip analysis of *Irx3*. Dotted line represents NISV = 1, a value of one indicates no change, greater than one an increase in expression, less than one a decrease in expression compared to the normalized median signal of *Irx3* from male (white bars) and female (black bars) gonads at E12, E12.5, E13, and E13.5. (B) *WISH* analysis of urogenital ridges using digoxigenin labeled *Irx3* probe at E11.5, E12.5, and E13.5. The gonad and mesonephros are indicated in two of the panels. Females (XX, top) are compared to males (XY, bottom).