Involvement of Homeobox Genes in Mammalian Sexual Development

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Key Words

Gametogenesis • Gonadogenesis • Homeobox • *Hox* genes • *Rhox* genes • Sex determination • Sexual development

‘Nobody will ever win the Battle of the Sexes. There’s just too much fraternizing with the enemy.’

Henry Kissinger

Abstract

In eutherian mammals, sexual development hinges on the differentiation of the gonadal primordia into either testes or ovaries, in turn determined by the activity of the Y-linked gene *Sry*. Organogenesis of the gonads requires input from a complex network of morphogenetic signals, many of which are still uncharacterised. Homeobox genes are known to play important roles in embryo morphogenesis and organogenesis in a number of systems. Here, we review current data relating to possible roles of the extended homeobox gene family on gonad development, sex-specific morphogenesis and gametogenesis, and speculate that these genes may play a broader role in these processes than is currently suspected.

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Of all the events that affect our lives purely by chance, arguably none is more profound than the random success of either an X- or a Y-bearing sperm in the race to reach an X-bearing oocyte. This event consigns each of us at fertilization to either female or male development, a dichotomy that reaches beyond biology to impact on many spheres including behavioural sciences, psychology, social sciences, politics, economics and recreation. The differences between males and females have exercised enquiring minds since the beginning of recorded history, and for many provide a lifelong source of fascination.

The primary function of the Y chromosome is to act as a vehicle for *Sry* which, in most mammals, causes the morphologically indistinct gonads of the fetus to differentiate into testes. In the absence of *Sry*, or more correctly of *Sry* function, the fetal gonads differentiate into ovaries. In turn, the differentiated gonads set in motion cascades of genetic and hormonal activity that dictate secondary sexual characteristics of the developing fetus; i.e., a male or female sexual phenotype. Thus, for the de-

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developmental biologist, a full understanding of the genesis of male and female characteristics rests on exposing the genetic networks leading to testis and ovary differentiation, and understanding how these bring about the organogenesis of other sex-specific ducts, tissues and organs.

The list of genes known to be involved in the regulation of sex differentiation and development has expanded significantly over the last decade. Similarly, our understanding of the intricate pathways leading to sexual identity has grown (Brennan and Capel, 2004; Wilhelm and Koopman, 2006). One theme that has emerged from these studies is that certain key gene families – *Wnt*, *hedgehog*, *Egf*, *Sox* and *Tgfb*, to name only a few – are consistently found to play roles in orchestrating development of diverse organ systems. Curiously, however, the role of homeobox genes in sexual development has remained relatively unexplored and unknown.

Homeobox genes encode homeodomain-containing transcription factors that act in a variety of contexts during development and are present in all animals examined. More than 200 homeobox genes have been characterised in vertebrates, grouped into numerous sub-families depending on sequence homologies. The canonical class of homeobox genes is the *Hox* subfamily, structurally and functionally homologous to the *Drosophila* homeotic complex (HOM-C), and in mammals represented by the 39 genes of the *Hox* complex. All remaining homeobox genes are grouped into smaller subfamilies and are collectively referred to as divergent homeobox genes (i.e. are not *Hox* genes).

Many homeobox genes show strong evolutionary conservation not only in homeodomain sequence but also in function, and seem to share a universal involvement in cellular specification and identity during embryogenesis. Body axis patterning of bilateral animals by spatio-temporal *Hox* gene expression is remarkably conserved across phyla, as is the regulation of nervous system development of distantly related organisms by LIM-homeodomain (LIM-HD) proteins. In general, the vast majority of developmental processes involve functional input from homeodomain transcription factors and, as will be discussed, the regulatory pathways governing sexual development also include members of the extended homeobox gene family.

This review focuses on the current state of knowledge regarding the involvement of homeobox genes in sexual development in mammals. We explore their disparate and often poorly understood roles in development and function of the reproductive system, as summarised in

table 1 and figure 1, and predict that a potentially more significant involvement of these important regulators remains to be uncovered. In particular, a picture is emerging of extensive involvement of homeobox genes in mammalian gametogenesis through the regulation of germ cell-specific gene expression.

Early Gonadal Development

LIM Homeobox Genes

Of the 12 characterized LIM-HD genes, 2 are known to play a regulatory role in early urogenital development. One of these, *Lhx1* (*Lim1*), is expressed in embryonic gonads, mesonephroi and kidneys (Barnes et al., 1994; Fujii et al., 1994; Karavanov et al., 1998), but its function in sexual development has been difficult to unravel because of early lethality of *Lhx1* mutants at 10 days post coitum (dpc) (Shawlot and Behringer, 1995). However, surviving *Lhx1*-null female neonates completely lacked oviducts, uterus, cervix and the upper part of the vagina, showing an essential role for *Lhx1* in female Müllerian agenesis. On the other hand, the female *Lhx1* nulls developed normal ovaries, indicating that *Lhx1* is dispensable for gonad formation (Kobayashi et al., 2004).

In rat, mouse and chick, expression of another LIM-HD member, *Lhx9* (*Lim9*), is evident in both sexes at early stages of gonadogenesis. Expression of *Lhx9* begins in mice in the presumptive gonadal region beginning around 9.5 dpc and subsides as gonadal somatic cells begin to differentiate (Birk et al., 2000; Mazaud et al., 2002). *Lhx9*^{-/-} mice show impaired somatic cell proliferation as well as a failure to form discrete gonads; however, no other major developmental defects are evident (Birk et al., 2000). The expression of steroidogenic factor-1 (*Sf1*) is significantly downregulated in *Lhx9*-deficient genital ridges, and LHX9 is evidently an upstream regulator of *Sf1* (Wilhelm and Englert, 2002). However, the molecular details of the role of LHX9 in early gonadal development, including the identification of interacting partners that mediate functional specificity and the full suite of genes regulated by LHX9, remain to be elucidated.

Emx2: An Important Transcriptional Regulator with Elusive Functional Specificity

Emx2 is another homeobox gene crucial for early gonadal development. Mouse *Emx2* is orthologous to the *Drosophila empty spiracles* (*ems*) homeobox gene, which regulates head morphogenesis. In vertebrates, *Emx2* function is known to be important for the developing

Table 1. Homeobox genes expressed during mammalian sexual development

Gene	Expression	Function	Reference
<i>Early gonadal development</i>			
<i>Lhx1</i>	Developing urogenital structures including gonads	<i>Lhx1</i> mutants are embryonic lethal 10.0 dpc. <i>Lhx1</i> essential for developing female reproductive tract, but dispensable for gonad development.	Shawlot and Behringer, 1995; Kobayashi et al., 2004
<i>Lhx9</i>	Early genital ridge	<i>Lhx9</i> ^{-/-} fail to form discrete gonads and show abrogated somatic cell proliferation. Upstream regulator of <i>Sf1</i> .	Birk et al., 2000; Mazaud et al., 2002; Wilhelm and Englert, 2002
<i>Emx2</i>	Early mesonephros and indifferent gonad	<i>Emx2</i> ^{-/-} show gross urogenital phenotype, including regressed gonads at 13.0 dpc. Also suggested to function in altering endometrial cell proliferation during female oestrous cycle.	Miyamoto et al., 1997; Pellegrini et al., 1997; Daftary and Taylor, 2004; Taylor and Fei, 2005
<i>Sex differentiation and development</i>			
<i>Rhox6/9</i>	Female-specific expression in foetal gonads, peaking at around 13.0 dpc. Also adult ovary and placenta	Knockout mice show no obvious phenotype. Functional redundancy between <i>Rhox6</i> and <i>Rhox9</i> is suggested.	Takasaki et al., 2000, 2001; MacLean et al., 2005
<i>Arx</i>	Interstitial of fetal male gonads, but not in Leydig cells	Leydig cells fail to differentiate in <i>Arx</i> ^{-/-} male gonads.	Kitamura et al., 2002
<i>Esx1</i>	Extraembryonic tissues and male germ cells	Suggested involvement in placenta-influenced foetal growth and spermatogenesis.	Branford et al., 1997; Li et al., 1997; Li and Behringer, 1998; Yeh et al., 2005
<i>Lbx2</i>	Genital ridge from 11.5 dpc	Unknown	Chen et al., 1999
<i>Mkx</i>	XY gonads from 11.5 dpc, localized to testis cords at 13.5 dpc	Unknown	Anderson et al., 2006
<i>HOXA7</i>	Ovarian follicles	Shown to be involved in the regulation of proliferative activities of developing human ovarian follicles.	Ota et al., 2006
<i>Hoxa11</i>	Stromal cells surrounding Mullerian and Wolffian ducts	Targeted mutations cause male and female sterility.	Hsieh-Li et al., 1995
<i>Hoxa13</i>	Genital tubercle	Mutations cause HFGS.	Reviewed by Scott et al., 2005
<i>Hoxd13</i>	Genital tubercle	Mutations cause SPD.	Reviewed by Scott et al., 2005
<i>Pbx1</i>	Throughout developing urogenital system	<i>Pbx1</i> ^{-/-} embryonic lethal 15/16 dpc. Histologically indistinguishable gonads at 14.5 dpc.	Selleri et al., 2001; Schnabel et al., 2003
<i>Oogenesis</i>			
<i>Irx3</i>	XX embryonic gonads, peaking at 13.5 dpc	Unknown	Jorgensen and Gao, 2005
<i>Nobox</i>	Primordial and growing oocytes	<i>Nobox</i> ^{-/-} shows disrupted postnatal follicle development. Knockout mouse shows downregulated <i>Oct4</i> and <i>Gdf9</i> expression.	Suzumori et al., 2002; Rajkovic et al., 2004
<i>Obox1–Obox6</i>	Adult gonads/germ cells. <i>Obox4</i> is testis-specific, whereas remaining five are ovary specific	Unknown	Rajkovic et al., 2002
<i>Eso-1</i>	ES cells and oocytes	Unknown	Li et al., 2006
<i>Lhx8</i>	Germ cells	<i>Lhx8</i> ^{-/-} have germ cell depleted ovaries.	Pangas et al., 2006
<i>Spermatogenesis</i>			
<i>Sprml</i>	Male germ cells in adult testis	Spermatogenesis. <i>Sprml</i> ^{-/-} male mice are hypofertile, but develop morphologically normal testis.	Pearse et al., 1997
<i>Rhox5</i>	Sertoli cells of adult testis, but also adult ovary and placenta	<i>Rhox5</i> ^{-/-} males are hypofertile. Suggested involvement in spermatogenesis.	MacLean et al., 2005
<i>TGIF</i>	Male germ cells in adult testis	Unknown (<i>TGIF</i> orthologous gene in <i>Drosophila</i> causes male sterility).	Ayyar et al., 2003
<i>Pbx4</i>	Adult testis	Suggested involvement in spermatogenesis.	Wagner et al., 2001
<i>Hox</i>	Several members expressed in adult testis	Suggested involvement in spermatogenesis.	Reviewed by Rao and Wilkinson, 2002

Fig. 1. Known or suspected roles of homeobox genes in embryonic and postnatal gonadal development. A simplified view of the genetic pathways governing gonadogenesis from the bipotential stage through to the differentiated testis and ovary (A) and post-natal regulatory and developmental events of testes and ovaries including gametogenesis to fertilisation (B). Some functionally important genes and molecules are indicated in black, with homeobox genes highlighted in red. Underscores indicate hypothesised roles based on evidence discussed in the text. Many genes with established function during the various developmental stages have been omitted for simplicity. RA = Retinoic acid.

central nervous system (Kastury et al., 1994; Pellegrini et al., 1996; Yoshida et al., 1997). In the urogenital system, *Emx2* is expressed in the epithelial components, with transcripts detected in the pronephric primordium at 9 dpc, and in the coelomic epithelium and mesonephros of both sexes at 10 dpc (Simeone et al., 1992a, b; Pellegrini et al., 1997). At 13.5 dpc, *Emx2* expression becomes very weak in male gonads, but strong expression remains detectable throughout the female gonad; however, expression is maintained in mesonephric and parameso-

nephric ducts of both sexes (Pellegrini et al., 1997). These data suggest an involvement of *Emx2* in urogenital development from the very earliest stages.

In support of this possibility, *Emx2* mutant mice fail to develop kidneys, ureter, gonads and urogenital tract, and die a few hours after birth due to renal anomalies (Miyamoto et al., 1997). In renal development, *Emx2* seems to be essential for metanephrogenesis, with deterioration of the ureteric bud in mutant mice and consequential failure to induce epithelialisation of the mesen-

chyme and its differentiation into the nephron (Miyamoto et al., 1997). The *Emx2* mutant phenotype is similar to those of *Lhx9*, *Wt1* and *Sf1*, and all these genes are expressed from an early stage before sex determination and gonadal differentiation. Common amongst these mutants are impaired thickening of the coelomic epithelium before 12 dpc, suggesting involvement in early cell proliferation and/or differentiation of coelomic epithelium cells.

In the developing central nervous system, *Emx2* appears to regulate proliferation and suppress differentiation, with *Emx2* transcripts downregulated upon cell differentiation (Gulisano et al., 1996; Tole et al., 2000; Gangemi et al., 2001; Galli et al., 2002, 2004). Persistent *Emx2* expression in the female gonads after sex determination and loss of expression in the male gonads may reflect the prolonged indifferent stage of the ovary as distinct from early differentiation of the testis. As *Emx2* expression is lost in male gonads at the onset of differentiation, so also is *Wnt4*, whereas both are maintained in the female gonad. *Emx2* has been reported to stimulate Wnt signalling in a mutual auto-regulatory loop in the occipital cortex and hippocampus, and reactivation of the Wnt signalling pathway partially normalizes the *Emx2*^{-/-} phenotype (Muzio et al., 2005). Thus, it is highly likely that *Emx2* is involved in regulating Wnt signalling which is known to play a role in ovary development and the formation of the female reproductive tract (Vainio et al., 1999).

Emx2 expression persists in adult reproductive organs, and experimental evidence suggests that EMX2 functions as a cell-specific anti-proliferative factor during the female oestrous cycle, ensuring endometrial receptivity to implanting blastocysts (Daftary and Taylor, 2004; Taylor and Fei, 2005). Here, EMX2 is temporally expressed in an inverse manner to HOXA10, which has been shown to directly suppress *Emx2* transcription by binding to its promoter (Troy et al., 2003).

Interestingly, expression of *Hoxa10*, *Hoxa9*, *Hoxa11* and *Hoxa13*, is also detected in the paramesonephric (Müllerian) duct of embryonic mice from 15.5 dpc (Taylor et al., 1997). However, it is not known whether HOXA10 transregulates *Emx2* during early development of the female reproductive system as it does in the adult ovary (Troy et al., 2003). Also, due to transcriptional and functional redundancy of HOX proteins, paralogous HOX members could also potentially transregulate the *Emx2* gene. It is possibly of interest that *Hoxa11* homozygous mutant mice show urogenital phenotypes, resulting in both male and female sterility (Hsieh-Li et al., 1995). Fi-

nally, to better understand EMX2 function both during embryogenesis and in the adult, downstream target genes need to be characterised.

Sex Differentiation

Rhox Genes: New Players in Reproductive Biology

One of the most exciting advances in the field of homeobox gene biology since the elucidation of the mouse and human genome sequences is the emergence of a previously undiscovered cluster of homeobox genes mapping to the X chromosome (MacLean et al., 2005). These reproductive homeobox X-linked (*Rhox*) genes show preferential expression in the reproductive organs in mice, with sex-specific expression in adult mouse gonads (MacLean et al., 2005), suggesting that these genes may be major players in the development or function of the reproductive tissues.

In mouse, the X-linked *Rhox* cluster consists of 32 genes. Many show strong homology to *Rhox2*, *Rhox3* and *Rhox4*, suggesting that they arose through duplication of an isolated region within the ancient *Rhox* cluster (MacLean et al., 2006; Morris et al., 2006; Wang and Zhang, 2006). Three of the *Rhox* genes (*Rhox3a*, *Rhox3c* and *Rhox3f*) are reported to encode premature stop codons, thus rendering them pseudogenes (Wang and Zhang, 2006). However, only *Rhox3c* contains a stop codon within the homeobox sequence, whereas *Rhox3a* and *Rhox3f* can make use of immediate downstream start codons for the transcription of full-length coding sequences. Thus, there are most likely 31 functional *Rhox* genes plus one pseudogene in the mouse genome. The structure of the *Rhox* cluster is depicted in figure 2.

Although MacLean et al. (2005) were the first to discover the entire *Rhox* cluster, a small number of family members have been described in previous studies. The first to be described was *Pem* (Wilkinson et al., 1990; Rayle, 1991; Sasaki et al., 1991), now known as *Rhox5*. Later, *Psx1* (Han et al., 1998; Chun et al., 1999) and *Psx2/Gpbox* (Han et al., 2000; Takasaki et al., 2000) were identified, renamed *Rhox6* and *Rhox9*, respectively, followed by *Tox* (testis and ovary specific homeobox) (Kang et al., 2004), now known as *Rhox8*. Studies of these genes offer some insight into their regulatory potential.

Expression of these 4 genes is highly specific to the reproductive organs. Although knockout mice for *Rhox5*, *Rhox6* and *Rhox9* produced no obvious abnormal phenotypes (Pitman et al., 1998; Takasaki et al., 2001), subsequent analyses have indicated a functional involvement

Fig. 2. The mouse *RhoX* gene cluster on the X chromosome. Functional *RhoX* genes are depicted as coloured squares, whereas an empty square depicts a postulated pseudogene. The three adjacent genes, *RhoX2*, -3 and -4, have undergone extensive duplication events in the mouse genome and are depicted as blue, red and green squares respectively. Based on sequence similarities, they are further denominated with additional suffixes a–g. Arrows indicate direction of transcription. An additional *RhoX* gene, *RhoX3f*, was reported by Wang and Zhang (2006), however more evidence is needed to conclusively state that it encodes a full transcript. No *RhoX* gene has been identified outside the X chromosome. The gene map is not drawn to scale.

of *RhoX5* in spermatogenesis (MacLean et al., 2005). Furthermore, because *RhoX6* and *RhoX9* show strikingly similar female-specific upregulation in germ cells at the onset of sexual dimorphism (Takasaki et al., 2000), lack of knockout phenotypes may be a result of functional redundancy. Therefore, a double knockout is required to characterise *RhoX6/RhoX9* function during early female gonad development.

Based on these observations, the *RhoX* genes are likely to play a broader role in the regulation of gametogenesis, both in male and female, and it will be of great interest to establish their detailed spatial and temporal expression patterns and functional specificity in fetal gonads.

Arx and *Esx1*

Also located on the X chromosome, but distal to the *RhoX* cluster, are the homeobox genes *Arx* and *Esx1* (*Spx1*). Both show striking sequence similarities to the *RhoX* genes and it may be speculated that all may have arisen from a common ancestral homeobox gene. Furthermore, all genes are expressed in the reproductive organs, although their expression patterns differ within these systems.

Arx is expressed in peritubular myoid cells, tunica albuginea, vessel endothelial cells and interstitial fibroblast-like cells in fetal male gonads from 12.5 dpc and appears to be important for Leydig cell differentiation during fetal testis development (Kitamura et al., 2002). *Arx* expression is undetectable in the Leydig cells themselves (as well as in Sertoli and germ cells), but the Leydig cell marker *Hsdb1* is diminished in the interstitial region of *Arx*-negative XY mutant testes (Kitamura et al., 2002), indicating that *Arx* functions through paracrine signalling pathways to promote Leydig cell differentiation. As yet, only *Pax4* has been identified as a target gene for ARX during pancreatic cell-lineage specification (Collombat et al., 2005), and although *Arx* plays a crucial role in the development of testis, pancreas and forebrain, and mutations in ARX are a common cause of X-linked disease in humans (Gecz et al., 2006), very little is known regarding how ARX functions in vivo.

Esx1 is expressed in extra-embryonic tissues during embryogenesis and is important for placental development. Through mutation analyses in mice, Li and Behringer (1998) showed *Esx1* to be an X chromosome-imprinted genetic regulator necessary for normal placen-

tal morphogenesis in that heterozygous mutant females would develop normally when inheriting a mutant *Esx1* allele from their father, but not when inheriting a maternal mutant *Esx1* allele, which would result in the same phenotype as homozygous mutant mice of both sexes; i.e., enlarged placenta and fetal growth retardation. In the adult mouse, *Esx1* is expressed in the testis and is thought to be involved in the regulation of spermatogenesis as expression seems to be limited to germ cells (Branford et al., 1997; Li et al., 1997; Yeh et al., 2005); however, any direct evidence for this is lacking.

Ladybird and Mohawk: Additional Regulators of Sexual Development?

Several studies implicate other members of the divergent homeobox gene family in sexual development. Expression of *Lbx2*, the murine orthologue of the *Drosophila ladybird* gene, in the developing eye and the brain has been reported; we note that *Lbx2* transcripts were also detected in the urogenital ridge from 10.5 dpc (Chen et al., 1999). Since that report did not specify the sex of the gonadal tissues examined, any sex-specificity is yet to be determined, and hence any potential significance during sexual development remains to be established.

In *Drosophila*, *ladybird* is responsible for maintaining expression of the *Wnt* homologue *wingless* in terminal and dorsal epidermis during embryogenesis (Jagla et al., 1997). In mice, *Wnt4* is thought to play a role in three distinct facets of female sexual development, namely the formation of the Müllerian duct, interstitial cell lineage differentiation and oocyte development involved in both male and female developmental pathways, and its expression is lost in the XY gonad as it differentiates into a testis (Vainio et al., 1999). By analogy with the role of *ladybird* in *Drosophila*, we hypothesize that *LBX2* in mice may also be involved in maintaining late *Wnt4* expression in XX gonads, thereby supporting late oocyte development, and thus independent of the hypothesised *Wnt4* regulation by *EMX2* during interstitial cell-lineage differentiation and the formation of the female reproductive tract.

Mkx (*Mohawk*) is a recently characterised gene closely related to the *Iroquois* class of the extended TALE-class (three amino acid loop extension) of homeobox genes, which during gonadogenesis is male-specifically expressed from 11.5 dpc, with expression restricted to testis cords at 13.5 dpc. Also, *Mkx* expression seems to closely follow that of *Sox9*, suggesting a possible regulatory relationship (Anderson et al., 2006). Extended expression profiling suggests that *Mkx* is likely to regulate diverse processes during development; because *Mkx* expression

generally precedes cell differentiation, it is hypothesised to act as a transcriptional trigger of differentiation events in several embryonic tissues. In mouse embryos, *Mkx* transcripts are detected from 9 dpc in the anterior somites, with expression extending to tail somites by 10.5 dpc. *Mkx* expression is also detected in the limb buds and kidneys at early embryonic stages (Anderson et al., 2006). To date, no functional analyses have been reported for *Mkx*.

The Complex Hox-Complex

The *Hox* complex is undoubtedly the most extensively studied sub-family of homeobox genes. The 39 mammalian *Hox* genes are organized into four paralogous clusters on four separate chromosomes, and are all orthologues of the *Drosophila* HOM-C complex genes (Scott, 1992). Their involvement in axial patterning of bilaterally symmetrical animals is well known, but they are also involved in an array of other morphoregulatory pathways. Despite their prominent place in molecular genetic studies of animal development, the transcriptional targets of mammalian HOX proteins mostly remain elusive and consequently their functional specificity has been difficult to unravel, as recently reviewed (Svingen and Tonissen, 2006).

Several *Hox* genes are expressed in both fetal and adult reproductive organs (Wolgemuth et al., 1987; Dolle and Duboule, 1989; Hsieh-Li et al., 1995; Adjaye and Monk, 2000; Pitera et al., 2001), however only during secondary sexual organogenesis and uterine implantation has HOX function been shown to be essential. Mutations in both *Hoxa13* and *Hoxd13* are linked to genitourinary malformations. As reviewed by Scott et al. (2005), mutations in human *HOXA13* cause the autosomal dominant disorder Hand-Foot-Genital Syndrome (HFGS), affecting the development of external genitalia, ureter and bladder. Similarly, *HOXD13* mutations are known to cause synpolydactyly (SPD), another autosomal dominant disorder affecting many of the same organs as HFGS, including external genitalia.

Hoxa11 mutations have been linked to defects in sexual development, with targeted mutations in mice causing both male and female infertility (Hsieh-Li et al., 1995). However, *Hoxa11* transcripts have not been detected in embryonic gonads. Female mutants produced normal oocytes, suggesting that female sterility was caused by uterine defects rather than improper gonadal development. Similarly, gonads of male homozygous mutants appear to develop normally, with infertility most likely caused by non-gonadal developmental defects such as

malformations of the vas deferens and perturbed spermatogenesis due to bilateral cryptorchidism.

HOXA10 and HOXA11 are also involved in embryonic implantation, with their expression in the endometrial glands and stroma regulated by sex steroids during the menstrual cycle (Taylor et al., 1998a, b). In addition to HOXA10 and HOXA11, HOXA9 and HOXA13 are also involved in female reproductive tract development, and their expression along the paramesonephric duct shows prototypic anterior to posterior expression boundaries, with HOXA9 limited to tissues developing into fallopian tubes and HOXA13 in the upper part of the vagina (Taylor et al., 1997). Much of how these transcription factors exert their effect is still to be elucidated, but some intricate regulatory pathways are starting to emerge. During decidualization, a high expression of the insulin-like growth factor binding protein-1 (IGFBP-1) is observed, which itself is progesterone-dependent (Bell et al., 1991). HOXA10 is known to activate *IGFBP1* expression in various types of endometrial cells in cooperation with the fork-head protein FKHR (Gao et al., 2002; Kim et al., 2003). Another HOX protein, HOXA5, again is reported to operate cooperatively with FKHR to stimulate the *IGFBP1* promoter (Foucher et al., 2005). To further add to this complexity, HOXA5 can also itself activate the progesterone receptor promoter (Raman et al., 2000).

It is curious that to date, no *Hox* genes have been found to play a role in gonadogenesis, either through expression studies or direct functional analysis. However, it is possible that functional redundancy among members of this large family may mask such a role. Overexpression of *Hox* genes in transgenic mice may be required to reveal a gonadal phenotype, as exemplified by skeletal phenotypes arising from *Hoxa7* knockout and transgenic studies (Balling et al., 1989; Chen et al., 1998).

PBX: Important HOX Co-Factors

PBX proteins are well-characterised HOX co-factors, and their transcripts are detectable in several reproductive organs. *Pbx1* is expressed in numerous embryonic tissues, and embryonic lethality at 15/16 dpc of *Pbx1*^{-/-} mice suggests an important developmental role for this gene (Selleri et al., 2001). In the urogenital system, *Pbx1* expression is detected within the coelomic epithelium and cells of the bipotential gonads and surrounding mesenchyme of the pronephric tubules, mesonephric tubules and Wolffian duct at 10 dpc. XX and XY gonads also show *Pbx1* expression in the interstitium at later stages (Schnabel et al., 2003). However, male and female gonads of *Pbx1*^{-/-} mice are histologically indistinguishable from

wild-type gonads at 14.5 dpc. Rudimentary gonadal structures do form in the mutant embryos as detected by laminin immunostaining, suggesting an arrest of sexual differentiation rather than failure to initiate gonad formation (Schnabel et al., 2003). Recently it was established that PBX1 forms a complex with HOX and PREP proteins to initiate *Sfl* gene expression during adrenal development (Zubair et al., 2006). Both *Pbx1*- and *Sfl*-deficient mice lack adrenal glands, and although separate from the gonads, the possible regulation of *Sfl* by PBX1/HOX complexes should be examined in the context of gonadal development.

Pbx genes are also expressed during mouse oogenesis. Although very little is known regarding HOX function during ovarian development and germ cell maturation, several *Hox* genes are co-expressed in the ovary with *Pbx* cofactors (Villaescusa et al., 2004), and future research will undoubtedly reveal HOX-PBX function in this tissue. Recently, HOXA7 was shown to be important for human ovarian follicular development, possibly by regulating cell proliferation (Ota et al., 2006). We speculate that this specific function may be achieved through interaction with TALE-class proteins, and further studies are necessary to appreciate where and how HOXA7 exerts its effects during folliculogenesis.

Gametogenesis

Oogenesis

As discussed above, published expression data point to the involvement of several *Rhox* genes in mouse oogenesis. Aside from *Rhox* genes, several other divergent homeobox genes show oocyte-specific expression in the mouse. Of the six murine *Iroquois* homeobox genes (*Irx1*–*Irx6*), *Irx3* has been shown to be highly expressed in XX gonads, reaching highest levels at 13.5 dpc, and prevailing until shortly after birth, in contrast to low levels of expression in XY gonads (Jorgensen and Gao, 2005). The stage at which *Irx3* expression reaches its plateau suggests an involvement in regulating the transition of female germ cells from mitotic proliferation to meiotic division.

At later developmental stages, another homeobox gene, *Nobox*, is preferentially expressed in primordial and growing oocytes (Suzumori et al., 2002). *Nobox* appears to be essential for postnatal follicle development as these processes are disrupted in knockout mice (Rajkovic et al., 2004). *Nobox*^{-/-} mice show downregulation of *Oct4* (a member of the POU class of homeobox genes),

Gdf9 and *Rfpl4*, suggesting that NOBOX acts early in the hierarchy of oocyte development.

A further homeobox gene family has been shown to be preferentially expressed in mouse female germ cells. Of the 6 identified *Obox* (oocyte-specific homeobox genes) members, all but *Obox4* (which shows preferential expression in the testis) are expressed exclusively in ovaries (Rajkovic et al., 2002). What functional role the reported expression of *Obox* genes plays during folliculogenesis remains to be determined, although their oocyte-specific expression is indeed intriguing.

More recently, a novel homeobox gene, *Eso1*, was characterised and shown to be preferentially expressed in mouse embryonic stem (ES) cells and ovaries (Li et al., 2006). The onset of gene expression coincides with the onset of meiosis in female germ cells at 13.5 dpc. With the additional observation that *Eso1* transcripts undergo cytoplasmic polyadenylation during oogenesis, Li and co-workers suggest that *Eso1* is involved in oogenesis and folliculogenesis.

In addition to the involvement of LIM-homeobox genes *Lhx1* and *Lhx9* in gonadogenesis as described above, a third member of this gene family, *Lhx8*, has been shown to be expressed specifically in germ cells in mice and is necessary for oogenesis. *Lhx8* expression is significantly downregulated in mice lacking *Sohlh1* (a basic helix-loop-helix transcription factor encoding gene essential for early folliculogenesis), and further experiments revealed that the *Lhx8*^{-/-} phenotype resembles that of *Sohlh1*^{-/-} mice, characterised by germ cell-depleted ovaries (Pangas et al., 2006). Thus, these transcriptional regulators can be added to the short list of known genes directing female germ cell development. It has been postulated that several hundred genes must be involved in the pathways driving oogenesis (Ko et al., 2000), and it is tempting to speculate that homeobox genes will play major roles in this regulatory hierarchy.

Spermatogenesis

A number of homeobox genes are known to be expressed in adult testis and further implicated in transcriptional regulation of spermatogenesis, including several *Hox* genes (Meijlink et al., 1987; Sharpe et al., 1988; Peterson et al., 1992; Watrin and Wolgemuth, 1993) and other divergent homeobox genes including *Cux1*, *Sprml*, *Pax5*, *Gtx* and *Rhox5* (Andersen et al., 1993; Lindsey and Wilkinson, 1996; Stein et al., 1996; Vanden Heuvel et al., 1996; MacLean et al., 2005). In this regard, it is interesting to note that *Pbx4*, a member of the divergent TALE-class of homeobox genes and well-characterised HOX co-

factors, shows specific expression in adult testis during gametogenesis (Wagner et al., 2001), and could operate synergistically with HOX proteins to transcriptionally regulate various events during spermatogenesis.

The POU-class homeobox gene *Sprml* is evidently important for proper development of spermatozoa in the adult testis as *Sprml*^{-/-} mice are hypofertile, however the knockout mice develop seemingly normal testes and spermatozoa numbers, and so the molecular mechanisms behind SPRM1 function during spermatogenesis remain unclear (Pearse et al., 1997). *Rhox5* knockout mice also show a hypofertile phenotype, possibly caused by increased apoptosis of meiotic germ cells in the testis (MacLean et al., 2005). In contrast to *Sprml*^{-/-}, however, *Rhox5*^{-/-} mice showed a weak epididymal and testicular phenotype.

In *Drosophila*, the TGIF-orthologous homeobox genes *achintya* and *vismay* are essential for sperm differentiation and *achi/vis* mutant flies are completely sterile (Ayyar et al., 2003). Ayyar et al. (2003) speculate that TGIF family genes may play a similar role in vertebrate spermatogenesis, and the hypothesis is substantiated by the observation that TGIF is expressed in spermatogonial stem cells and mitotic spermatogonial cells in adult mouse testis. However, the presence of several TGIF homeodomain proteins in vertebrates may reflect more intricate control mechanisms making their exact function more difficult to unravel. Indeed, this might be the case for numerous homeobox genes known to be expressed in testicular germ cells, exemplified by several viable and fertile *Hox* knockout mice where transcripts are clearly present in the germ cells (reviewed by Rao and Wilkinson, 2002).

Concluding Remarks

Sex determination and sexual development are complex processes involving many distinct but interconnected cellular events governed by a complex hierarchical network of regulatory genes and proteins. As we have shown, a growing number of homeobox genes are now known to be expressed in mammalian reproductive tissues at various stages from early embryogenesis to adulthood; in some cases the roles of these have been confirmed by mutational analysis, but for many a direct role remains to be established experimentally. In the case of *Hox* and *Rhox* genes however, gene knockout and/or knockdown approaches in mice are not going to be straightforward. Because these are large gene families

with members showing overlapping gene expression patterns, functional redundancy will likely obscure the phenotypes of individual gene targeting experiments. Therefore, multiple knockouts achieved by breeding or multiple targeting, or overexpression in transgenic mice may be required to reveal the functions of these genes in sexual development.

Evidently, both *Hox* and non-*Hox* homeobox genes are expressed in mammalian reproductive systems, with functional importance both during early gonadogenesis and the development of secondary sex organs. Also, the complete expression profile of the entire *Hox* cluster during separate time-points in the developing gonads and mesonephroi is yet to be determined. Both sexual development and *Hox* regulation and functioning are heavily influenced by endocrine signalling (Cummings and Kavlock, 2004; Daftary and Taylor, 2006), and thus it can be speculated that *Hox* genes might operate within cross- and auto-regulatory pathways controlling various facets of sexual development. Indeed, several *Hox* genes and non-*Hox* homeobox genes such as *Pax2* and *Cux* are believed to be important for proper male reproductive tract

development as thoroughly reviewed elsewhere (Rao and Wilkinson, 2002).

Another emerging trend regarding the homeodomain transcription factors, their expression patterns and suggestive functional specificity, is a bias towards gametogenesis, as indicated in figure 1. Whether this bias will be upheld as the functional specificity of these proteins continues to be characterised remains to be seen. Certainly, the emergence of increasing numbers of homeobox genes, such as the *Rhox* and *Obox* families, expressed specifically in reproductive tissues is encouraging for future efforts towards elucidating the molecular mechanisms controlling the events of sex determination and subsequent development of the reproductive organs, including postnatal gametogenesis and fertility.

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