Slugs and snails, or sugar and spice?

Sex determination and sexual differentiation

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Mammalian existence relies upon pregnancy producing either male or female offspring. Under normal circumstances, this 'polarized' outcome is determined by the chromosomal constitution of the spermatozoan that successfully fertilizes the ovum — a Y chromosome acts dominantly to produce a male foetus. This concept of sex chromosome action has been with us for almost a century[1]. However, understanding the genetic steps between fertilization and the development of male or female appearance proved less forthcoming for a long time. Building upon the landmark experiments of Jost in the 1940s[2], the previous decade has witnessed significant advances in the understanding of male and female development[3]. For the most part, information has arisen either by genetic manipulation of laboratory mice or by careful clinical and molecular genetic analyses of human individuals with sex reversal. In both circumstances, chromosomal constitution (46,XY or 46,XX) fails to correspond to the sexual phenotype (male or female).

During life in the womb, the passage of human sexual development post-fertilization can be broken down into three phases: (i) formation of the bipotential gonad, (ii) its determination to either a testicular or an ovarian fate, and (iii) its ensuing differentiation. The last step generates the foetal hormonal milieu that defines the sexual phenotype of the offspring.

Early formation of the gonad

Morphologically, gonadal formation starts during the fifth week of human development when condensation of the intermediate mesoderm and coelomic epithelial proliferation either side of the gut mesentery form the mesonephroi (a primitive kidney system) and the gonadal ridges (Figure 1A)[4]. Insight into the genetic regulation of this process in humans has largely been inferred from gene expression and targeted disruption analyses in mice. Accordingly, the genes encoding the LIM homeobox proteins LHX1 and LHX9, WT1, SF-1, and EMX2, all play key roles during early development of the bipotential mouse gonad[3]. More detailed investigation of the precise relationship between these transcription factors is complex. For instance, *WT1* encodes four major isoforms with different intracellular functions. Other gene knockouts are complicated by significant pathology in different organs. More recently, isoform-specific (e.g. for WT1) and conditional (e.g. targeted disruption of SF-1 in the anterior pituitary) transgenic animals have been created[5,6], and the use of these approaches should significantly advance our current understanding.

In humans, until 41 days post-conception, the gonadal ridge is morphologically indistinguishable in 46,XY and 46,XX embryos[7]. This bipotential (or 'indifferent') gonad, whose size is increasing, is thought to provide instructive signals that lead to the migration of primordial germ cells into the gonadal ridge from the posterior endoderm of the yolk sac (Figure 1A). The mechanism underlying this miraculous journey remains largely unknown but, coupled with cell proliferation, it results in the presence of approximately 6×10^5 germ cells within the gonad by the eighth week of human development. Ultimately, these fascinating cells provide the gametes for the next generation. This propensity to form all tissue types has been harnessed, at least partially, by laboratory groups searching for human stem cells[8,9]. Upon isolation, diploid primordial germ cells are capable of deriving pluripotent cells in culture that give rise to all three major lineages characteristic of the early human embryo, and from which all subsequent cell types arise.

Sex determination: translating genetic into gonadal sex

Between 44 and 52 days post-conception, the bipotential 46,XY gonad undergoes profound morphological change. This process

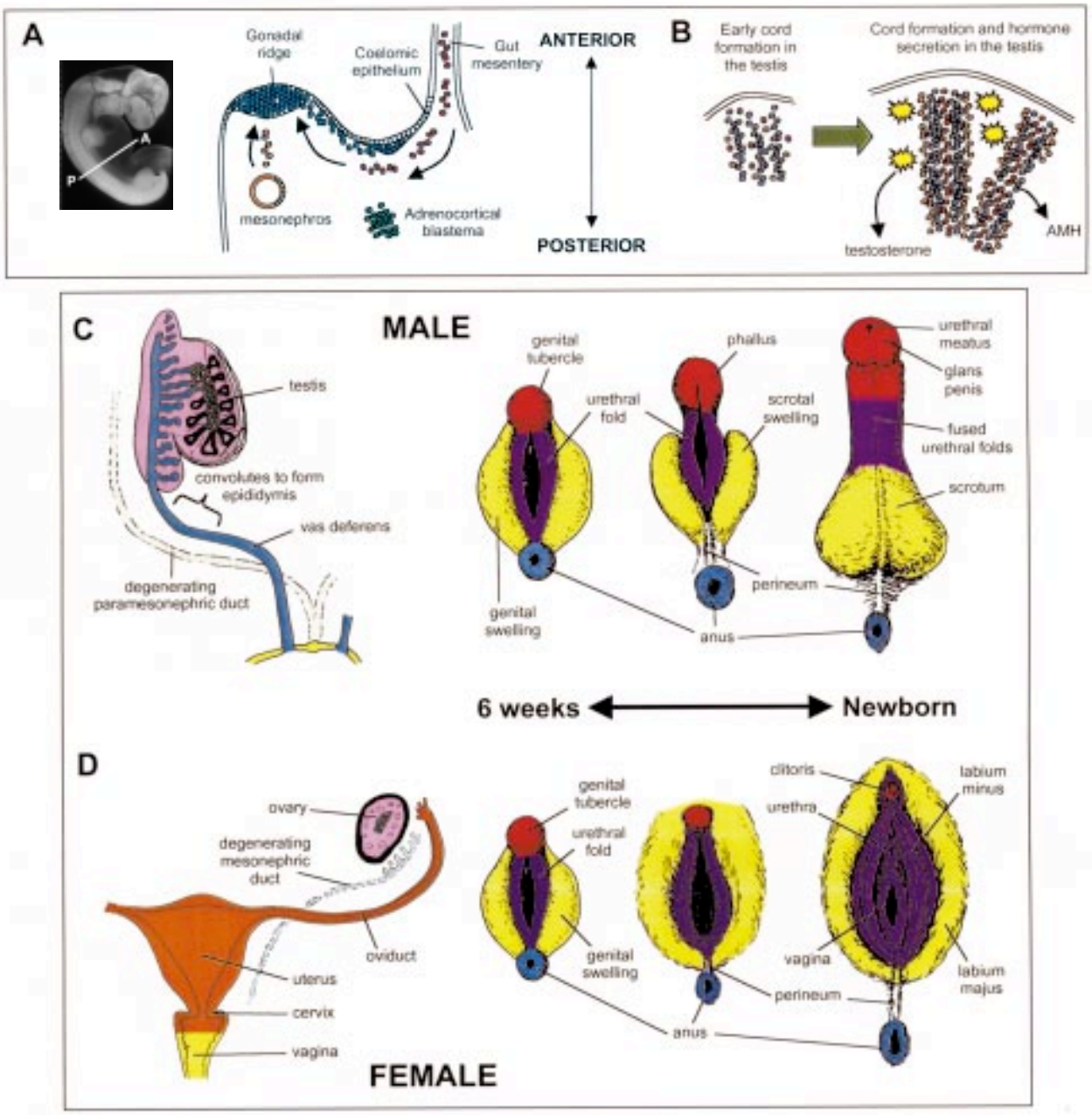


Figure 1. Formation of the bipotential gonadal ridge, testicular differentiation and sexual differentiation. (**A**) A transverse plane is shown through the left gonadal ridge of a human embryo at approximately 6 weeks gestation. A, anterior; P, posterior; blue, proliferating coelomic epithelium; red, migrating primordial germ cells; green, developing adrenal cortex. (**B**) Differentiating Sertoli cells in the presumptive testis form cords, which subsequently envelop the primordial germ cells and secrete AMH. Orange, mesonephros-derived cells; yellow, Leydig cells. (**C**) In males, the paramesonephric duct (pale green) regresses with development of the mesonephric system (epididymis and vas deferens, blue). (**D**) In females, the paramesonephric duct (brown) forms the oviducts, uterus, cervix, and upper third of the vagina, whereas the mesonephric system regresses (pale blue). The external genitalia are shown from 6 weeks gestation to the newborn period. Red, derivatives of the genital tubercle; purple, derivatives of the urethral folds; yellow, derivatives of the genital swellings.

(sex determination) irrevocably commits the gonad to a testicular fate owing to precisely regulated expression of several key genes. Coelomic epithelial cells differentiate into Sertoli cells, which subsequently surround the primordial germ cells to form the earliest testicular cords (Figure 1B)[10]. This anatomical arrangement induces mitotic arrest within the germ cells at a stage where they are primitive spermatogonia. This process requires unknown factors. The differentiation of Sertoli cells also stimulates the migration of mesonephric cells into the testis, and these cells contribute to the cell lining that surrounds the testicular cords (Figure 1B)[11].

Understanding the genetic regulation of this developmental cascade in males obviously focuses attention on the Y chromosome, and in 1990 the testis-determining gene, SRY was identified[3]. This gene induces differentiation of the Sertoli cell lineage, which steers the bipotential gonad away from an ovarian fate towards testicular development. Other genes have now been discovered that regulate differentiation of the Sertoli cell lineage. These genes, which act downstream of SRY, encode numerous transcription factors, which regulate the hormone expression that characterizes the early human testis.

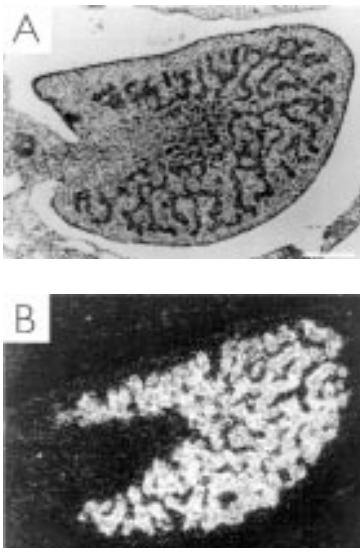


Figure 2. AMH expression at 52 days post-conception in the developing 46,XY human testis. Serial transverse sections are shown. (**A**) Haematoxylin and eosin staining of the darker sex cords within the lighter interstitium. (**B**) A dark-field photomicrograph showing AMH mRNA tissue expression as detected by *in situ* hybridization with an AMH anti-sense probe. Dense white/silver grains overlying the Sertoli cells demonstrate gene expression. Scale bar=80 μm.

"Several cases of familial sex reversal have been described that map to unexplained areas of the human genome."

During the period of testicular determination and early differentiation, little morphological change occurs within the gonadal ridge of 46,XX human embryos. This absence of testicular determination indicates an ovarian fate. One caveat is the presence of germ cells, as their absence leads to ovarian regression. Without SRY, 46,XX coelomic epithelial cells differentiate into follicular cells rather than Sertoli cells and the germ cells continue to proliferate. At approximately 11 or 12 weeks of gestation and while surrounded by follicular cells, the germ cells enter meiotic prophase, demarcating the transition from oogonia to oocytes. At this stage, ovarian morphology *per se* (rather than absence of testicular differentiation) is more apparent[12].

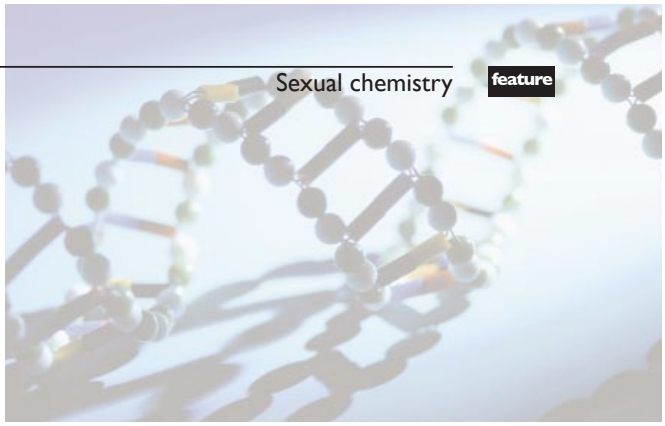
Sexual differentiation

The secretion of two hormones from the differentiating testis defines the early dimorphism in sexual phenotype. Sertoli cells secrete anti-Müllerian hormone (AMH, also called Müllerian inhibiting substance) under the transcriptional activation of SF-1, WT1, SOX9, and possibly GATA4 (Figure 2)[3]. Another transcription factor, DAX1 is proposed to repress these effects. Slightly later in testicular development (at approximately 8 weeks of gestation) Leydig cells differentiate in the interstitial spaces between the cords and secrete the hormone testosterone. Again, SF-1 is a key transcriptional activator of the genes responsible for testosterone biosynthesis[13].

This combined genetic regulation of hormone secretion orchestrates the development of 46,XY internal and external genitalia. The absence of these hormones in either 46,XX foetuses or aberrant 46,XY testicular differentiation results in the default development of female genitalia (Figures 1C and 1D). AMH suppresses development of the paramesonephric (Müllerian) duct, which if unhindered would give rise to the oviducts, uterus and the upper third of the vagina. In contrast, growth and development of the mesonephric system (derived from the mesonephros) under the influence of testosterone ultimately forms the vas deferens and epididymis (Figure 1C). In humans, dihydrotestosterone, produced via the peripheral conversion of testosterone by 5α-reductase, acts on the external genitalia. It stimulates complete fusion of the urethral folds, growth and fusion of the genital swellings to create the scrotum into which the testicles descend, and elongation of the genital tubercle to form the phallus (Figure 1C)[12].

Sex reversal

It stands to reason that any disruption of these events will perturb normal gonadal development, sex determination and sexual differentiation. Failure at the bipotential gonad stage leads to the



default female differentiation of the internal and external genitalia (Figure 1D). Further downstream, loss of function mutations in SRY result in 46,XY gonadal dysgenesis and phenotypically, male-to-female sex reversal. Mutation of the genes that encode transcriptional activators of AMH (SF-1, SOX9, WT1) is also associated with persistent Müllerian structures in 46,XY individuals[14]. Similarly, duplications of the X chromosome including the *AHC* gene, are proposed to result in excessive action of its product DAX1, potentially over-repressing AMH and resulting in a similar persistence of Müllerian derivatives (dosage-sensitive sex reversal). In contrast, duplication of the SOX9 locus in a 46,XX individual resulted in absence of the uterus, suggesting that SOX9 excess can over-ride the lack of SRY by causing Sertoli cell differentiation[15].

The effects of androgen under-representation in 46,XY individuals are more protracted. The earlier that the deficiency of testosterone occurs, the fewer androgen-dependent phenomena take place and the external genitalia will have a more female appearance. In this instance the urethral folds fail to fuse, but develop into the labia minora. The genital tubercle undergoes only minimal enlargement as the clitoris, the genital swellings expand to form the labia majora, and the gonad remains intra-abdominal (Figure 1D). A later deficiency in androgen, once scrotal fusion has occurred, results in more subtle defects of essentially male external genitalia, such as hypospadias where the urethral folds fail to fuse completely.

Unanswered questions

In keeping with virtually all aspects of biological research, knowledge of the genomic sequence that instructs our existence offers an unparalleled opportunity for rapid progress in understanding human sex determination and sexual differentiation. Several cases of familial sex reversal have been described that map to unexplained areas of the human genome[14]. By focusing on these regions armed with complete sequence data, we can confidently expect the discovery of new genes important in gonadal development. Similarly, advances in both microarray technology and transgenic manipulation of the laboratory mouse should serve as both the means to screen, on a genome-wide level, for differences in expression between the developing 46,XY and 46,XX gonad and as powerful tools to unravel the function of candidate genes.

Material used in Figure 2 forms part of the Newcastle University collection of the National Developmental Biology Resource, jointly funded by the MRC and Wellcome Trust (www.wellcome.ac.uk/en/1/biovendev.html).

Abbreviations used in this article

LHX	limb homeobox
WT1	Wilms tumour 1
SF1	steroidogenic factor 1 (NR5A1)
EMX2	homologue of Drosophila empty spiracles gene 2
SRY	Sex determining region of the Y chromosome
SOX9	SRY-related HMG-box gene 9
GATA4	GATA-binding protein 4
DAX1	DSS-AHC critical region on the X chromosome gene 1 (NROB1)
AHC	adrenal hypoplasia congenita gene

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