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SEX DETERMINATION AND SEXUAL DIFFERENTIATION

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SUMMARY

• //;. humans and other mammals, sex is determined by the presence or absence of Y chromosome. If Y chromosome is present, it will channel the genital ridge of the embryo into the pathway oftestis development, while in the absence of Y chromosome ovaries develop. Once testes have formed, they secrete anti-Miillerian hormone and testosterone, which masculinize the reproductive tract. By contrast, the female reproductive tract develops in the absence of fetal gonadal hormones. Testis development is brought about through the action of the sex-determining region located on the short arm of the Y chromosome (Siy gene), but correct doses of other genes on autosomes as well as the X chromosome, are also required. Sry appears to be widely expressed in human fetuses, suggesting the possibility that its influence on development is not confined to the testes. There is additional evidence of a difference in developmental rates between XY and XX cleaving embryos, in which Sry and another gene in the sex-determining region namedZfy, for the zinc finger protein it encodes, are already expressed. These findings are consistent with the possibility that Y-chromosomal genes affect somatic sex differences prior to the formation of steroid hormones. (Biomed Rev 1997; 7: 75-83)

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INTRODUCTION

• How does it come about that babies are born either male or female? The question has been debated since civilization began and has given rise to literally hundreds of hypotheses. Two and a half millennia before Broca (1) developed his idea of the lateralized brain, Greek philosophers put forward the view that the sex of the embryo was determined by the side of the body from which it arose. The rule: males on the right, females on the left, could be applied both to the position in the mother's womb as well as to the origin of the semen in the father's testes (2, 3). Although Aristotle objected to it, the theory persisted until recent times, but by the 19th century, it had lost its dominant position and was replaced by the view that sex is determined by environmental factors, particularly the mother's nutrition. A poor nutrition was thought to give rise to lean males and ample nutrition to plump females.

With the advent of the 20th century came a radical change in viewpoint. Environmental factors lost some of their preeminence as causes of health and disease, while genetic factors came to the fore. The year 1900 saw the rediscovery of Mendel' s paper (4, 5), and in the years that follows sex chromosomes were discovered in insects (6). In the order Hemiptera, two types of sex chromosome mechanism, giving rise to two types of dimorphic sperm, were found. In some species, both classes of sperm had the same number of chromosomes, but one pair differed in size, while in other species, the number of chromosomes in the two classes of sperm differed by one. When two

chromosomes were present, the larger chromosome was called "X chromosome", and the smaller one, "Y chromosome", and both were called "sex chromosomes". Sperm bearing X chromosome were found to give rise to female insects, while Y-hcaring sperm, and those without a sex chromosome, gave rise to males.

Attempts to find sex-determining genes on the sex chromosomes were destined to be inconclusive. In tnc fruitfly,Z)rojo-/)A;Vo mg&iMogojfer, males were likewise found to have one X und one Y chromosome, while females were XX. Exceptional XXY flies were fertile females, whereas "XO" flies, i.e. having one X and no Y chromosome, turned out to be male, albeit sterile. These findings excluded the possibility that the Y chromosome in Z)mjop/;;7o carried genes for malcness and gave rise to the idea that the sex of the flies is determined by the number of X chromosomes, two X chromosomes giving rise to a female, while a single X chromosome per cell results in a male. This simple scheme had to be modified when it was found that Iriploid flies with three sets of autosomcs and two X chromosomes were not females but intcrsexes with a mixture of female and male characteristics, and it was concluded that the sex of a riy is determined by the ratio of the number of X chromosomes to that of autosomes. Bridges (7) proposes that the X chromosome of Drojop/zzVa carries genes for femaleness while the autosomes carry genes for male characteristics.

In recent years, molecular studies have led to the isolation of lour "numerator genes", .?»fe;Ye.M-o,6,c and nmf, but only to one autosomal "denominator gene", ^ea(/po«. These genes interact both with the X: autosome ratio and the^ex-/gfAaZ f&t/) gene in such a way that &?/ is switched on in flies destined to become females. The fact that only one denominator gene has been identified has lee! to the suggestion that the male-determining function of the autosomcs may be mediated by factors such as cell size rather than genes (8).

In contrast to the situation in Dfmop/z;'/o, the Y chromosome ol' the flowering plant &7^/;e f^m/co and some other species was Ibund to be male-determining (9).

THE HUMAN Y CHROMOSOME: KLINEFELTER'S SYNDROME AND TURNER'S SYNDROME

H Large-scale studies on human chromosomes began in the second half of this century, following the development of two techniques. One of these was the ability to grow cells in culture, so that enough material could be obtained from small biopsies or specimens obtained at operation. The other important progress was pre-treating the material with hypotonic saline solutions, which causes the chromosomes to be dispersed within the cell, instead of being crowded together with many overlaps. As a result of these techniques it became established that the human diploid chromosome number is 46, and that the sex chromosomes of males are XY and those of females XX. But some human individuals have different sex chromosome constitutions.

Men with Klinefelter's syndrome have small testes, which in the adult lack germ cells, as well as reduced testosterone levels and an increased incidence of mild mental retardation (10, 11). The chromosome constitution of the majority of patients is 47,XXY, i.e. they have three instead of two sex chromosomes. Some of them are 48.XXXY or 49.XXXXY, but those in the last group are hypogonadic and suffer from severe mental retardation and multiple malformations as well.

Patients with Turner's syndrome are females with infantile genitalia and so-called "streak gonads", i.e. the gonads are replaced by streaks of fibrous tissue. They also have certain somatic abnormalities, as well as short stature. The most common chromosome constitution is 45,X; because of the absence of the second sex chromosome, it is sometimes referred to as XO. Most fetuses with this chromosome constitution are spontaneously aborted, and a large proportion of the survivors carry a second cell line, for instance XX or XY (11).

The fact that patients with Klinefelter's syndrome arc male, in spite of having two X chromosomes, whereas patients with Turner's syndrome are female, even though they have only one X chromosome, indicates that sex determination in humans does not depend on the number of X chromosomes, but on the presence or absence of Y chromosome. If present, Y chromosome presides over male sex differentiation, whereas in the absence of Y chromosome, female sex develops. Thus the hu- ! man system of sex determination, in common with that of other mammals, resembles that of flowering plants rather than that of insects. We shall return to the human Y chromosome after discussing the topics of sex reversal and the developmental origin of the sexual apparatus.

SEX REVERSAL

* In addition to Klinefelter's and Turner's syndromes, there are other abnormalities of sexual development, which have played an important role in the elucidation of the genetic basis of sexual development. In present-day medical genetics, the term "sex reversal" is applied to conditions in which individuals with XY sex chromosome develop as females, or those with XX sex chromosomes develop as males.

Patients with complete XY sex reversal, a condition referred to as XY gonadal dysgenesis or "pure gonadal dysgcnesis", resemble those with Turner's syndrome in having female genitalia and streak gonads, but they differ from Turner patients

in not having any somatic abnormalities, and their height is not impaired (12). There is, however, an increased susceptibility to gonadal tumours. XY females are known also in other species, in some of which fertile XY females occur as part of natural populations (13, 14).

Incomplete sex reversal can result in the condition known as true hermaphroditism, in which testicular and ovarian tissue coexists in the same individual. Patients may have an ovary on one side and a lestis on the other, or they may have one or two ovolestes (15, 16). The genitalia are typically ambiguous, i.e. intermediate between those of normal males and females; and the sex of rearing may be either male or female. It may be noted in passing that in patients with hermaphroditism, most ovaries arc situated on the left, while testes and ovotestes are preferentially present on the right side. This fact is more in line with the thinking of the philosophers of ancient Greece (2) than with that of present-day molecular biologists!

True hermaphroditism can be associated with other sex chromosome constitutions. Indeed in human hermaphroditism, 46,XY is relatively rare, the majority of patients being either 46,XX, or they are mosaics or chimeras with more than one cell line, such as 46,XX/46,XY. Mosaics originate from a single /.ygolc, whereas chimeras have a more complex origin, such as fertilization of the egg nucleus and a polar body by two spermatozoa, or by fusion of two fertilized eggs (17).

While molecular investigations have shown that most non-mosaic 46,XX human hermaphrodites lack Y chromosomal sequences, a different situation is presented by 46,XX males. Phcnotypically, these patients resemble those with Klinefelter's syndrome, i.e. males with testes that lack spermatogenesis, but in XX males there is no increased risk of mental retardation, and they are less tall than men with Klinefelter's syndrome. In the majority of XX males, one of the X chromosomes carries DNA sequences of varying lengths, derived from the short arm of the Y chromosome. XX males without Y-derived DNA sequences ometimes occur in the same families as patients u ith 'rue hermaphroditism, suggesting that they represent differi, piviii ilypic manifestations of the same spectrum.

Belori i vjui^ing the origin of XX males, it will be appropria! ' o lake a look at the early events in development during whk-[1]. i he embryo or fetus assumes the phenotype of one or an-

DEVELOPMENTAL ORIGINS OF THE SEXUAL APPARATUS

• In humans and oilier mammals, the principal anatomical features thai distinguish males and females - testes, vasa dcfcrcntia, seminal vesicles, prostate, and male external genitalia, as opposed to ovaries, Fallopian tubes (oviducts), uterus, vagina and female external genitalia - derive from common structures in the embryo. The somatic cells of testes and ovaries originate from genital ridges, which form in apparently identical fashion on the mesonephros of XX and XY embryos, while the primordial germ cells migrate into these ridges from an extra-embryonic site (18, 19). During this stage, two duct systems develop: the Wolffian, or mesonephric, duct, capable of developing into the male reproductive tract and associated structures (vas deferens, seminal vesicles and epididymis), and the Miillerian, or paramesonephric, duct which has the potential of becoming the female reproductive tract, i.e. oviduct, uterus and upper part of the vagina (Fig. 1). Which of these alternatives comes into existence depends on whether the genital ridges develop into testes or ovaries.

The first histological sign of sexual differentiation of the gonad is the appearance of Sertoli cells in developing testes (20). Fetal testes secrete two active substances, anti-Mullerian hormone, also referred to as Mullerian-inhibiting substance, which demolishes the Miillerian duct as well as testosterone, which stabilises the Wolffian duct. As a result of the action of both substances, the embryo assumes a male phenotype. Fetal ovaries are not actually required for the establishment of a female reproductive tract, since the Mullerian duct will develop and the Wolffian will duct regress in the absence of any hormones (21).

In accordance with the need for its early function, the fetal testis differentiates earlier than the ovary. It has long been known that the first sign of a gonad becoming an ovary is that it has failed to show signs of testicular differentiation at a time when a developing testis would have shown histological evidence of its future development (18). "At a definite stage, usually referred to as the stage of gonadal sex differentiation (day 15 in rabbits, stage 15-17mm in human fetuses) testicular differentiation rapidly becomes discernable in the male fetuses (early seminiferous tubules), whereas nothing happens in the female" (22). Evidently, the process of gonadal differentiation is an asymmetrical one. During development, the underlying decision is not: "testis *versus* ovary", but "testicular differentiation" versus "no differentiation". By the time the ovary differentiates, the time for testis differentiation is past.

The pivotal role of the fetal testis in controlling sexual development implies that the crucial decision whether the embryo develops into a male or female depends on whether or not the genital ridge develops into a testis; and given the cytogenetic evidence relating the presence of Y chromosome with male (and absence of a Y chromosome with female) development, we must conclude that the Y chromosome is responsible for the differentiation of the genital ridge into a testis. But what is the mechanism that allows the Y chromosome to fulfil this task?

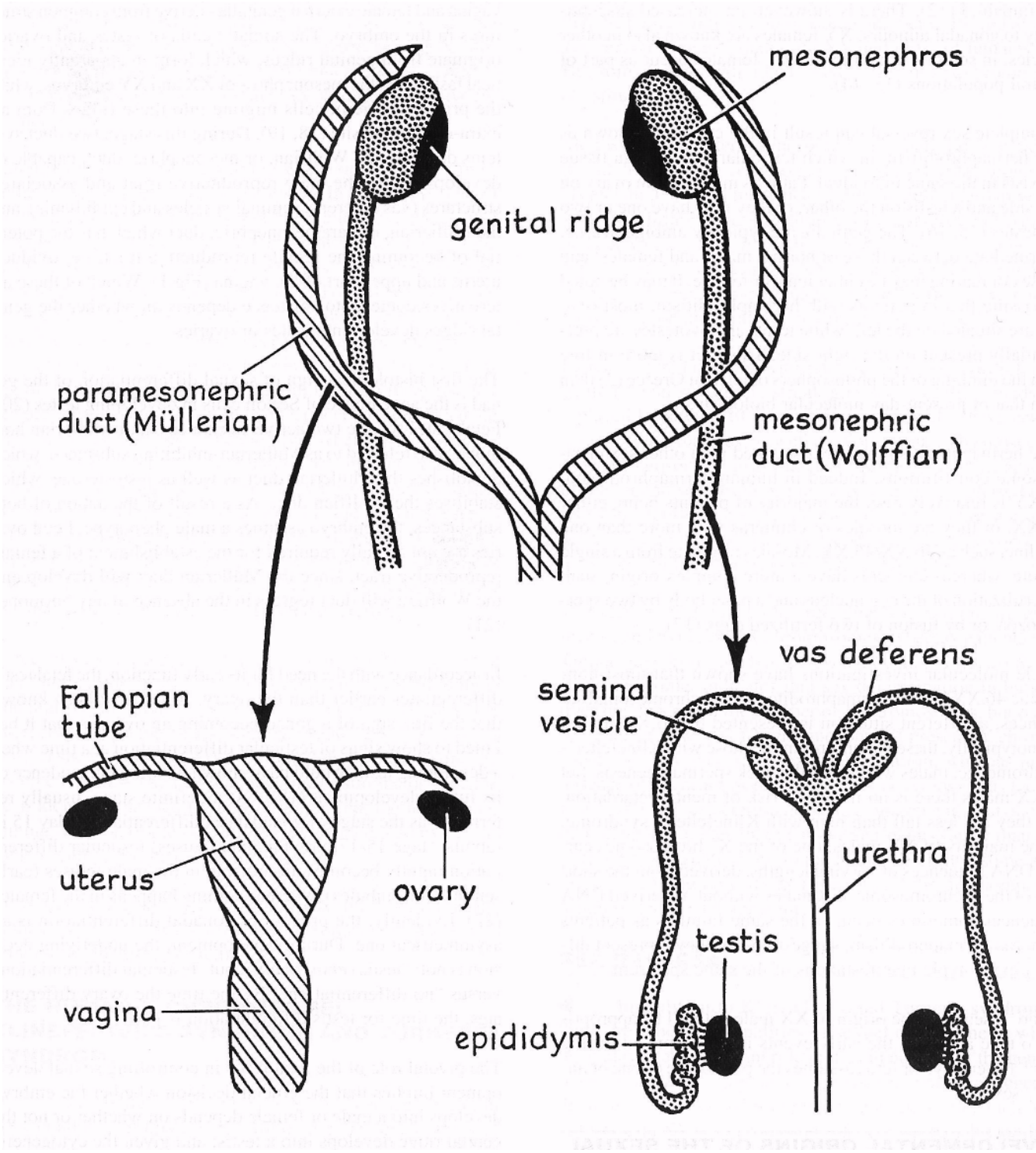

Figure 1. *Differentiation of a male and female phenotype from a bipotential genital ridge and a double set of ducts. If the genital ridge differentiates into a testis, its hormonal secretion will demolish the Milllerian duct and induce the Wolffian duct to develop into vas deferens, seminal vesicle and epididymis. If testis development does not occur and an ovary is formed, in the absence of fetal gonadal secretion, the Wolffian duct regresses and the Miillerian duct differentiates into Fallopian tubes, uterus and upper part of the vagina.*

THE TESTIS-DETERMINING GENE, *Sty*

• The Y chromosome differs from all other chromosomes in not having a pairing partner in meiosis. However, at the tip of its short arm, referred lo as Yp, there is a short region that is homologous to a similar region on the X chromosome (Fig. 2). This is known as the "pseudoautosomal region" (23). In this region pairing occurs during male meiosis, and crossing over takes place (24).

Evidence obtained from patients with deleted Y chromosomes indicates that male development requires the presence of the short arm, hut not of the long arm, of the Y chromosome (25). This in turn suggests that there are one or more genes on the short arm of the Y chromosome whose function converts the hipotential gonad into a testis. Molecular geneticists envisage the existence of a single gene named testis-determining factor *(Tdf);* in mice, this gene is known as *Tdy. A* concentrated search to isolate this gene culminates in the identification *of Sry* (26).

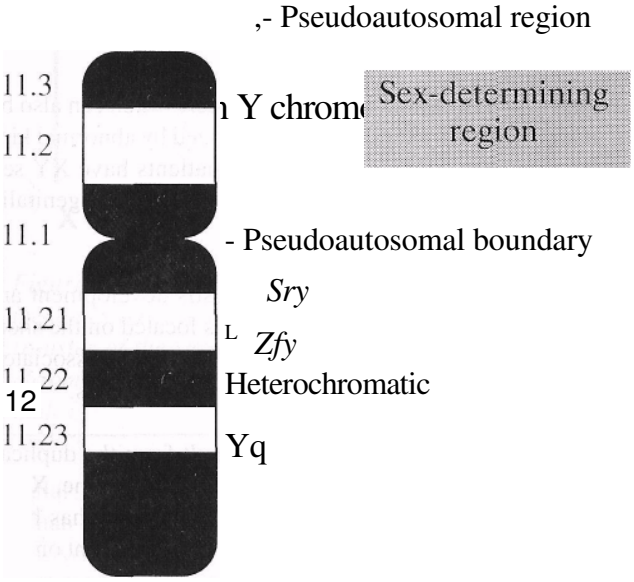

Figure 2. Organization of the human Y chromosome. The terminal portion of the short arm contains the pseudoautosomal region, which pairs with a similar region on the X chromosome during meiosis. The short arm contains the sex-determining region, including Srv. The long arm contains Azf, comprising genes required for spermatogenesis.

A previously isolated candidate gene for Tdf, named *Zfy,* for the zinc finger protein, which it encodes (27), subsequently falls out of favor. TheZ/y gene is located on Yp at a distance of 145 Kb from the pseudoautosomal boundary, whereas the distance between *Sry* and the pseudoautosomal boundary is only 36 Kb. This gene is generally accepted as being identical to *Tdf* (28, 29). Chief among the reasons for this view is the finding that the mouse homologue, *Sry,* when inserted into genetically female blastocysts, induces some, though not all, of them to develop as males (30). The transgenic males are .sterile for two reasons. One is that genes on the long arm of the Y chromosome are required for spermatogenesis; the other is that the presence of a second X chromosome is incompatible with spermatogenesis, as seen in Klinefelter's syndrome in humans.

The important role played by *Sry* in human male development is emphasized by the fact that mutations in the gene can cause sex reversal and the development of XY females. About 15% of XY patients with complete gonadal dysgenesis have mutations in *Sry.*

Given the asymmetrical nature of the process of sex differentiation, the sequence of events can be simply represented by the model in Fig.3. In the presence of *Sry,* the genital ridge initiates testicular differentiation, while in the absence of the gene the genital ridge remains undifferentiated until a later stage, when the beginning of meiosis in the germ cells heralds the differentiation of the ovary.

The *Sry* gene is a regulatory gene encoding a protein with a high mobility group (HMG) box protein-binding domain. It is postulated that the gene product acts on the somatic cell of the genital ridge causing the differentiation of Sertoli cells. These, in turn, initiate the formation of the early seminiferous tubules, and, therefore, the differentiation of the testis. In the mouse, transcripts of *Sry* have, indeed, been observed specifically in the somatic cells of the genital ridge, as well as in germ cells of the adult testis. On the other hand, the human gene has been found to be expressed in all fetal tissues examined, i.e. adrenal, brain, liver, pancreas, small intestine, spleen, thymus and heart, whereas in the adult, *Sry* expression was found in heart, liver, kidney and testis, but not in lung (31). Furthermore, transcription of the gene has been described in very early human and murine embryos, long before the formation of the genital ridge. The expression of *Sry* in organs outside the genital ridge suggests the possibility that it has functions other than in testis determination, but this has not yet been established.

The identification of *Sry* has helped in elucidating the origin of most cases of XX males (32). About 80% of patients carry Y-derived DNA sequences, including *Sry,* on one of their X chromosomes as a result of accidental crossing over outside the pseudoautosomal region (Fig.4). Whereas crossing over be-

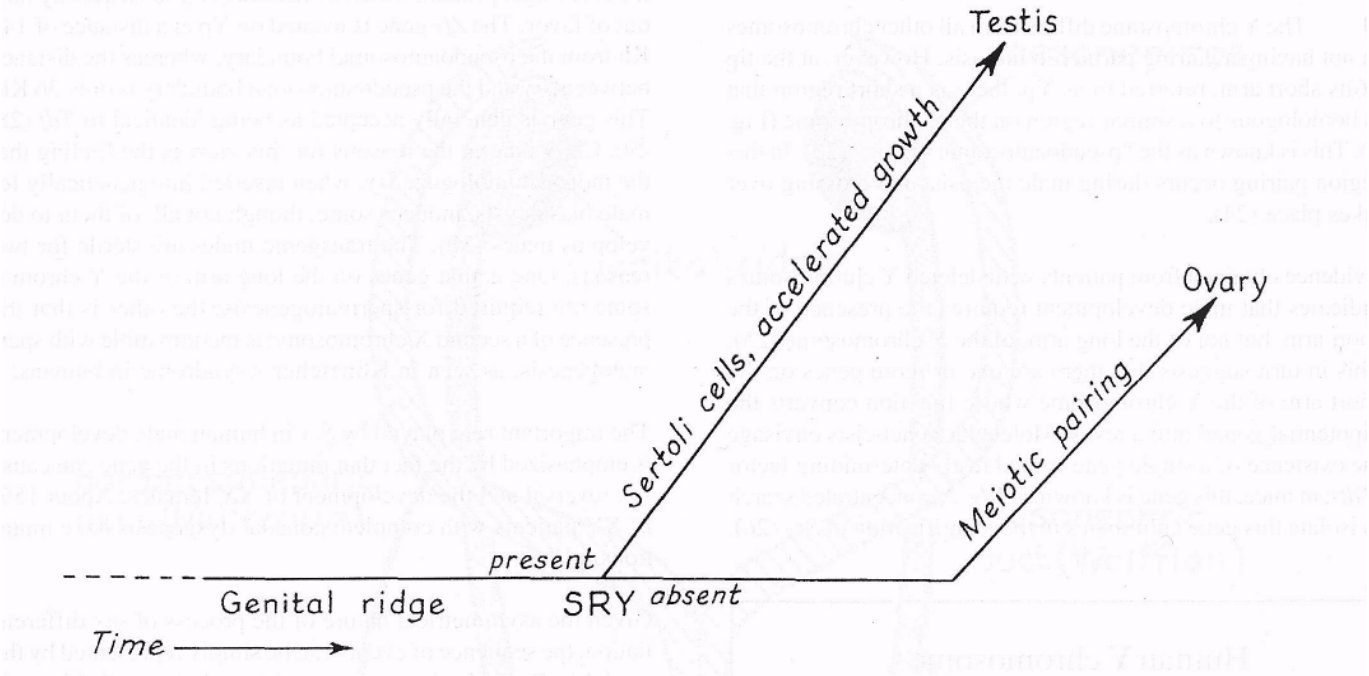

Figure 3. Asymmetrical model of gonadal sex determination. Under the influence ofSry the genital ridge initiates testis differentiation. In the absence ofSry, the gonad remains undifferentiated, until it later initiates ovarian differentiation.

twecn the X and Y chromosomes is normally confined to the pseudoautosomal region, an exceptional cross over event may lake place beyond the pseudoautosomal boundary, thus transferring varying lengths of DNA from the short arm of the Y to the short arm of the X chromosome.

OTHER GENES INVOLVED IN SEX DETERMINATION

• In addition to *Siy,* there are several autosomal and X-chromosomal genes whose normal functioning is required for testis differentiation. The human malformation syndrome known as "campomelic dysplasia" is a serious disorder of bone development, which causes the death of most affected infants in the neonatal period. It has long been known that the majority of patients are female, even if their sex-chromosome constitution is XY. The gene causing the condition was found to be localized on chromosome 17q24.3-q25 and named *Sral,* referring to its effect on sex reversal. It has since been cloned and Ibund to be a member of the *Sox* series of Sry-related genes, *So.\9* (29, 32, 33). The *Sox* genes, like *Sry,* encode a protein with an HMG domain. It appears that normal male sex differentiation requires a double dose of the *Sox9* product.

Another autosomal gene with a role in testis differentiation is the Wilms tumor gene, *Wtl.* Mutations of this gene give rise to Wilms tumor, a childhood kidney cancer, which can also be part of Denys-Drash syndrome characterized by abnormal kidney development. Some of the female patients have XY sex chromosomes and gonadal dysgenesis; the external genitalia can be ambiguous (29, 32).

Other autosomal regions involved in testis development are likely to come to the fore. One of these is located on the short arm of chromosome 9, since deletions of 9p can be associated with XY sex reversal or genital ambiguities in males.

Male-to-female sex reversal can also result from the duplication of a region on the short arm of the X chromosome, Xp21 (33). The putative gene thought to be responsible has been named "dosage sensitive sex reversal" *(Dss).* If present on two different sex chromosomes, the gene does not cause sex reversal, presumably because one will be inactivated. A gene, named *Daxl,* which is responsible for the X-linked form of adrenal hypoplasia, is located in this region, but its relationship to sex reversal is not yet certain. The *Dss* duplication is associated with mental retardation and multiple malformations in addition to sex reversal.

The Sry-related gene, *Sox3,* is located on the long arm of the X chromosome. A haemophilic male patient in whom this gene has been deleted has mental retardation and partial primary tes--

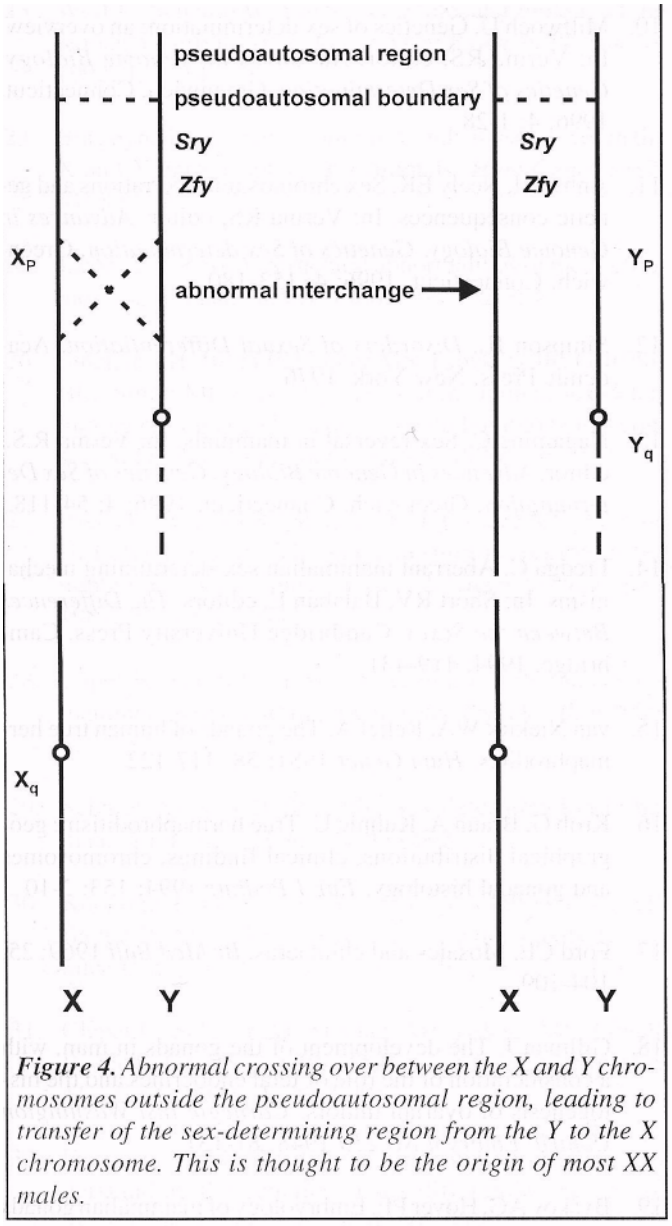

Figure 4. Abnormal crossing over between the X and Y chromosomes outside the pseudoautosomal region, leading to transfer of the sex-determining region from the Y to the X chromosome. This is thought to be the origin of most XX males.

ticular failure. The gene is found to be widely expressed in human and mouse fetuses in the genital ridge and central nervous system (34).

SPERMATOGENESIS GENES

• More than 20 years ago, the results of a chromosome study on men with azoospermia have suggested that the long arm of the Y chromosomes carries factors necessary for spermatogenesis, and the existence of the azoospermia factor *(Azf)* has been postulated (35). The correctness of this hypothesis has since been confirmed by molecular studies, which have shown that azoospermia or severe oligozoospermia may also be associated with interstitial microdeletions, which are not visible in the microscope. The critical region has been localized to Yql 1.23 and is likely to contain more than one gene, including two candidate genes, deleted in azoospermia *(Daz)* (36), and RNA-binding motif 1 *(Rbml)* (37), which was previously known as *Yrrm* (38). The latter gene is present on the Y chromosome of all eutherian mammals tested as well as in marsupials.

In the mouse, a candidate spermatogenesis gene, *Ubely,* that encodes the ubiquitin-activating enzyme Ubel, has been identified on the short arm of the Y chromosome, while a homologue, *Ubelx,* is present on the X chromosome (39). Whereas *Ubelx* is ubiquitously expressed, the Y-encoded transcripts are found predominantly in germ cells, and the authors suggest that the *Ubely* gene serves to increase Ubel production in times of high demand.

SEXUAL DIFFERENTIATION BEFORE GONAD FORMATION

• Although it has been widely assumed that genetically male and female embryos develop in identical fashion until a switch acts on the hitherto bipotential gonad, causing it to develop into either a testis or an ovary, it has become apparent in recent years that the sex chromosomes exert their effect long before the genital ridge is formed. Data on accelerated development of male preimplantation embryos in mice and cattle have been soon followed by findings of sex differences in blastocysts(10, 40).

In human embryos produced by *in vitro* fertilization, differences in developmental rates between genetic males and females have been found virtually from the beginning of zygote formation (41, 42). Males have a higher mean cell number than females on the second day after insemination, and it has been reported that embryos transferred with four or more cells give rise to more males than do embryos with fewer cells. Male embryos between days 2-5 have a higher pyruvate uptake, and on days 4 and 5, a higher glucose uptake and lactate production, indicating that even at this early stage, the metabolic rate of male embryos is higher than that in female embryos. Another unexpected finding is that the Y-chromosomal genes of the sex-determining region, *Sry* and *Zfy,* are already transcribed in the 2-cell stage of mice (43) and at the zygote stage in human embryos (44).

CONCLUSION

• It is evident even from a brief survey that the last decade has witnessed striking advances in the elucidation of the genetics of sex determination, including the identification of the long-sought-after "testis-determining" gene, .Sry. However,

the way in which *Siy* functions in the process of sexual differentiation is still unknown. It has even been suggested that an understanding of the process may be more easily achieved by cloning autosomal sex-determining genes than by identifying genes that possess Sry-binding sequences (45).

At the same time it has become apparent that, on the phenotypic level, the function of Srv is likely to extend beyond the confines of the genital ridge, since the gene has been shown to he expressed in early human embryos as well as in a variety of human fetal organs, including the brain. It is equally remarkable that there appears to be a difference in metabolic rates between genetically male and female embryos from the earliest time of their existence, with males having the higher metabolic rate.

Though the significance of these findings is not fully understood, they indicate the likelihood that the process of somatic sexual differentiation begins before that of the gonads and the production of sex-specific steroid hormones. This in turn suggests the possibility that the sexual differentiation of the brain may originate through the activity of the genotype and is later modified by hormonal action.

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