Aul 6. Determinação do sexo. Mamíferos

(texto parcial extraido de Umesh & Brototi)

Sex Determination and Differentiation in Vertebrates

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The concept of sex determination and differentiation has been baffling mankind since time immemorial. While Aristotle hypothesized in 335 B.C. that sex was determined by the heat generated during conception, Andreas Vesalius in 1543 A.D illustrated that women were but males, their genitalia turned inside out. In numerous cultures since then, women have been considered as the "default state of men". However, in the 17 th century gradually it was discovered that the females produced eggs that transmitted parental traits. Later, in the twentieth century Geddes and Thomson came forward with the hypothesis that constitution, age, nutrition and environment of parents decide the sex determination of the offspring. This environmental view of sex determination was again challenged by the discovery of sex chromosomes by McClung in 1902. Following this, more evidences gradually clinched to suggest the chromosomal concept of sex determination. Numerous experimental studies in vertebrates have now established beyond doubt that sex is determined either by chromosomal factors, environmental influences, or interplay of both, depending on the species/groups.

Chromosomal Sex Determination (CSD):

Before the karyotyping of human chromosomes, it was considered that sex is determined by the number of X chromosomes present in an individual. It was observed that in drosophila, males had single X chromosome (XY or XO) and the presence of 2 or more X chromosomes (XX, XXX, or XXY) always conferred female phenotype. It was thus considered that the Y chromosome was a null chromosome. However, in 1966, a landmark observation was made by Jacobs and Ross. They described two sisters who had female external genitalia but 46 XY karyotype in which the Y chromosome consisted only of its long arm. It seemed therefore, that the testicular determining region of the Y chromosome normally resides in its short arm. This was confirmed by high resolution banding studies of an XX male which revealed that some material from the short arm of Y chromosome had been translocated to one of his X chromosomes. With advanced techniques, it became apparent that sex determining gene on short arm of Y chromosome is responsible for the development of testis in mammals. Also, testicular development is seen impaired in several clinical syndromes resulting from autosomal deletions or mutations. Now autosomal genes such as *SOX* 9, *WNT* 4, *SF*1, *DMRT* 1 etc are shown to play a crucial role in the downstream events of sex differentiation initiated by *SRY.*

SRY (Sex determining region on Y chromosome): *SRY* gene is located near the tip of the short arm of Y chromosome that encodes a transcription factor of 204 amino acids. The central 79 amino acids encode the HMG (high mobility group) box. In the entire SRY protein, only HMG domain shows sequence conservation. HMG box functions as DNAbinding, and DNA-bending domain and also has two nuclear localization signals essential for translocation of protein into the nucleus. However, the non conserved regions outside the HMG box are also essential for SRY function, since a truncated SRY protein lacking the carboxy end is unable to induce male development in XX transgenic mice. As an architectural transcription factor, SRY unwinds the DNA and bends it to almost 80 degree thereby, bringing the other distantly bound transcription factors in close contact. The exact binding site of SRY on the DNA and the mechanism function of SRY is the up regulation of SOX 9. It is interesting to note that *SRY* is absent in monotremes (egg laying mammals: platypus and echidna) and in non-mammalian vertebrates.

SOX 9 (SRY-related high-mobility group box 9): *SOX* 9 present on chromosome 17 in human is a highly

conserved autosomal gene responsible for testicular differentiation. Like *SRY*, *SOX* 9 encodes a transcription factor that also contains a HMG box and a transactivation domain in the C-terminus. *SOX 9* was discovered in an investigation of campomelic dysplasia (CD), a disease involving bone and cartilage disorder. XY patients with this disease developed as phenotypic females. Mutational analysis revealed that absence of *SOX 9* is responsible for CD as well as XY sex reversal.

In mouse, at 10.5 days post conception (dpc), just before or around the same time as *Sry* transcripts are first detected, *Sox* 9 is expressed at low levels in the developing gonads of both sexes. By 11.5 dpc, *Sox* 9 is robustly expressed in the XY gonads and is completely absent from XX gonads. Although its expression is up regulated by *Sry* expression, *Sox* 9 remains active in embryonic testis long after *Sry* expression has ceased. SOX 9 along with other transcription factors activate the expression of *Amh* gene (anti Müllerian hormone gene). The binding of SOX 9 HMG box bends the DNA which bring SF1 and GATA 4 in close proximity to each other and along with WT1 and HSP 70 form a tightly associated protein complex that activates transcription of the *Amh* gene. The complete absence of AMH transcripts is seen in XY mice mutant for HMG box (DNA binding domain) of *Sox 9* gene suggesting that *Sox 9* is required for AMH expression.

Although experimental evidences in non mammalian vertebrates show that *Sox 9* has a conserved role in sex determination, it is important to mention here that its expression in alligator and chicken begins well after pre Sertoli cell differentiation and AMH expression. It seems that *Sox 9*, in non mammalian vertebrates may be involved in Sertoli cell organization, rather than early testicular determination (Pask & Graves, 1999).

SF1 (Steroidogenic factor 1): *SF1* gene transcribes a protein, otherwise known as Ad4BP, belonging to the orphan nuclear receptor family. Initially, SF1 was described to regulate the production of cytochrome P-450 steroid hydroxylase enzymes that are necessary for synthesis of steroids, and thus, are expressed in many steroidogenic tissues, including adrenal gland, ovary and Leydig cells of the testis. Subsequently, SF1 transcripts were detected in the mouse urogenital ridge even at the stage of the indifferent gonad (9-12 dpc) and mutation in *Sf1* gene was shown to cause complete dysgenesis of gonad in both sexes. This suggests its role in early formation of the indifferent gonad and, thereby, *Sf1* is placed upstream of *Sry* in sex determination pathway. However, *Sf1* also plays an important role in the downstream testicular differentiation pathway initiated by Sry. *Sf1* activates testicular differentiation by influencing both, Leydig and Sertoli cells. SF1 in Leydig cells regulates steroid biosynthesis and in Sertoli cells it binds to Amh promoter region and activates the expression of AMH in collaboration with other transcriptional factors. The importance of *SF1*, located on chromosome 9, for testis development and *AMH* regulation in humans is demonstrated by XY patient heterozygous for *SF1* where the individual has malformed fibrous gonads and retains fully developed Müllerian duct structures (Achermann *et al.*, 1999).

WT1 (Wilms' tumor 1)**:** The *WT1* gene first came into focus in patients with Wilms's tumor where mutation of this gene led to embryonic kidney tumor. Later, mutation in this gene was seen associated with the disruption of bipotential gonadal development. The presence of WT1 transcripts in developing gonads substantiates its role in early bipotential gonadogenesis. *WT1* encodes variant transcripts by alternative splicing, alternative translation start sites, and RNA editing..

GATA4/FOG2: GATA4, a member of the GATA family of transcription factors, contain a zinc finger DNA-binding domain that binds to the consensus sequence WGATAR in the 5'-flanking region of target genes. In mice, it is detected as early as embryonic day 11.5 in the somatic cells of primitive gonads of both sexes and therefore, GATA4 in conjunction with other transcription factors play role in development of bipotential gonad (Viger *et al.,* 1998).

DMRT 1 (double sex-and mab-3 related transcription factor): *DMRT 1* belongs to the family of genes that encode proteins containing DM-domain, a novel DNA-binding motif. It is one of the most conserved genes in sex determination, since its presence is observed across the phyla from invertebrate to vertebrate. Although its expression coincides with *Sry/Sox 9*, maximum expression of *DMRT 1* is seen in Sertoli cells during postnatal testis development.

DAX 1 (DSS-AHC critical region on X chromosome, 1): *DAX 1* maps on short arm of X chromosome and encodes for a protein that belongs to the orphan nuclear receptor family. Duplication of this gene causes male-female sex reversal **(**dosage-sensitive sex reversal, DSS), whereas its deletion results in adrenal hypoplasia congenita (AHC). *DAX 1* is initially expressed in genital ridges of both sexes. Its expression persists in case of developing ovary while it is drastically down regulated with testis differentiation. *DAX 1* suppresses testis differentiation at two levels: one, by inhibiting SF1-induced *SRY* expression in a bipotential gonad, and two, by repressing synergistic action of SF1 and WT1 and thereby, suppressing downstream genes, *e.g. Am*h and other steroidogenic genes. Thus, it is considered as ovary– determining gene.

WNT 4**:** *WNT 4* is another important ovary determining gene. Like *DAX 1*, its expression is turned off with the differentiation of testis. *WNT 4* knock out XX mice shows masculinization as ovarian differentiation ceases and its cells express testis-specific markers, including *Amh* and testosterone producing enzymes (Vainio *et al.*, 1999). This suggests that *WNT 4* is obligatory gene for female sex differentiation.

In marsupials too, the control of testis determination is vested in the Y chromosome, though it is the smallest of any mammals (Graves and Shetty, 2001). However, the number of X chromosome plays a critical role in other aspects of sex differentiation (Table.1). Single X chromosome in XO animals leads to the development of empty scrotum (without testis), whereas scrotum fails to develop in XXY animals having testis. In fact, the presence of two X chromosomes leads to the development of pouch and mammary glands in lieu of the scrotum (Manolakou et al., 2006). Thus, marsupials are different from other mammals with regard to the accessory sex organ differentiation since the formation of scrotum, pouch and mammary glands in marsupials are dependent on genes present on X chromosomes rather than on gonadal hormones as in eutherian (Pask and Benfree, 2001).

Table 1: X-linked secondary sexual differentiation in marsupials

	Y chromosome (testis)	**Absence of Y chromosome (no testis)**
Single X chromosome (scrotum)	**XY** (males with scrotum and testis)	**XO** (males with empty scrotum)
Double X chromosome (pouch and mammary glands)	**XXY** (males with testis, pouch and mammary gland)	**XX** (females with pouch and mammary glands)

However, the monotremes are unusual because they have multiple set of sex chromosomes rather than the single pair usually found in marsupials and eutherians. Recent report demonstrates the presence of 10 sex chromosomes in platypus arranged as X1Y1X2Y2X3Y3X4Y4X5Y5 (Rens *et al.*, 2004). Interestingly, genes present on X chromosome situated at one end of the 'sex chromosome chain' are orthologous with those on the human-X chromosome, while genes on chromosome situated at the other end of the chain are homologous with those on the bird-Z chromosome. Hence, the platypus provides an important link between the chromosomal sex determination in mammals and birds.

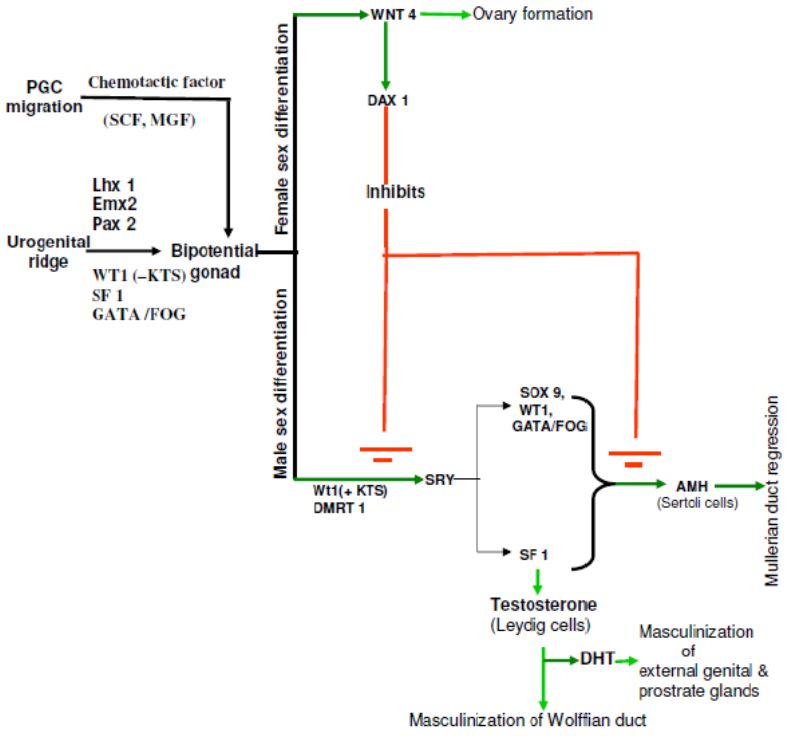

Fig. 1. Schematic representation of chromosomal sex determination and differentiation in mammalian model

Sex Differentiation:

The sexually indifferent bipotential gonad develops from the ventromedial surface of the mesonephros near the kidneys at around 4 weeks in the human fetus and 9.5 days post-coitum (dpc) in the mouse. The somatic cells of gonad are derived from the mesonephros and coelomic epithelium that covers the coelomic surface of the gonadal ridge. These cells proliferate in the gonadal primordia and form the sex cords. Several factors such as Lhx1 (LIM Homeobox gene1), Emx2 (homolog of empty spiracles homeobox gene 2), Pax 2 (paired box gene 2), WT1and SF1 are involved in cell proliferation and development of the bipotential gonad.

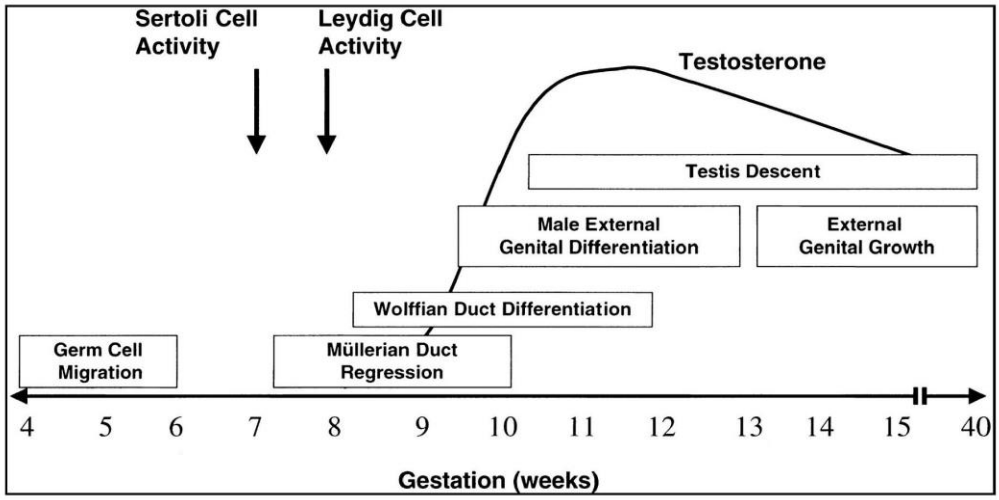

Fig 3. Embryologic events in male sex differentiation depicted in temporal fashion. The line depicts the increase in fetal serum testosterone concentrations. The word activity refers indirectly to the action of AMH in causing Müllerian duct regression and androgens to induce male sex differentiation (Hughes,

2001)

The gonad is subsequently colonized by primordial germ cells (PGC) that originate from epiblast-derived cells present in the yolk sac near the base of allantois and migrate through the hindgut to invade the indifferent gonad. Germ cell migration is under the influence of stem cell factor (SCF), mast cell growth factor (MGF), and extra cellular matrix proteins like fibronectin and laminin (Bendel-Stenzel *et al.,* 1998). During migration the germ cells proliferate but do not differentiate. The primordial germ cells are distinguishable from the other cell type because of their large size and large round nuclei. Histologically, they are identified by high alkaline phosphatase activity and glycogen. PGCs along with the somatic cells form the "gonadal ridge". The formation of gonadal ridge is completed within 5 to 6 week of gestation in human embryos. No sexual difference can be observed in the gonads until the 6week of embryonic life in humans and 11.5 dpc in mice.

Male Sex Differentiation

In XY fetus of 6-7 weeks, the first sign of testis differentiation is seen with the aggregation of the pre Sertoli cell (derived from mesonephros) around the germ cell now called gonocytes, to form the testicular cords. These cords lose contact with the surface epithelium and become separated from it by a thick extra cellular matrix, the tunica albuginea**.** By the end of 9 week, the mesenchyme that separates the seminiferous cords gives rise to interstitial cells. Later these are differentiated into steroid secreting Leydig cells. Although differentiation of Leydig cells in the initial phase is independent of gonadotropin action, its proliferation and differentiation in the first and second trimesters of the fetal life depends on placental hCG, and thereafter, controlled by fetal pituitary LH (Josso *et al.,* 2005).

The masculinization of the genital tract starts with the regression of Müllerian duct under the influence of anti Müllerian hormone (AMH) secreted from Sertoli cells**.** Shortly after the Müllerian duct regression, the portion of Wollfian duct adjacent to testis is differentiated into epididymis, the central portion becomes vas deferens, and the distal end of the duct near the urogenital sinus develops into seminal vesicle. The prostrate gland develops as a series of outgrowths from the urogenital sinus (Fig. 4). The virilization of the Wollfian duct is under the control of testosterone as the phenomenon is seen inhibited after the administration of anti-androgen or testosterone antibody at the critical period of sex differentiation (Elger *et al*., 1970).

Under the influence of androgen, the male external genitalia start differentiating around the 9week of gestation in case of human. The genital tubercle elongates to form the phallus and scrotum, and the urethral folds fuse over the urethral groove. Although testosterone plays primary role in differentiation of Wolffian duct into epididymis, vas deferens and seminal vesicle, there is evidence that it is not the active masculinizing hormone in certain tissues. It is dihydrotestosterone (DHT) that masculinizes urogenital sinus and genital tubercle into prostrate and penis,respectively (Fig. 4).

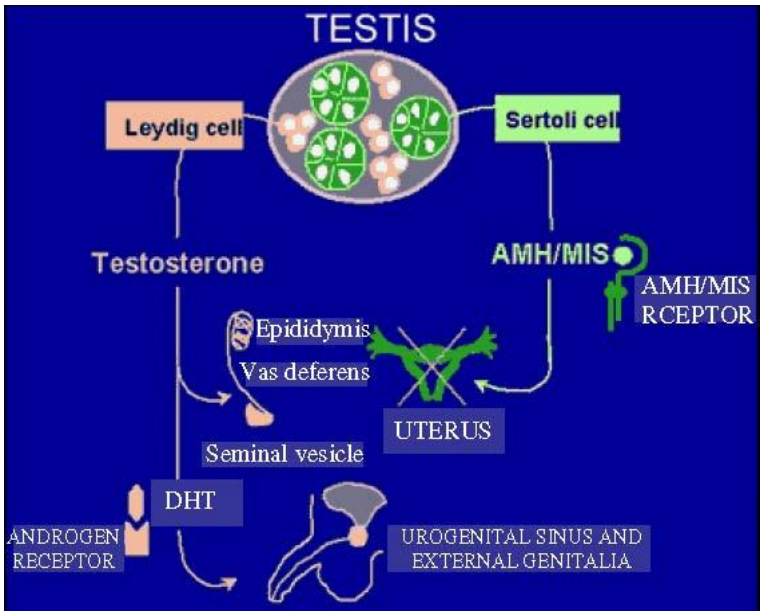

Fig 4: Role of different hormones in male sex differentiation (Josso *et al.*, 2005)

when XY children lacking a functional gene for 5-α-reductase, the enzyme that converts testosterone to DHT, were reported to have a blind vaginal pouch and enlarged clitoris. However, they have male internal anatomy: developed testis, epididymis, vas deferens and seminal vesicle (Thigpen, 1992). Administration of 5-α-reductase inhibitor in rats is shown to severely affect the masculinization of external genitalia.

Testicular Descent:
The factors controlling testicular descent has been the subject of much controversy. Previously, AMH was considered to be associated with testicular descent as decreased AMH level is usually seen in cryptorchid patients. Later, testicular dysgenesis was cited as the cause of cryptorchidism. Recently, Insulin like factor 3 (INSL3) secreted by fetal Leydig cells and belonging to the insulin/relaxin super family has been shown to be involved in the gubernaculums development in INSL3 mutant mice (Nef & Parada ,1999). Mutation of this gene has been detected in cryptorchid patients. Moreover, androgens mediate the disappearance of the cranial suspensory ligament (van der Schoot & Elger, 1992) and are required for the inguinoscrotal phase of testicular descent.

Female Sex Differentiation:
In females, the primary sex cords undergo degeneration and a new set of sex cords is then produced by the epithelium. These sex cords reside in the periphery and hence, are called cortical sex cords. The primordial germ cells proliferate by mitosis and give rise to oogonia that enter into meiosis by 10week in human fetus and form the oocyte. The cortical cords surround the oocytes and form granulosa layer. The formation of primordial follicle, oocyte surrounded by a single layer of flattened granulosa cells, commences around 16week of gestation. This is the pool from where Graffian follicles are formed (primordial-primary-secondary-tertiary/Graffian) by week 23-24 in human fetus. Oocytes proceed to the diplotene stage and remain arrested till the time of puberty.

Female differentiation of the internal genital tract is characterized by the regression of the Wollfian duct that disappears at 90 days of human fetal development. Vestiges remain in the form of Gartner canals and Rosenmüller organs. The Müllerian duct differentiates into oviducts, uterus, cervix, and upper vagina. Although the role of estrogen in female fetal development was obscure earlier, in recent years it is seen indispensable for Müllerian duct differentiation using null mouse model. The secretion of estradiol in XX gonads starts at the same time when testosterone synthesis begins in XY gonads (George & Wilson, 1978). Nonetheless, the female sex differentiation in eutherian mammals takes place in a sea of factors from the placenta, maternal circulation and the fetal gonads. Therefore, one should be cautious while describing the role of any factor in female sex

differentiation.

Anomalies of Sex Determination and Differentiation:
The anomalies at any step of sex determination/differentiation leads to disorders. The alterations might be chromosomal, gonadal or phenotypical. The opportunities for mishaps are considerably high in males than females since the male sex development is very active and highly complicated process. The chromosomal abnormalities occur due to numeric changes in the sex chromosomes. For example, only one X chromosome is present in females with Turner's syndrome (XO). The bilateral streak gonads and incomplete development of sexual characteristics with primary amenorrhea at puberty is reported in these females. On the other hand, an extra X chromosome in males due to non-disjunction of X chromosomes during oogenesis leads to Klinefelter's syndrome (XXY). Males with this syndrome have small testis, azoospermia resulting in infertility, low concentrations of testosterone, high levels of gonadotropins and poor virilization. Similarly, triple X (XXX) syndrome is reported in females.

In case of gonadal anomalies, genetically females (XX) generally have male external genitalia. This results either due to defects in sex determining genes on autosomes or linking of a testis determining gene, otherwise present on Y chromosome, with autosome. These females have either cryptorchid testis or atleast one ovo-testis. The gonadal dysgenesis is also true for 46 XY males with defects in Y chromosome or sex determining genes on autosomes.

Phenotypical anomalies arise due to imbalance in hormone milieu or its responding machinery during development. In this case, the chromosomal sex and the gonadal sex match up, but the ambiguity in external phenotype results in pseudo-hermaphroditism. Female (XX) pseudo-hermaphroditism occurs as a result of excess androgens during embryo development. The masculinization of external genitalia is prevalent, though these females have normal ovaries. In case of XY males, pseudo-hermaphroditism might arise due to androgen insensitivity syndrome of embryo. They develop feminine characteristics due to lack of masculinization.

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