IJNRD.ORG ISSN : 2456-4184

INTERNATIONAL JOURNAL OF NOVEL RESEARCH AND DEVELOPMENT (IJNRD) | IJNRD.ORG

An International Open Access, Peer-reviewed, Refereed Journal

SEX DETERMINATION IN MAMMALS: IN STARTLING EVOLUTIONARY APPROACHES

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ABSTRACT

The finding that sex chromosomes in one lineage may be homologous to autosomes in other lineages are consistent with the hypothesis that sex chromosomes evolved independently from autosomes that have acquired a sex determining allele. Therian mammals (marsupials and placental) have an XX female: XY male sex chromosome system, which is homologous to autosomes in other vertebrates. The testis-determining gene, SRY, is conserved on the Y throughout therians but is absent in other vertebrates, suggesting that the mammal system evolved about 310 million years ago (MYA). The profoundly different journeys of male and female life are thus decided by a genetic coin toss. Male bipotential gonad develops into testes containing type A spermatogonia with influences from SRY, SOX-9, SF-1. SF-1 acts on sertoli cells, which secretes Mullerian Inhibiting Substance. It also acts on Leydig cell too to exert testosterone secretion. This male specific hormone influences the Wolffian duct to structure internal genitalia (seminal vesicle, epididymis, vas deferens). In the absence of SRY, the female foetus results in decreased expression of SF -1. Granulosa & thecal cells of ovary secrete oestrogen under the influence of RSOP -1 & WNT-4. Mullerian duct will be going to form female internal genitals and genital tubercles form external genitalia.

Keywords: Sex determination, SRY, SOX9, Wnt, Testis determination, Ovary determination.

INTRODUCTION

In mammals, sex is determined by the Y chromosome, which encodes Testis Determining Factor (TDF). This factor causes undifferentiated embryonic gonads to develop into testis rather than ovaries. The testis subsequently produces male sex hormones that are responsible for male sexual characters. If castrated rabbit foetuses are reimplanted in the uterus during early development, they went on to develop ducts and external genitalia of female pattern [1]. It concludes that testis plays an important role in sex determination, whereas development of femaleness represents the "default" state [2]. According to this conceptual framework, testes development corresponds to the first sex determination step. Once testes are formed, they produce two major hormonal effectors, Testosterone and Anti Mullerian hormone (AMH). First AMH causes the mullerian duct to regress. Next testosterone causes differentiation of the Wolffian duct into the male internal genitalia like epididymis, vas deferens, seminal vesicle etc. In the urogenital region, testosterone is converted into Dihydrotestosterone (DHT), which causes morphogenesis of penis and prostate gland. In absence of AMH, the mullerian duct develops into female internal genitalia like uterus, oviducts, cervix, upper vagina and genital tubercles develops into female external genitalia like labia, clitoris, lower vagina. In 1959, with the description of the karyotype of patients with Turner syndrome (45, X) [3] and Klinefelter syndrome (47, XXY) [4], the critical role of Y chromosome was established. In 1990, a gene responsible for sex determination which could encode TDF identified on the short arm of Y chromosome, it's SRY (Sex determining region on Y chromosome) [5]. Several genes playing a role in sex determination were subsequently identified. In mammals, SRY has been identified in only marsupial and eutherian mammals [6]. This means that SRY evolved in an ancestor of Theria (marsupial and eutherian mammals). This is consistent with the observation that SRY does not exist in Monotremata (monotremes) such as platypuses and echidnas [7]. Although the eutherian SRY gene is expressed mainly in the testis and brain, the wallaby SRY gene is expressed in a broad range of tissues including testis, brain, kidney and mesonephros [8].

THE CHROMOSOMAL BASIS OF MAMMALIAN SEX DETERMINATION

In mammals, primary sex determination is directed by whether an organism has an XX or an XY karyotype. In most cases male is XY and female is XX. The Y chromosome carries a gene that encodes TDF that organizes the bipotential gonads into testis. If the SRY gene is present, it acts in testis formation. And in absence of SRY, the ovary forming genes will function. The conversion of genital ridge into bipotential gonad requires SF1, WT1, LHX9, GATA4 etc. genes. The bipotential gonad appears to be moved into female pathway by FOXL2, RSPO1, WNT4 genes and in male pathway by SOX9 and SRY gene. The ovary makes thecal cells and granulosa

cells, which together are capable of synthesizing oestrogen. Under the influence of oestrogen, the mullerian duct differentiates into female internal genitalia like uterus, oviducts, cervix, upper vagina. Testis makes two major hormones involved in sex determination. The first is AMH causes mullerian duct to regress. The testosterone, causes differentiation of wolffian duct into male internal genitalia like epididymis, vas deferens, seminal vesicle. In urogenital region, testosterone is converted into DHT which causes morphogenesis of male external genitalia like penis, scrotum and prostate.

INVOLVEMENT OF SRY GENE IN SEX DETERMINATION

The gene SRY was isolated from a region of Y chromosome close to the PAR. SRY gene has DNA binding properties that are mediated by high mobility group (HMG) motif. HMG box containing proteins may be classified into two groups. One binds to three-dimensional DNA structures, without sequence specificity. The other which includes SRY, is able to recognise DNA with sequence specific requirements [9]. SRY binds with a similar affinity to its linear consensus sequence (AACAATG) and to four-way junctions [10]. SRY belongs to a family of HMG box proteins named SOX (SRY box), binding to the same consensus DNA target as SRY. The first action of SRY as the male sex determinant may be to promote cell proliferation. SRY has been found to induce cell division in mouse foetal gonads immediately prior to testis differentiation [11] and the accelerated growth of males compared to females has been noted in preimplantation embryos of mice, humans, and cows. The male specific proliferation of cells in the gonad is critical for testis determination, as disruption of this process results in male-to-female sex reversal [11]. In humans SRY expression also surges in the male gonad immediately prior to Sertoli cell and testis determination and expression persists in the testis into adulthood [12]. Similarly, dog, sheep and pig SRY is up-regulated during the critical window of sex determination; SRY expression is maintained in the adult testis in dog and sheep but down-regulated in pig [13]. An additional role for SRY in the brain has been intensively debated because of the possibility that this gene may be responsible for sex differences in behaviour. SRY transcription has been reported in the brain of rodents and humans, but no difference in mating behaviour has been noted in XX male sex reversed mice [14]. Specific SRY knockdown in the brain of rats, however, results in loss of coordination [15]. In particular SRY is proposed to up-regulate the testis-determining gene SOX9. The up-regulation of SRY in pre-Sertoli cells is closely followed by the up-regulation of SOX9 expression, a gene capable of initiating successful testis development in the absence of SRY [16]. Although SRY has been generally supposed to be a transcription activator, the poor conservation of this gene (outside of the HMG box SRY orthologues cannot be aligned) suggested that it might act, instead, as a repressor of a repressor [17]. SRY was proposed to function as a repressor in a double inhibition of a putative factor Z to explain rare familial XX male syndromes [18]. The putative intermediate gene was later proposed to be the X-borne gene, SOX3, from which SRY evolved [19].

THE EVOLUTION OF SRY FROM SOX3 GENE

The closest relative of SRY is SOX3, found on the X chromosome in all therian mammals and proposed to be the gene from which SRY evolved [20]. SOX3 belongs to a family of genes that share the SRY HMG box DNA binding domain, for which they were named SOX (SRY-like HMG box). SOX3 mutations result in mental retardation, growth hormone deficiency and failure of spermatogenesis, although not sex reversal [21]. SOX3 is expressed in the indifferent gonad in humans and mice [22], but not marsupials [23]. However, the expression of SOX3 in the developing gonads of Xenopus and chicks [24] suggests that SOX3 expression in the urogenital ridge is a conserved vertebrate trait that has been subsequently lost in marsupials. It has been suggested that a truncation mutation of SOX3 resulted in a dominant suppressor that assumed the role of the male sex-determining switch in the same way that truncation of the related SOX gene, SOX9, results in dominant suppression [25]. The evolution of SRY presumably defined the therian X and Y as sex chromosomes; a mutation of one SOX3 allele on the autosome pair that was the ancestor of the therian X and Y would have defined the proto-Y, whereas the other copy of SOX3 remained unaffected on the partner chromosome, the proto-X. Non-mammalian vertebrates have no orthologue of SRY, and SOX3 is autosomal, consistent with a causative role for SRY in the differentiation of the therian XY pair. It has been thought, therefore, that the evolution of SRY, and the beginning of XY differentiation in mammals, must have occurred shortly after the divergence of mammals and reptiles 310 MYA. However, recent studies of sex and SRY in the most basal mammals, the egg-laying monotremes, have completely changed our perspective on when and how this occurred.

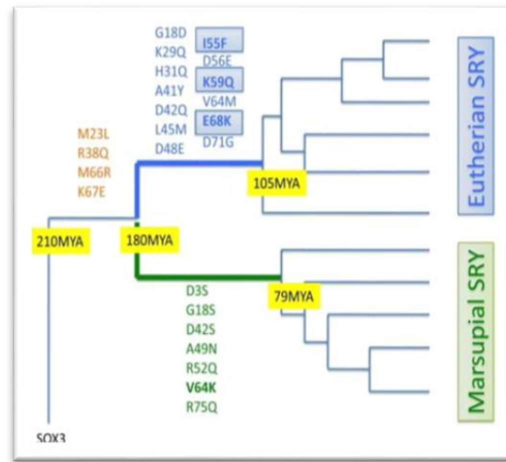

fig 1: Inferred ancestral substitutions are shown on the tree by parsimony. Four substitutions were specific to the branch that SRY differentiated from SOX3 in the ancestor of Theria. 13 substitutions were on the branch containing the eutherian SRY, and seven substitutions were on the branch containing the marsupial SRY. The divergence time on the tree, 79 or 105 MYA coincides with the radiation of marsupials or eutherians, and 210 180 MYA is the divergence time of monotremes and Theria. [26]

AUTOSOMAL GENES INVOLVED IN SEX DETERMINATION

The Wilms tumour gene, WT1, initially identified as an oncogene responsible for a paediatric kidney tumour, was shown to be involved in early gonadal development. WT1 expression pattern studies reveal that it is expressed in genital ridge of male and female. Moreover, knockout mice homozygous for the WT1 null mutation have kidney and gonadal dysgenesis. The absence of gonads caused by gonadal degeneration, during embryogenesis suggests a role of WT1 in the maintenance of gonadal development. Mutations in WT1 were identified in patients with Denys-Drash syndrome and Frasier syndrome, who have renal failure and sexual ambiguity [27].

Steroidogenic factor 1 (SF1) is an orphan nuclear receptor that was initially shown to be regulator of expression of cytochrome P450 steroid hydrolases. It is expressed in the adrenal cortex, testis, ovary, hypothalamus and pituitary. Disruption in SF1 in mouse results in gonadal and adrenal agenesis, resulting in post-natal death due to adrenal insufficiency.

An SF1 response was found in DAX1 promoter and it was shown that SF1 upregulates the expression of DAX1 in an adrenocortical carcinoma cell line. WT1 antagonizes DAX1 inhibition of SF1 mediated transactivation. SF1 also seen to regulate MIS via an SF1 response element present in is promoter. The fact that SF1 expression declines only in female gonads suggests that SF1 may play a major role in Mullerian duct regression.

Another autosomal gene involved in sex determination is a member of SOX family gene, named SOX9 associated with campomelic dysplasia, a congenital skeletal malformation syndrome in which most of the XY patients are XY females with gonadal dysgenesis [28]. SOX9 is involved in both chondrogenesis and gonad development.

table: 1 [29]

Gene	Localization	Family	Function	Phenotype of mutation
SF1	9q33	Orphan Nuclear Receptor	Transcription factor	Gonadal and adrenal agenesis in mouse
WT1	11p13	Zinc Finger Protein	Transcription factor	Dens-Drash and Frasier syndromes
SRY	Yp11	HMG Protein	Transcription factor	XY gonadal dysgenesis
DAX1	Xp21.3	Orphan Nuclear Receptor	Transcription factor	XY gonadal dysgenesis
SOX9	17q24	HMG Protein	Transcription factor	Campomelic dysplasia with XY gonadal dysgenesis
MIS	19q13	TGF beta	Growth factor	Persistent Mullerian Duct syndrome

DIFFERENTIATION OF MAMMALIAN TESTIS

Testis differentiation is induced by the expression of SRY in a subset of somatic cells that are induced to differentiate into Sertoli cells. Sertoli cells are believed to act as the organizing centre of the male gonad.

1. **SERTOLI CELLS**

Sertoli cells are somatic cells that associate with germ cells and nurture their development into sperm, so that they are called nurse cells. They are the first cell type known to differentiate within the gonad from bipotential precursors of the supporting cell lineage and are therefore the first indicator that the gonad has passed from the indifferent stage into testis development. In situ hybridization [30] and RNase protection studies [31] showed that SRY is expressed in the gonad at 11.5 dpc (days post coitum) and that expression is associated with the somatic cells of the genital ridge and not the germ cells [32]. These cells become positive for SRY mRNA only after delaminating from the coelomic epithelium, indicating that SRY is not the cause of this delamination [33]. However, definitive evidence that SRY is exclusively expressed in Sertoli cells, or more accurately the pre-Sertoli cells, were hampered for many years by the lack of a molecular tool to detect endogenous mouse SRY expression in situ. Transgenic mouse models, expressing either GFP or an epitope-tagged SRY [34], under the control of the SRY promoter, suggested that SRY expression is restricted to the Sertoli cell lineage. More direct studies recently became possible through the generation of a mouse SRY antibody and demonstration that the subset of somatic cells that expresses SRY almost immediately start to co-express SOX9, which in turn is a reliable lineage marker of developing Sertoli cells [35]. The differentiation from pre-Sertoli cells into Sertoli cells is marked by the polarization of the cells when they form epithelial aggregates that assemble into testis cords. Concurrently there is a change in the expression of certain extracellular matrix proteins; desmin is downregulated, whereas cytokeratins are upregulated. On the basis of these findings, pre-Sertoli cells are defined as nonpolarized, dispersed somatic cells that express SRY and/or Sox9, whereas a Sertoli cell is polarized, resides within the testis cord structure, and expresses Sox9 (Fig.2).

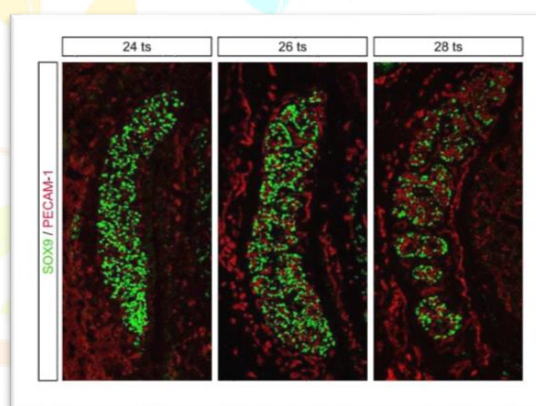

fig 2. Differentiation of pre-Sertoli cells into Sertoli cells. Nonpolarized, dispersed somatic cell visualized by SOX9 immunofluorescence (green) represent pre-Sertoli cells at the 24-tail somite (ts) stage. A few hours later, by 28 ts, these cells become polarized, forming epithelial aggregates that assemble into testis cords; at this stage they are referred to as Sertoli cells. PECAM-1 counterstaining (red) marks PGCs and endothelial cells. [36]

2. **PERITUBULAR MYLOID CELLS**

One of the three cell types that migrate from the mesonephros into the male gonad is the peritubular myoid (PM) cell. These cells form a single layer of flattened cells surrounding the Sertoli cells, circumscribing the testis cords. They are thought to have two main functions: 1) to contribute structurally to the formation of the testis cords in conjunction with Sertoli cells and 2) to promote the movement of mature sperm through the seminiferous tubules of the adult testis for export to the seminal vesicles, a function mediated by their smooth muscle-like character. PM cells represent the only cell type in the testis so far for which no counterpart can be identified in the ovary. This might be due to their origin from immigrating cells from the mesonephros, which only occurs in an XY gonad after the expression of SRY [37].

3. **TESTIS CORDS**

Under the light microscope, the first signs of testicular differentiation appear in the mouse at 12.5 dpc with the formation of cylindrical cords, the precursors of the adult spermatogenic tubules. They are composed of clusters of germ cells enclosed by a layer of Sertoli cells, which is in turn surrounded by a layer of PM cells. Interestingly, testis cords can form in the genetic or pharmacologically induced absence of germ cells [44], demonstrating a negligible role of germ cells in this process. Subsequently, PM cells and the Sertoli cells collaboratively induce the deposition of a basal lamina between their respective layers, thus defining the boundary between the testis cords and the interstitial tissue.

4. **LEYDIG CELLS**

Within the second compartment of the testis, the interstitium, steroidogenic Leydig cells differentiate Leydig cells often lie in clusters close to blood vessels, in line with their steroidogenic role. In mammals there are two types of Leydig cells. The foetal Leydig cells originate, at least in part, in the mesonephros, and are responsible for the production of androgen for the foetal masculinization; these cells probably degenerate postnatally. The adult Leydig cells, which differentiate after birth, appear to

be unrelated to their foetal counterparts. Studies indicated that they arise from undifferentiated precursor cells that are part of the mesenchymal cells of the interstitium [38].

5. **VASCULAR AND OTHER INTERSTITIAL CELLS**

Although Leydig cells are often considered the main component of the testicular interstitium, probably because of their essential and obvious male-specific endocrine roles, several other interstitial cell types can be found. These include endothelial cells, fibroblasts and blood-derived cells such as macrophages, lymphocytes, plasma cells, monocytes and mast cells. Endothelial cells, alongside PM and Leydig cells, represent a third cell type that migrates into the testis from the mesonephros [39]. They form the male-specific vasculature with the prominent coelomic vessel on the surface of the gonad and side branches in between the testis cords. Inhibitor experiments and mutant analysis so far suggest that the formation of the vasculature and the testis cords are intimately interrelated.

DIFFERENTIATION OF MAMMALIAN OVARY

The ovary has two main functions: 1) the production of steroid hormones and 2) the generation of mature oocytes that are capable of being fertilized and developing into an embryo. If SRY gene is not present, the interactions between paracrine and transcription factors in the developing genital ridge activates Wnt4 and RSpo1(R-Spondin1). Wnt4 activates canonical Wnt pathway which is made more efficient by RSpo1. The Wnt pathway causes the accumulation of beta catenin, stimulates further Wnt activity. The continued production of beta catenin induces both the transcription of ovary producing genes and blocks the testis determining pathway by interfering with SOX9.

1. DIFFERENTIATION OF OVARIAN GERM CELLS

Female-specific gene expression has been reported as early as 11.5dpc [40]. Furthermore, detailed histological analysis revealed that there is a phase from around 13.5 to 15.5 dpc, in which the poorly differentiated structure undergoes remodelling (Fig.3). The presumptive oocytes develop as interconnected cysts that are linked by cytoplasmic bridges. There is also a high degree of vascularization, with a dense network of small vessels that become visible only by using molecular markers. These vessels demarcate strings of germ cells, also known as ovigerous cords. [41].

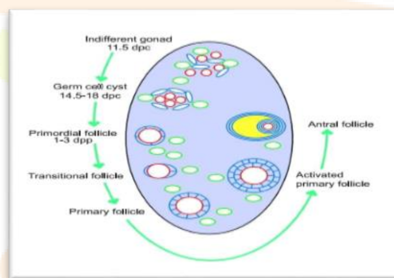

fig3. Ovary and follicle development and differentiation. Schematic representation of the stages of cellular organization in the foetal and postnatal mouse ovary, leading to primordial, primary, and antral follicle formation. Oocytes are shown in red, supporting pre-granulosa cells in blue, steroidogenic thecal cells in green, and antral fluid in yellow. [41]

2. FORMATION OF PRIMORDIAL FOLLICLES

Follicles form when the oocytes are blocked in prophase I of meiosis, from 14–15 weeks of gestation, in the human species. This process begins when the germ cells have colonised the gonads and divide to form clusters called cysts (or syncitia). These cysts are organised in ovigerous cords, where cytoplasmic bridges, surrounded by somatic ovarian cells, connect the oocytes to each other. The formation of primordial follicles results from the fragmentation of the ovigerous cords. These primordial follicles then consist of an oocyte surrounded by a few flattened somatic cells, the future granulosa cells. The different stages of follicle maturation are controlled by many factors and require a very precise dialogue between the oocyte and the somatic cells that surround it. Among the important factors involved in the formation of primordial follicles are FIGLA factor, SOHLH factors, neurotrophins and FOXL2. In the ovaries of Foxl2 -/- mice, the growing oocytes are (and remain) surrounded by a single layer of granulosa cells. This indicates that granulosa cell differentiation has been altered from the early stages of folliculogenesis. In addition, the oocytes remain clustered (up to 10 oocytes) and are surrounded by a layer of support cells. These structures persist for up to 8 weeks after birth as clusters of unfragmented oocytes and ovarian cords. These data show that FOXL2 is necessary for the fragmentation of cysts and the formation of primordial follicles [42].

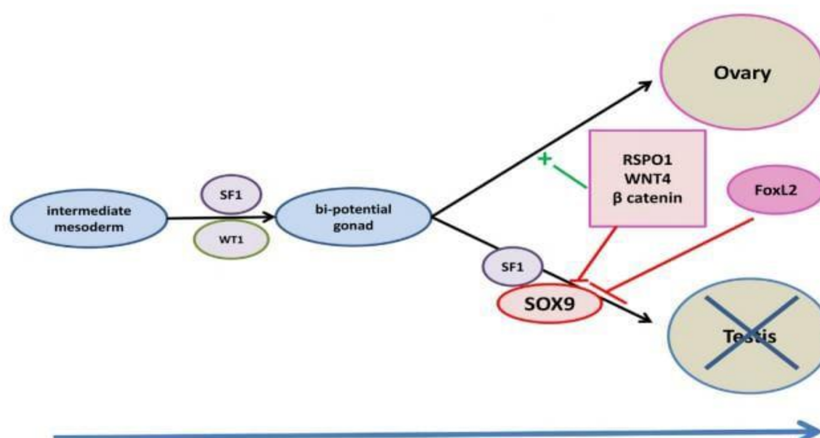

Fig: Factors involved in ovarian development over time before birth [43]

CONCLUSION

Comparisons amongst vertebrates have revealed a variety of sex-determining mechanisms, surprising for such a vital function. Whereas genes downstream in the sex-determining pathway appear to have been conserved across vertebrates, the sex-determining trigger shows extreme variation between vertebrate taxa. The evolution of SRY from the autosomal SOX3 gene 166–148 MYA initiated the differentiation of a new X and Y sex chromosome pair early in therian evolution. In case of ovary differentiation, Rspo1 plays a critical role in inhibition of SOX9 gene. On the other hand, in addition to its role in determining and differentiating Primordial germ cells in follicular cells, Rspo1 is inhibited after birth allowing maintenance of homeostasis in the adult ovary.

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