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Mammalian X Chromosome Dosage Compensation: Perspectives From the Germ Line

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Sex chromosomes are advantageous to mammals, allowing them to adopt a genetic rather than environmental sex determination system. However, sex chromosome evolution also carries a burden, because it results in an imbalance in gene dosage between females (XX) and males (XY). This imbalance is resolved by X dosage compensation, which comprises both X chromosome inactivation and X chromosome upregulation. X dosage compensation has been well characterized in the soma, but not in the germ line. Germ cells face a special challenge, because genome wide reprogramming erases epigenetic marks responsible for maintaining the X dosage compensated state. Here we explain how evolution has influenced the gene content and germ line specialization of the mammalian sex chromosomes. We discuss new research uncovering unusual X dosage compensation states in germ cells, which we postulate influence sexual dimorphisms in germ line development and cause infertility in individuals with sex chromosome aneuploidy.

1. Introduction: Mammalian Sex Determination

The ancestors of mammals (synapsids) diverged from those of birds/reptiles (sauropsids), around 310 million years ago (Mya; **Figure 1**). Mammals subsequently separated into three groups: the prototherians (egg-laying mammals), diverged from the therian ancestors 166 Mya, and the therians subsequently separated into the metatherians (marsupials) and eutherians 148 Mya. With some exceptions,[1] prototherians and therians have an XY male/XX female sex chromosome system. In eutherians, the sex of the gonad is determined by the Y-encoded gene *Sry*.[2] When *Sry* is present a testis is formed, and when it is absent an ovary is formed. Secondary sexual characteristics develop later in response to sex hormones secreted by the gonad.[1,3] Birds have a distinct sex chromosome system in which females are ZW and males, ZZ. Sex determination in birds is controlled by the Z-encoded *DMRT1* gene, which in double-dose drives testis development[4] (Figure 1; also see section 4).

The pathways governing sex determination in metatherians have not been deciphered, primarily because genetic manipulation has not yet been performed in these mammals. However, clues to the chromosomal basis of sex determination in metatherians have been provided from individuals with naturally occurring sex chromosome aneuploidy. As in eutherians, gonadal sex in metatherians is dictated by the presence or absence of the Y chromosome. A metatherian Y-encoded *Sry* orthologue has been identified and may trigger testis development through a similar molecular pathway to that observed in eutherians.[5] Intriguingly however, development of secondary sexual characteristics such as the scrotum and processus vaginalis initiates prior to gonadal differentiation, and is thus independent of gonadal sex hormone secretions.[6] Instead, secondary sexual characteristics are governed by the number of X chromosomes, with one X chromosome driving male and two X chromosomes, female characteristics. As a result, sex chromosome aneuploid metatherians exhibit a mismatch between their primary and secondary sexual characteristics. Individuals with only a single X chromosome develop ovaries and male secondary sexual characteristics, while individuals with two X chromosomes and a Y chromosome develop testes but female secondary sexual characteristics.[7] It has been suggested that this X dosage dependent mechanism was the ancestral form of sex determination in mammals.[8] The identity of the X dosage dependent sex determinant/s nevertheless remains unknown, and is an exciting avenue for future study.

Prototherians have five X and five Y chromosomes, none of which are homologous to the therian XY pair.[9] *Sry* is therefore not the sex determination trigger in these mammals. However, one of the prototherian Y chromosomes carries *AMH*.[10] This gene is implicated in sex determination in several fish species,[11] and directs degeneration of the female duct primordia during male sex determination in mammals.[12] It therefore represents a promising candidate sex determination gene in prototherians.

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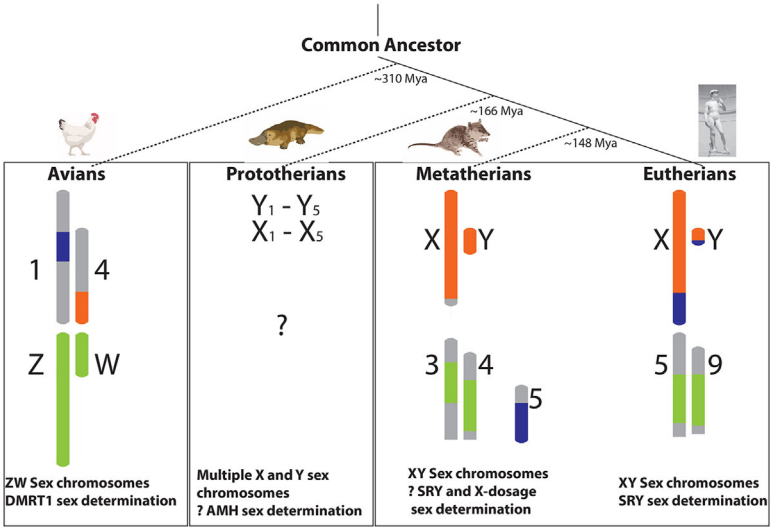

Figure 1. Evolution of mammalian sex chromosomes and sex determination systems. The origin of therian sex chromosomes from ancestral autosomes is shown with estimated times of their divergence. The eutherian X chromosome shares ancestral sequence synteny with chicken chromosome 4 (X-conserved region (XCR), orange) and chicken chromosome 1 (X-added region (XAR), dark blue). The metatherian X chromosome has the X-conserved but not X-added region. Corresponding XAR sequences in metatherians are found on chromosome 5. Syntenic sequences to the avian sex chromosomes Z and W are on the autosomes in eutherians (chromosomes 5 and 9) and metatherians (chromosomes 3 and 4).

2. Mammalian Sex Chromosome Evolution

The therian X and Y chromosomes evolved from a pair of ancestral autosomes[13–15] approximately 148–166 MYa, prior to the divergence of eutherians and metatherians (Figure 1). The acquisition of *Sry* likely initiated sequence divergence of this autosomal pair, to form the proto-Y and proto-X chromosomes. Subsequently, appearance of sexually antagonistic alleles near *Sry*, coupled with chromosome inversion events encompassing this locus, caused suppression of meiotic recombination between the proto-Y and proto-X chromosome.[10,13,14,16,17] XY recombination in eutherians is restricted to one or two small regions of homology termed the pseudoautosomal region (PAR).[18] These regions permit crossover formation between the X and Y chromosomes during meiosis, which is a prerequisite for correct sex chromosome segregation at the first meiotic division.[19] However, in metatherians, recombination between the X and Y chromosomes has ceased completely, and alternative mechanisms to ensure accurate XY segregation have evolved.[20]

As a result of recombination suppression, the Y chromosome has lost most of its ancestral genes through genetic drift.[21,22] The extant human Y chromosome contains only 17, and the mouse Y chromosome 9, of the 639 ancestral genes for which it shared X orthologues.[23]

3. Specialization of the Y Chromosome for Reproduction

The Y chromosome is unique among mammalian chromosomes in that it is inherited exclusively through the male lineage, and thus has acquired genes with specialized roles in spermatogenesis. Y chromosome microdeletions are among the commonest genetic causes of infertility, affecting 10–15% of individuals with azoospermia (i.e., no sperm in ejaculate[24]).

The region of the human Y chromosome that does not recombine with the X chromosome, referred to as the "male-specific" (MSY) region, comprises 95% of the chromosome's length.[25,26] Over 99% of the MSY is euchromatic, and can be divided into three sequence classes: X degenerate, ampliconic, and X transposed.

X degenerate sequences are remnants of genes that were present in the ancestral autosomal pair. Comparison of these sequence with their X-encoded homologs has confirmed that genetic decay of the human Y chromosome can be attributed to at least four separate events that suppressed X-Y recombination.[14] These events have created four evolutionary strata of Y genes that show increasing divergence from their X homologs from the short to the long arm of the X chromosome. X degenerate genes on the MSY are typically present in single copies, and show remarkable conservation across mammals. Most are expressed ubiquitously, and fulfill important dosage sensitive functions, for example in transcription and translation.[10,27]

Ampliconic sequences comprise about 30% of the human MSY. Sixty Y-ampliconic genes belonging to nine gene families have identified. Ampliconic genes are often arranged in palindromes, whose high arm-arm identity (>99.99%) is maintained by frequent intrachromosomal gene conversion events.[28] These genes had evolved from a variety of genomic sources and means, including transposition from the autosomes and/or X chromosome, and retroposition. In mice, ampliconic sequences dominate the MSY, accounting for 98% of its gene content.[26] It has been proposed that the MSY acquired and maintained ampliconic genes that specifically enhance male fitness.[22,25] Consistent with this prediction, ampliconic genes show testis-biased expression, and in mice are essential for correct sperm maturation.[29,30]

X transposed sequences are 99% identical to DNA sequence on the long arm of the human X chromosome. Their presence on the human MSY is the result of an X transposition event that occurred 3–4 Mya, after the divergence of the human and chimpanzee lineages.[31] As a result, these sequences are not present on the mouse Y chromosome. X transposed sequences are relatively gene poor but are enriched for repeats, for example LINE1 elements (long interspersed nuclear element 1).

4. Specialization of the X Chromosome for Reproduction

The eutherian X chromosome comprises the "X conserved region" (XCR), which is similar across all therians, and the "X added region" (XAR); an autosomal segment that was added to X chromosome after the eutherian – metatherian divergence.[32] The XCR contains sequence that is syntenic to chicken

chromosome 4, while the XAR contains sequence that is syntenic to chicken chromosome 1. Conversely, sequences that are syntenic to the avian Z and W sex chromosomes are found presently on the therian autosomes (Figure 1). Unlike the Y chromosome, the X chromosome has retained almost all ancestral genes due to protection offered by X–X recombination during female meiosis. Susumo Ohno thus predicted that the gene content of the X chromosome should be conserved among eutherians.[17] Despite this conservation, X chromosome genes are predicted to evolve specialized functions for the germ line. Dominant mutations benefiting female reproduction are more likely to become fixed on the X chromosome, because this chromosome spends twice the amount of time in females as it does in males. Conversely, since the X chromosome is hemizygous in males, it should also accumulate recessive mutations that benefit male fitness and are otherwise neutral to female fitness.[33] Genomic and transcriptomic studies have revealed that both scenarios are true: the X chromosome is enriched for genes involved in oogenesis as well as in spermatogenesis.[34,35,36–38] To date there has been a greater research focus on X chromosomal spermatogenesis than oogenesis genes.

In mice, X spermatogenesis genes function at two principle stages: before meiosis and after meiosis. Genes regulating the premeiotic stage of spermatogenesis are expressed predominantly in spermatogonia, and most are single copy.[38] Genes regulating postmeiotic stages are expressed in developing spermatids and many are multiple copy, arranged as tandem or palindromic repeats within large complex ampliconic regions.[36,37] Ampliconic X genes have evolved independently in different eutherian mammals and therefore exhibit limited conservation.[37] As such, they represent exceptions to Ohno's law that the gene content of the X chromosome is conserved among eutherians. Ampliconic genes comprise at least 13% and 17% of all genes on the human and mouse X chromosome, respectively.[36,37,39] Given their abundance and restricted expression in the male germline, these genes are excellent candidates for unexplained male infertility. However, with some exceptions,[40] their functions are not known, principally because ampliconic regions are difficult to manipulate using conventional genetic approaches. Ampliconic genes are also are an important consideration when computing somatic X dosage compensation states (see next section).

Although rich in genes expressed in spermatogonia and spermatids, the X chromosome is under-populated with genes expressed during male meiosis.[34,41] This is likely an evolutionary consequence of meiotic sex chromosome inactivation (MSCI), the silencing of the X and Y chromosomes at pachynema of meiosis. MSCI has been documented in eutherians and metatherians but not in other vertebrates so far studied, and therefore may have evolved after the prototherian – therian divergence.[42] Although it is essential for male fertility, the function of MSCI, and in particular why it is restricted to therians, is unknown. MSCI is retained after meiosis, during spermatid differentiation, albeit in a manner that is less efficient than observed during meiosis.[43,44] A hypothesis not yet explored is that X genes expressed in spermatids are amplified in order to ensure optimum expression levels in the face of this later phase of XY repression.[36] A similar explanation could explain the massive amplification of post-meiotically expressed genes on the mouse Y chromosome.[26,30] Alternatively, or in addition, gene amplification on the X and the Y chromosome may facilitate meiotic drive, in which the X and Y chromosome compete for preferential transmission.[29]

5. X Chromosome Dosage Compensation Mechanisms

In therians one of the most striking consequences of Y chromosome decay is an imbalance in the dosage of X-linked versus autosomal genes. After individual genes were lost from the proto-Y chromosome, their corresponding alleles on the proto-X chromosome were present in males as single copies. The resulting haploinsufficiency for X-linked gene products would have compromised male fitness. Evolution of a mechanism to up-regulate X-linked gene expression two-fold to match autosomal expression, resulting in an X chromosome to autosome expression ratio (X:Autosome; abbreviated hereafter to X:A) of 1, would therefore have been required.

The existence of such a mechanism of X chromosome upregulation (XCU) was first proposed by Susumu Ohno.[17] He also posited that while XCU in males would ensure dosage parity between the X and the autosomes, in females it would result in a two-fold excess of X- over autosomal products. Thus, in females a second dosage compensation mechanism, in which one of the two X chromosomes is silenced, would have evolved. This process is X chromosome inactivation (XCI), the silencing of one of two X chromosomes, which results in the formation of the Barr body.[45] XCI is mediated by the non-coding RNA *Xist* in eutherians[46] and *Rsx* in metatherians.[47] As a result of dosage compensation, males therefore have one active upregulated X chromosome, while females have one active upregulated X chromosome, and one inactivated X chromosome.[48,49]

XCI in mammals is well described, and numerous techniques allow this process to be monitored in vivo and in vitro. Cytological approaches, for example antibody staining and RNA FISH, assay inactive X-associated epigenetic marks and expression of specific X-linked genes or reporters. "Omics" approaches, for example RNA-seq, ChIP-seq, and chromosome conformation capture, provide information on X chromosome wide changes in gene expression and chromatin architecture, even in single cells.[50,51,52]

In comparison, mammalian XCU is less studied, and although predicted by Ohno in the 1960s, experimental evidence for XCU at an X locus was not acquired until the 1990s.[53] Unlike XCI, which is mediated at the chromatin level, XCU is achieved via transcriptional and post-transcriptional mechanisms. These include elevated transcription of X-genes via H3K4me3,[54] RNA Pol2 phosphorylation and MOF-mediated H4K16 acetylation,[55] increased X transcript half-life,[55,56] increased ribosome density[56] and decreased autosomal transcript half-life.[57] Cytological approaches have not yet been used to monitor XCU, and while RNA FISH can be used quantitatively,[58] it is more commonly applied to assay the "on-off" states observed in XCI, than the fine-tuning of gene expression resulting from XCU. For this reason, XCU has been most extensively studied using omics approaches such as RNA microarrays,[48] RNA-seq[49,54,59,60–62]

and proteomics.[49,62] In these instances, XCU is examined by calculating the X:A ratio, which in turn is derived by dividing the median expression from the X chromosome by the median expression from the autosomes.

A second complication is that X:A ratio estimations differ depending on which X and autosomal genes are included in the analysis, and which cell type is assayed. XCU, like XCI, is not expected to affect all X chromosomal genes, because not all of these genes are dosage sensitive: studies suggest that dosage compensation is more critical for genes exhibiting ubiquitous expression and housekeeping functions than for those exhibiting tissue-specific expression and functions.[63,64] This distinction becomes critical when one considers the unusual gene content of the X chromosome. As outlined, the mouse and human X chromosomes are over-represented in genes that are highly expressed in reproductive tissues and lowly expressed, or silent, elsewhere.[34,36,37,39] When assaying XCU in the soma, including these reproduction-related genes artificially lowers the median X expression level and, consequently, the X:A ratio. Using this approach, studies have concluded that the X:A ratio is close to 0.5, and therefore that XCU does not take place in eutherians.[61,62] Conversely, when assaying XCU in reproductive tissues, including reproduction-related genes increases the X:A ratio above 1, because tissue-specific genes are expressed at higher levels.[65] Only when lower and upper thresholds of expression are imposed does the presence of XCU, that is X:A ratios of 1, become apparent.[49,54,60,64–66] It is noteworthy that in the single metatherian in which XCU has been examined, the opossum *Monodelphis domestica*, somatic X:A ratios approximate 1, even when these expression limits are not imposed.[61] This finding could be explained if the X chromosome of metatherians has not undergone the same extent of germ line specialization as the X chromosome of eutherians. Sequencing of representative metatherian X chromosomes will resolve this point.

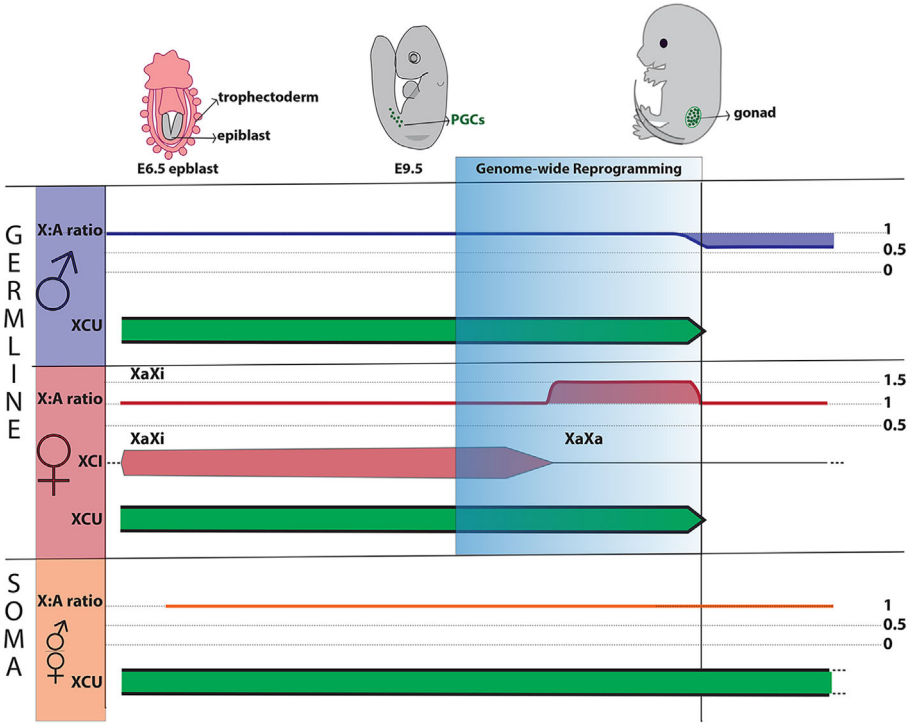

Figure 2. Contrasting X chromosome dosage compensation states in the early embryo, germ line and soma. X dosage compensation states are reset in the germ line in both males and females during GWR, characterised by the reversal of XCU in both sexes and reversal of XCI in females. Following a period in which X:A ratios exceed 1, X dosage balance is reinstated in females when XCU is also reversed. In contrast, loss of XCU in the male germ line culminates in X dosage decompensation (X:A ratio <1) which persists thereafter. Female and male somatic tissues, which do not experience GWR, remain X dosage compensated.

6. Reconfiguring X Dosage Compensation States in the Germ Line

The importance of balancing gene dosage during development is evident from analysis of aneuploidy. In humans, aneuploidy is associated with severe developmental consequences and its incidence in utero decreases with gestational age.[67] Exceptions that can survive embryogenesis and reach adulthood include trisomy 21 (Down syndrome), and the sex chromosome aneuploidies Turner syndrome 45 (XO), Triple X syndrome 47 (XXX), Klinefelter syndrome 47 (XXY), and Double Y syndrome 47 (XYY). That the somatic effects of sex chromosome aneuploidy are less deleterious than those of autosomal aneuploidy attests to the existence of XCU and XCI.

Nevertheless, a common finding in sex chromosomally aneuploid patients, particularly those with Turner syndrome or Klinefelter syndrome, is hypogonadism and infertility.[68] The germ line is therefore particularly sensitive to X chromosome imbalance in these conditions. Because the X chromosome contains a preponderance of genes expressed only in reproductive tissues,[34,36,37,39] it is tempting to attribute the infertility phenotype to an imbalance in the dosage of these genes. However new work has shown that X housekeeping genes exhibit unusual dosage compensation states during germ cell development, making them alternative candidates for sex chromosome – related infertility.[65]

In mice, germ cell development initiates around embryonic day (E) 6.5, when primordial germ cells (PGCs) are specified from the posterior proximal epiblast (**Figure 2**). At this stage, PGCs are X dosage compensated. In females one X chromosome is active and upregulated, while the other X chromosome is inactive. In males, the single active X chromosome is upregulated.[65,69,70] PGCs proliferate and migrate along the hindgut endoderm and thereafter colonise

the gonad between E10.5 and E11.5. During this time, they undergo genome wide reprogramming (GWR), when epigenetic marks are erased.[71–75] Contemporaneously, the X dosage compensation state of PGCs is reconfigured. In female PGCs, upregulation of the active X is erased, and the silent X reactivates.[65,70,76] However these events do not occur simultaneously; loss of XCU occurs later than loss of XCI, resulting in an unusual X dosage compensation state in which the combined X chromosome output exceeds that of the autosomes, that is the X:A ratio exceeds 1. This state of X-hypertranscription lasts around three days, and is corrected to an X:A ratio of one only at E15.5, when oocytes reach zygonema of meiosis. X-hypertranscription also occurs during human female gametogenesis.[65]

In female germ cells X dosage compensation is ultimately reinstated because erasure of XCU from the active X chromosome is buffered by reactivation of the inactive X chromosome. However, in the male germ line, there is no second X chromosome to provide such buffering. How then does X dosage compensation play out during male GWR? Interestingly, XCU is also erased in male mouse and human germ cells, resulting in a state of X dosage decompensation (X:A ratio <1) as germ cells cease dividing and enter quiescence.[65] Subsequently, the X:A ratio remains low when germ cells reinitiate mitotic divisions and enter meiosis, before decreasing further at pachynema, as a result of MSCI. Germ cells therefore exhibit X dosage compensation states that differ both between the sexes, and from somatic cells, and that are thus exceptions to Ohno's Law.[17] Importantly, the sex differences in X dosage compensation states established after GWR persist during the remainder of germ cell development. As yet there is no evidence that reprogramming per se is responsible for these changes in X dosage compensation states. This possibility could be investigated by assaying active X chromosome-associated chromatin marks and transcript half-life in instances where GWR is intact and perturbed.[72]

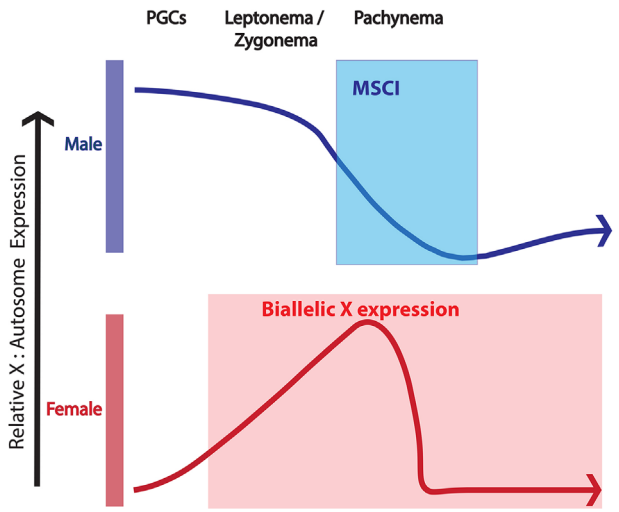

Figure 3. Relative X chromosome activity during meiosis in male and female germ cells. X chromosome activity is fundamentally different in the male and female germ line during meiosis. Relative to PGCs, X:A ratios rise during leptonema/zygonema in females and thereafter fall during pachynema. In males, the X:A ratio in leptonema/zygonema is lower relative to PGCs, and then drops even lower levels in pachynema, due to MSCI.

7. Contribution of X Dosage Compensation States to Germ Cell Development and Sexual Dimorphisms

Are the unusual X dosage compensation states observed in female and male germ cells essential for their development (**Figure 3**)? There is already evidence that reducing expression from the X chromosome is critical for the male germ line. Almost or all X genes, including those with housekeeping functions, are silenced as a result of MSCI, and with the exception of those that are multicopy,[36] they remain repressed during spermatid maturation.[43] A minority of X housekeeping genes have given rise to autosomal, retroposed duplicate copies that are expressed during and after meiosis, and that can functionally compensate for their silenced X-encoded paralogues.[77] However, most X housekeeping genes have no autosomal "backups", and misexpression of other sex-linked genes during meiosis is lethal to male germ cells.[78] The new findings demonstrate that this period of X-hypoexpression initiates much earlier than when MSCI initiates, being evident from the point at which sex differences in germ cell development first appear. Thus, low X:A ratios may be a prerequisite for development of the male germ line.

In female germ cells, loss of XCI and XCU are important in order to erase parental epigenetic marks on the two X chromosomes before reinstatement of X dosage compensation in the embryo. Removal of XCI and XCU could also facilitate pairing and genetic exchange between the two X chromosomes during meiosis. However, the significance of X-hyperexpression during early meiosis is unclear. The current view is that genes expressed during meiosis are under-represented on the X chromosome.[34] However, this conclusion is based on transcriptional data from male germ cells, and thus may not apply to the female germ line. Indeed, male and female meiosis differs in many respects,[79] and so may have distinct transcriptional requirements. If oocytes capitalize on X-hyperexpression to receive a higher dose of X-linked transcripts, one could envisage that the X chromosome would be a favored site for the evolution of genes regulating female meiosis. This hypothesis could be tested by applying transcriptomic analysis to female germ cells.

Studies on subfertile sex chromosome aneuploid mice support the model that atypical X dosage compensation states are required for germ cell development. Klinefelter syndrome variant XX mice are phenotypically male but carry two X chromosomes. Interestingly, following GWR, germ cells from these males exhibit X-hyperexpression, as is observed in wild type females.[65] Conversely, in Turner syndrome XO mice, which carry a single X chromosome, germ cells exhibit X-hypoexpression after GWR, as observed in wild type males.[65] X dosage compensation states are thus determined by the X chromosome complement rather than the phenotypic sex of the gonad. The mismatch in X dosage compensation in gonadal sex could contribute to the aetiology of infertility in Klinefelter and Turner syndrome models.

In mammals the sex of germ cells is ultimately determined after they have colonised the gonad. In males, *Sry* expression in the somatic gonadal supporting cells causes germ cells to embark on male development, while in females the absence of *Sry* induces germ cells to follow a female pathway. However, since male and female cells are genetically distinct, they may exhibit differences in sexual identity prior to this time. There is already evidence that sex chromosome complement influences germ cell development.[80] The Y chromosome is an obvious potential source of sex differences, because it is found only in males and contains many germ cell expressed genes. Sexual dimorphisms in X dosage compensation state could also contribute. In females, reactivation of the inactive X chromosome in germ cells is evident from as early as E7.0–8.0, that is before gonadal colonization.[70] X dosage compensation states nevertheless remain similar between males and females until E9.5.[65] However, the status of X dosage compensation between this point and that at which *Sry* is expressed has not been examined. More broadly, the contribution of sex chromosome complement versus gonadal environment to germ cell identity could be dissected using sex chromosomally aneuploid mouse models. This approach may reveal gene expression signatures shared between XX male and XX female germ cells, or between XY male and XO female germ cells.

8. Conclusions

Though the existence of XCU was originally contested, it has now gained acceptance as a biological process intrinsic to somatic X dosage compensation. During gametogenesis, XCU, like XCI, is erased, and this creates sex differences in X dosage compensation states that may promote sexual dimorphisms in germ line development, and shed light on sex chromosome-related infertility. A priority now is to track when following fertilization X dosage compensation mechanisms are reinstated. The ontogeny of XCI during embryogenesis shows striking differences between eutherians. In mice, XCI occurs rapidly, from the four cell stage,[81] in rabbits it occurs from the early blastocyst stage,[82] and in humans it occurs even later.[52,83] In mice, XCU has been observed using RNA microarrays from the zygote stage[48] and single cell RNA-seq from the four cell stage.[51,84] Thus XCU and XCI are temporally linked, suggesting a requirement for tight regulation of X dosage from very early in development in this species. However, relationships between XCI and XCU in other eutherians have not been examined. More strikingly, X dosage compensation states during gametogenesis and embryogenesis have never been investigated in a metatherian species. The analysis of diverse model systems will prove essential in defining broad principles that regulate X dosage compensation in mammals.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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