REVIEW

# Epigenetic regulation of mammalian sex determination

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**Abstract : Epigenetics is the study of changes in gene function that cannot be explained by changes in DNA sequence. A mammalian body contains more than two-hundred different types of cells, all derived from a single fertilized egg. Epigenetic gene regulation mechanisms essentially contribute to various processes of mammalian development. The essence of epigenetic regulation is the modulation of gene activity through changes in chromatin structure. DNA methylation and histone modifications are the major epigenetic mechanisms. Sex determination is the process of establishing a gender. *Sry*, the sex-determining gene in therian mammals, initiates testis differentiation. Recent studies have provided evidence that epigenetic mechanisms contribute to *Sry* regulation.**
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## 1. INTRODUCTION

The regulation of gene expression without changes in the DNA sequence is governed by epigenetic mechanisms. Epigenetic mechanisms contribute to numerous biological processes, not only in higher eukaryotes but also in single cell eukaryotes. For example, epigenetic mechanisms control mating type silencing in yeast, temperature-dependent vernalization in plants, position-effect variegation in insects, and germline imprinting and X-chromosome inactivation in mammals. DNA methylation, histone modification, non-coding RNA and chromatin remodeling are the major players in epigenetic regulation (1). Among them, DNA methylation and histone modification have been most extensively studied.

DNA methyltransferases (DNMTs) catalyze methylation at the 5' carbon of a cytosine (5mC) next to a guanidine (CpG). Mammals have two types of DNMTs. DNMT1 acts as a maintenance methyltransferase that catalyzes the methylation of hemimethylated DNA sequences, while DNMT3 acts as a *de novo* DNA methyltransferase that catalyzes the methylation of unmethylated DNA sequences (2). Recently, the ten-eleven translocation 1-3 (Tet1-3) proteins have been found to possess DNA hydroxylase activity toward 5mC. The Tet1-3 proteins can convert 5mC into 5-hydroxymethyl cytosine (5hmC), which is considered to be an intermediate in the process of active DNA demethylation (3).

The nucleosome is the fundamental unit of chromatin, and consists of 147 base pairs (bp) of DNA wrapped around a core histone octamer (two each of H3, H4, H2A and H2B) (4). The tail regions of the core histones are susceptible to a variety of covalent modifications, including acetylation, phosphorylation, methylation, and ubiquitination (5). These modifications can be reversed by the corresponding deacetylase, phosphatase, demethylase and deubiquitinase. Distinct combinational sets of histone modifications are considered to regulate unique biological outcomes. This concept is referred to as the "histone code hypothesis" (6).

The structure of the epigenome can be modulated by environmental changes. For example, vernalization in flowering plants requires the methylation of specific histone arginine and lysine residues (7). In animals, the nutrition status during development can lead to locus-specific changes in the epigenome. For instance, methyl donor supplementation of pregnant female mice induces the CpG hypermethylation of a specific allele in their offspring (8). On the other hand, the activities of chromatin modification enzymes are dependent on high-energy metabolites as cosubstrates. DNMTs use *S*-adenosyl methionine as the methyl donor. The kinases, acetyltransferases and methyltransferases acting on histones require ATP, acetyl-CoA and *S*-adenosyl methionine as the phosphoryl, acetyl and methyl donors, respectively. Chromatin modification enzymes sensitively monitor environmental and metabolic events, and thus function as sensors of changes in these conditions (9).

In mammals, a single fertilized egg differentiates into more than two-hundred different types of cells during development. Epigenetic mechanisms essentially contribute to this process, by regulating gene expression in spatial and temporal manners. Sex determination is the genetic or environmental process by which the gender (male or female) of an individual is established, in a simple binary fate decision. A gene called sex determining region Y chromosome (*Sry*) was identified as a candidate for a mammalian sex determining gene (10). The introduction of a genomic fragment containing *Sry* generated male mice, although they were chromosomally female mice, indicating that *Sry* is necessary and sufficient for testis induction (11). The SRY protein is the founding member of the SOX (SRY-related HMG box) family of transcription factors. *Sry* expression is restricted to a subset of gonadal somatic cells from embryonic day (E) 10.5 to E12.5 in mice. This spatial and temporal *Sry* regulation is critical for testis differentiation (12, 13). In this review, I will particularly focus on the epigenetic regulation of *Sry* expression.

## 2. ROLE OF DNA METHYLATION IN MAMMALIAN SEX DETERMINATION

*1) DNA methylation profiles of the Sry promoter in developing mice embryos*

Sixteen CpG sites exist in the 4.5-kb 5'-flanking region of the mouse *Sry* locus (14). A sodium bisulfite sequencing analysis revealed that the CpG sequences of the *Sry* promoter region were hypermethylated in E8.5 embryos, in which *Sry* was not yet

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expressed. However, this region became hypomethylated specifically in the XY gonad at E11.5, while it was still hypermethylated in the other tissues where *Sry* was not expressed (14). Thus, an inverse relationship exists between the *Sry* expression levels and the extent of DNA methylation. Hypomethylation of promoter region DNA is generally associated with actively transcribed genes. The cause and effect relationship between the expression of *Sry* and the hypomethylation of its promoter region is currently unclear, and deserves further study.

*2) Do Gaadd45 family proteins contribute to the DNA demethylation of the Sry locus?*

The GADD45A, B, G proteins are a family of stress-response proteins. GADD45 mediates diverse cellular processes, such as DNA repair, apoptosis, cell cycle arrest and senescence (15, 16). The GADD45 proteins also function in gene activation, by promoting DNA demethylation and MAPK signaling. The Gadd45 proteins are considered to recruit DNA repair proteins to specific loci, in order to initiate DNA demethylation (17).

*Gadd45g*-mutant mice display complete male-to-female sex reversal. *Sry* expression is reduced in the undifferentiated gonads of *Gadd45g*-mutant embryos, suggesting that GADD45G positively regulates *Sry* expression (18, 19). Unexpectedly, a bisulfite sequencing analysis revealed that the CpG sequences within the *Sry* promoter region of undifferentiated gonadal somatic cells were still hypomethylated in the *Gadd45g*-mutant embryos. These facts suggested that GADD44G activates *Sry* expression in a different manner than by the CpG demethylation of *Sry*. Alternatively, GADD45 activates *Sry* in a different manner other than CpG demethylation of *Sry*. GADD45G binds and activates MAP3K4. The activated MAP3K4 sequentially activates p38MAPK, resulting in the direct or indirect activation of GATA4, which is implicated in the regulation of *Sry* expression (Figure 1) (20).

## 3. ROLE OF HISTONE METHYLATION IN MAMMALIAN SEX DETERMINATION

Histone methylation was previously considered to be an irreversible modification that could only be removed by histone exchange or dilution during replication. The identification of the Lysine-specific demethylase 1 (LSD1) and Jumonji C (JMJC) histone demethylase enzyme families resulted in a quite different viewpoint of the regulation of histone methylation (21, 22).

The methylation of histone H3 lysine 9 (H3K9) is a hallmark for transcriptionally silenced heterochromatin, and is conserved from fission yeast to mammals (23, 24). JMJD1A (also called TSGA/JHDM2A/KDM3A), an enzyme that demethylates H3K9, plays an important role in gene activation in spermiogenesis and metabolism (25-28). Recently, Kuroki *et al.* reported that XY mice deficient in JMJD1A exhibit male-to-female sex reversal. The development of external and internal genitalia in XY *Jmjd1a*-mutant mice was variable. Approximately 20 % of the XY *Jmjd1a*-mutant mice had male external genitalia, and the others had ambiguous or female external genitalia (Figure 2). *Sry* expression is perturbed in *Jmjd1a*-mutant XY gonads at E11.5 (29). Three different approaches were employed to determine the critical step(s) in the testis-developing pathway controlled by JMJD1A. First, a microarray analysis revealed that the expression levels of the known positive regulators of *Sry* were not compromised by the *Jmjd1a* mutation. Second, a rescue of the sex-reversal phenotype was attempted by experimentally restoring *Sry* function, by crossing the *Hsp-Sry* transgenic mouse line (30) into the *Jmjd1a*-deficient background. Consequently, the forced expression of the *Hsp-Sry* transgene rescued the sex-reversal phenotype of the *Jmjd1a*-deficient mice. Finally, a chromatin immunoprecipitation analysis revealed that JMJD1A accumulates on the *Sry* locus in undifferentiated XY gonads, and mediates its H3K9 demethylation. Taken together, these results revealed that JMJD1A specifically contributes to the *Sry* activation

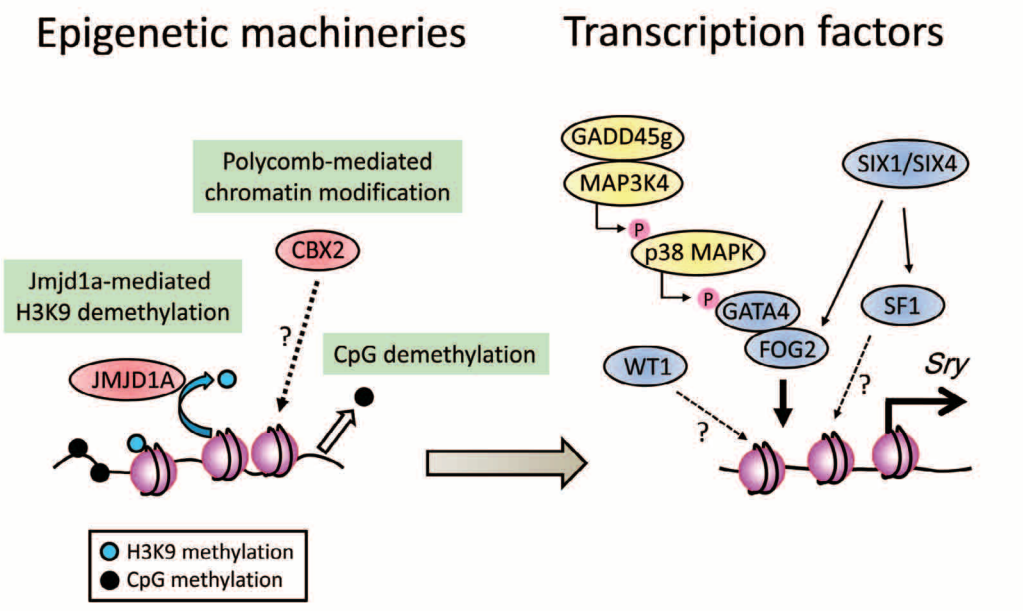


Figure 1. Epigenetic machineries and transcription factors involved in Sry regulation in mice
*Sry* expression is defined through the cross-talk between its epigenetic status (left) and the functions of specific transcription factors (right). JMJD1A erases H3K9 methylation, which is a hallmark of transcriptionally suppressed chromatin (29). DNA demethylation may be one of the epigenetic mechanisms responsible for *Sry* expression (14). CBX2, a subunit of Polycomb-repressive complex 1, is involved in *Sry* regulation, although the molecular mechanism remains to be determined (33). GADD45G activates the GATA4 transcription factor via the activation of p38 MAPK (18, 19). Recently, it was demonstrated that the SIX1/SIX4 transcription factors control *Sry* expression, by positively regulating *Fog2* and *Ad4BP/Sf1* expression (46). The roles of the other transcription factors in *Sry* expression have been discussed in another review (47).

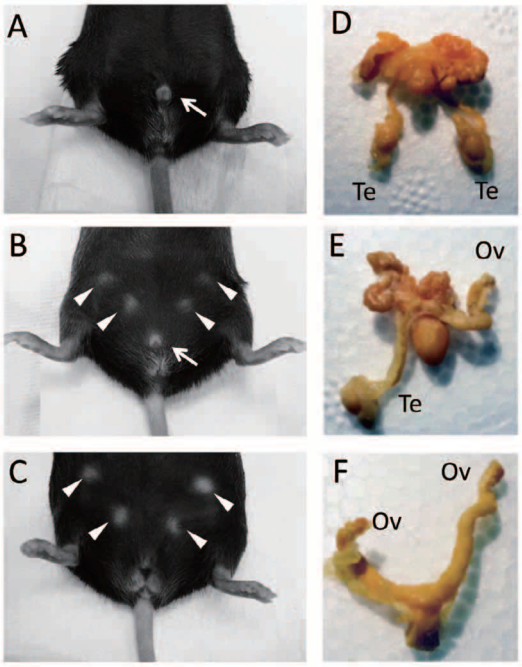


Figure 2. Genitalia development in XY *Jmjd1a*-mutant adult mice
External and internal genitalia of 3-month old *Jmjd1a*-mutant mice are shown on the left and right, respectively. Among 58 XY *Jmjd1a*-mutant animals examined, 11 have male external genitalia (A), 14 have ambiguous external genitalia (B) and 33 have female external genitalia (C). The development of internal genitalia in XY *Jmjd1a*-mutant mice was also variable. Among 58 XY *Jmjd1a*-mutant animals examined, 7 have two testes (D), 12 have a testis and an ovary (E) and 34 have two ovaries (F). The arrows and the arrowheads represent the penis and the mammary gland, respectively. Te, testis ; Ov, ovary.

step during the testis-development pathway, by directly catalyzing H3K9 demethylation (Figure 1) (29).

## 4. ROLE OF POLYCOMB GROUP PROTEINS IN MAMMALIAN SEX DETERMINATION

The polycomb group (PcG) proteins were identified as molecules required for maintaining the repressed state of homeotic genes in *Drosophila* (31). The functions of the PcG proteins are highly conserved, from *Drosophila* to mammals. In vertebrates, the PcG proteins assemble into two distinct complexes, polycomb-repressive complex 1 (PRC1) and polycomb-repressive complex 2 (PRC2). The PRC complexes at least partially exert their functions through chromatin modification, because both of the PRC complexes possess histone modification activities. PRC1 and PRC2 catalyze the ubiquitination of H2AK118 and the methylation of H3K27, respectively (32).

Chromobox homolog 2 (CBX2) (also referred to as M33) is one of the four core subunits of PRC1. *Cbx2*-mutant mice suffer from multiple defects, such as anterior vertebral shifting (33) and adrenal and spleen hypoplasia (34). In addition, the gonads of both sexes were hypoplastic, and the XY gonad displayed male-to-female sex reversal (33). Microarray analyses revealed that the expression levels of not only *Sry* and *Sox9*, but also the genes encoding transcription factors essential for gonadal development, such as *Lhx9*, *SF-1 (also called Ad4BP)*, *Dax-1*, *Gata4*, *Arx*, and *Dmrt1*, are affected in *Cbx2*-mutant gonads (35). Considering the fact that *Gata4* is required for *Sry* expression (20), CBX2 may regulate *Sry* expression indirectly, by positively regulating *Gata4* expression. In embryonic stem (ES) cells, the genes required for gonadal development, such as *Lhx9*, *SF-1*, and *Gata4*, are negatively regulated by PRC complexes. Chromatin immunoprecipitation analyses indicated that these genes were the direct targets of both PRC1 and PRC2 in ES cells (36, 37). Interestingly, these genes were positively regulated by CBX2 in developing gonads (35). Further studies will be required to understand the molecular mechanisms underlying the contributions of CBX2 and the CBX2-containing PRC1 complex to the activation of these genes in gonadal cells.

## 5. PERSPECTIVES

Two major types of sex-determination exist in the animal kingdom : genotypic sex determination (GSD) and environmental sex determination (ESD). In the latter case, the sex is determined after fertilization, depending on environmental cues. Temperature-dependent sex determination (TSD) is a form of ESD observed in some fish and reptiles (38). An indispensable role of epigenetic mechanism for ESD has been demonstrated recently. The European sea bass employs a unique polygenic system of sex determination, in which genetics and temperature contribute equally to sexual fate (39). In this species, exposure to high temperature during a certain larval period increased the DNA methylation of the *Cyp19a1* gene, encoding an aromatase that converts androgens into estrogens. The acquisition of DNA hypermethylation at this locus resulted in the induction of masculinization, even in chromosomally female fish (40). These findings constitute the first molecular examination of an epigenetic mechanism that mediates the effects of temperature on sex ratios in vertebrates.

Dysregulation of the epigenetic machineries is associated with several human diseases (41, 42). Disorders of sex development (DSDs) are congenital conditions, in which chromosomal, gonadal, and/or anatomical sex is atypical (43). Mammals employ GSD, where sex is determined at conception due to the genetic differences of zygotes. However, it seems likely that the epigenetic machineries also play important roles in the regulation of sex-determining genes in mammals, as reviewed in this article. In addition, more than half of the human DSDs cannot be explained by alterations in the characterized genes required for sex determination and gonadogenesis (44). Collectively, alterations of the epigenetic machineries and/or epigenetic states may be responsible for the onset of DSDs. Accordingly, the CBX2 gene mutation was found in a human exhibiting a male-to-female sex reversal phenotype (45). Given the accumulating studies in the epigenetics research area, many new insights will emerge to reinforce the links between epigenetic mechanisms, sex determination, and gonadogenesis.

## DISCLOSURE OF CONFLICT OF INTEREST

The author declares no conflict of interest.

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