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X-Chromosome Inactivation: A Complex Circuits regulated by Non-coding RNAs and Pluripotent Factors

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ABSTRACT

X-chromosome inactivation is one of the most complex events observed in early embryo developments. The epigenetic changes occurred in female X-chromosome is essential to compensate dosages of X-linked genes between males and females. Because of the relevance of the epigenetic process to the normal embryo developments and stem cell studies, X-chromosome inactivation has been focused intensively for last 10 years. Initiation and regulation of the process is managed by diverse factors. Especially, proteins and non-coding RNAs encoded in X-chromosome inactivation center, and a couple of transcription factors have been reported to regulate the event. In this review, we introduce the reported factors, and how they regulate epigenetic inactivation of X-chromosomes.

(Key words : X-chromosome inactivation, X-chromosome inactivation center, Pluripotent factors)

INTRODUCTION

Dosage Compensation is an essential process to balance the expression levels of genes in sex chromosomes between males and females (Graves, 2015). There are three typical mechanisms to equalize dosages of genes located in sex chromosome. Genes in one X-chromosome transcribed two-fold high in male flies (Park and Kuroda, 2001) and both X-chromosomes are partially inactivated in worms (Meyer, 2000). Contrary to the non-vertebrates species, placental mammals apply inactivation of one X-chromosome in females and balancing the dosage of X-linked genes between males and females (Finestra and Gribnau, 2017). The epigenetic process to equalize the expression levels of X-linked genes between male and female eutherians is called X-chromosome inactivation (XCI). After the first suggestion of the event by observing condensed chromatin structure in nucleus called 'barr-body' (Lyon, 1961), numerous studies have been performed and helped to understand the event comprehensively. Fifty years on, the mechanisms of XCI in developing embryos has been studied and various factors have been reported to be associated with XCI.

Although the female-specific epigenetic change in X-chromosome has been suggested over 50 years ago, its relation to normal embryo development and stem cell biology has been highlighted strongly recent 10 years. Unsuccessful development of embryos which are generated *in vitro* is accompanied with unbalanced expression of X-linked genes (Inoue *et al*., 2010; Park *et al*., 2012). Increased numbers of X-linked genes are abnormally silenced or down-regulated chromosome-widely in the preimplantation embryos. The limited developmental competence could be rescued by preventing ectopic inactivation of X-chromosome (Matoba *et al*., 2011). The process is occurred during short developmental period, for example, from morular to epiblast stage in mice, with strict regulation of genetic networking. The embryonic stage-dependent characters of XCI are good indicators to evaluate status of embryonic stem (ES) cells (Brons *et al*., 2007; Nichols and Smith, 2009). The association of XCI with stem cells and embryo developments has increased the importance of understanding the epigenetic event.

The effort to find master gene inducing XCI revealed that certain genomic region in X-chromosome manages silencing of X-chromosome. The genomic region was named to XIC, X-chromosome inactivation center (Rastan, 1983). Ten years later from the identification of the XIC, one non-coding RNA (ncRNA) encoded in the genomic region was defined to initiate XCI as a master gene, named to *XIST*, X-chromosome inactivation spe-

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cific transcript (Brockdorff *et al*., 1992; Brown *et al*., 1992). Including the *XIST*, other ncRNAs and proteins encoded in XIC has been reported to be associated with initiation of XCI. And, in 2008, one study suggested that pluripotent factors delay the initiation of XCI (Navarro *et al*., 2008). This study firstly demonstrates the relevance between XCI and pluripotency in developing embryos and ES cells.

Even though numerous factors have been reported to regulating XCI, the detailed procedures of XCI in developing embryos and mechanisms how chromatin status is changed during the XCI are still unclear. Therefore, in this review, we introduce the regulators which are involved in XCI, and elucidate the molecular circuit of the epigenetic events.

X-CHROMSOME INACTIVATION CENTER

XIC harbor multiple regulators regulating XCI. Comparative genomics revealed that the XIC is conserved in vertebrates (Duret *et al*., 2006). This 0.5 to 1 Mb length of synteny encodes genes with fixed order in the vertebrates (Fig. 1). However, eutherian XICs have unique genomic region compared with those in non-eutherian vertebrates. A few ncRNAs are encoded only in the eutherian XICs (Chureau *et al*., 2002; Duret *et al*., 2006). Although eutherian XIC is conserved in diverse taxa, the genomic region is considered to be translocated from autosome to sex chromosome, X during the evolution. In non-eutherian vertebrates, the counterpart region of eutherian XIC are placed in autosome, and the region is settled down in X chromosomes together

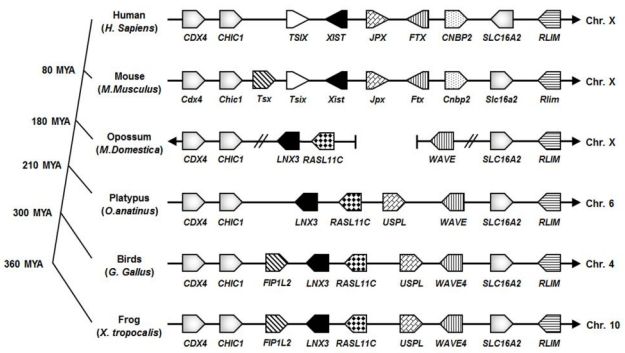

Fig. 1. Evolution of X-chromosome inactivation center in vertebrates. Arrows and triangles indicate protein coding and non- coding RNA (ncRNA) genes, respectively. The components with same patterns in each XIC and XIC-homologue region are orthologs or partially homologue. The diagram and pyrogenic tree is not scaled.

with appearance of ancestral mammals (Duret *et al*., 2006; Hore *et al*., 2007). Interestingly, the translocated region has been disrupted, and assembled again during evolution of therians (Grützner *et al*., 2004; Hore *et al*., 2007). In this period, certain protein-coding genes which are conserved in non-eutherian vertebrates were disappeared and finally ncRNA genes have been evolved in eutherians. The evolutionary history suggests that the ncRNAs in XIC regulates XCI which is a eutherian-specific event. Therefore, after the first identification of *XIST*, the relevance of neighboring genes to XCI has been investigated, and individual regulators will be elucidated.

XIST

The ncRNA was identified first in human by female-specific hybridization of a human cDNA probe and confirming the probe is located in XIC (Brown *et al*., 1992; Brown and Lafreniere, 1991). After identification of the orthologs in various species (Brockdorff *et al*., 1992; Chureau *et al*., 2002; Hwang *et al*., 2013), comparative genomic analysis revealed that this gene has been originated from *LNX3* by its psedugenization (Duret *et al*., 2006; Elisaphenko *et al*., 2008). *XIST* orthologues express 15 to 30 kb lengths of transcript from the conserved genomic structure composed with long first and last exons and multiple intermediate exons. The ncRNA is the major factor to initiate XCI in eutherians. Differentiating *Xist*-null ES cells were failed to inactivate X-chromosomes (Penny *et al*., 1996). And also, embryos with mutated paternal *Xist* allele failed to develop extraembryonic tissues because of abnormal imprinted XCI (Kalantry *et al*., 2009; Marahrens *et al*., 1997). These results clearly demonstrate that *XIST* is major factors inducing XCI. This gene consists of several repeat sequence regions (repeat A to F) and one of them, repeat A has been reported to essential compartment to silence the X-chromosome (Wutz *et al*., 2002). Although numbers of the repeats are different, the monomers which are 24 bp lengths with double hairpin structure are conserved among the species (Hwang *et al*., 2013). Interestingly, this region expresses short transcript independent to *Xist* (Rep A), and this short transcript is reported to recruit PRC2, which induce repressed chromatin status by modifying histone (Zhao *et al*., 2008). Another repeat region, repeat C, which is dominantly present in mouse *Xist* (14 copies) but less popular in human *XIST* (1 copy) is reported to be associated with localization of *Xist* to future inactive X-chromosome (Sarma *et al*., 2010). This biochemical features also support the importance of this ncRNAs in XCI.

TSIX

One study observed unexpected XCI in XO individual of which *Xist* 3′ region was deleted (Clerc and Avner, 1998). They suggested the 3′ region of *Xist* would have roles to prevent XCI. From the hypothesis, an anti-sense transcript to *Xist*, *Tsix*, has been identified (Lee *et al*., 1999). The transcript which is expressed from *Xist* antisense strand is transcribed on active X-chromosome preferentially and diminished from the future inactive X-chromosome in differentiating mouse ES cells. Some studies revealed that this gene is related to the imprinted and random XCI in mouse (Lee, 2005; Lee and Lu, 1999; Sado *et al*., 2001). Embryos with mutated maternal *Tsix* are lethal because extraembryonic lineages failed to be formed with unexpected inactivation of the maternal X-chromosome. And *Tsix*-mutated X-chromosomes are preferential inactivated, and *Tsix* deficiency induces drastic elevation of *Xist* expression in mouse ES cells. These results support that *Tsix* regulates *Xist* expression accurately to choose the future inactive X-chromosome in mouse XCI. Several studies suggested that the repression of *Xist* through *Tsix*. One report suggested that *Tsix* transcript prevent the recruitment of the *RepA* transcript, which is connected with PRC2 protein, to the *Xist* promoter region, preventing *Xist* expression (Zhao *et al*., 2008). Other reports suggested that *Tsix* transcripts methylate *Xist* promoter by recruiting DNMT3a (Navarro *et al*., 2006), or modifying the chromatin structure of 5′ region of *Xist* (Sado *et al*., 2005; Sun *et al*., 2006). However, compared to the mice, which are required the accurate regulation of monoalleleic *Xist* expression in developmental phase, other species like human and rabbit showed less coordinated monoalleleic expression of *XIST* in their embryonic development because both species didn't showed tightly regulated monoallelic *XIST* expression in developing embryos (Okamoto *et al*., 2011). Although the candidate *Tsix* was suggested in human (Migeon *et al*., 2001), the conserved functions and sequences are unclear in other eutherians (Chureau *et al*., 2002; Escamilla-Del-Arenal *et al*., 2011; Migeon *et al*., 2002). Therefore, conserved function and orthologs of *Tsix* in other eutherians are needed to be examined further.

JPX

This gene, which is also called Enox, is encoded in 10 Kb upstream region from 5′ region of *Xist* (Johnston *et al*., 2002). This gene is considered to be originated from the ancestral protein coding gene, *USP1*, and the first exon of the *USP1* showed sequence homology with promoter region of *JPX* gene in human. Most compartment of the ncRNA is mobile element and the sequences of the orthologs are variable among the species (Hwang *et al*., 2015). The results support that this gene is evolved through species-specific manner like *XIST* orthologs (Chureau *et al*., 2002). A few studies demonstrated that the gene is positive regulator of *Xist* expression in mice. *Jpx* gene expresses highly in the differentiating male and female ES cells. This means that the gene is XCI-escaping gene and its expression is not affected by the *Xist*. Although deletion of the gene did not influence on the male ES cells differentiation, in female, it led to cell death and *Xist* down-regulation. Exogenous *Jpx* expression in heterozygotic female ES cells showed rescued XCI rate and normal growth of the differentiating ES cells (Tian *et al*., 2010). The gene is considered to work with dose-dependent manner to induce XCI. The study also suggested that *Jpx* transcript would compete with CTCF, which is *Xist* repressor by binding to the promoter region. Consistently, it is confirmed that CTCF could bind *Jpx* transcript (Sun *et al*., 2013). Therefore, *Jpx* is a *Xist*-activator and its functions would be associated with CTCF closely. However, as only a few studies confirmed the detailed roles of *Jpx* in mouse and the sequences of the orthologs are less conserved, it is required that the functions of the gene are examined in various species.

FTX

This ncRNA is posited 5′ to *Xist,* and considered to be evolved from ancestral protein coding gene, *WAVE4* (Elisaphenko *et al*., 2008). The five exons at the 5′ region of the gene are conserved between human and mouse orthologues (Chureau *et al*., 2002). Interestingly, microRNAs, *miR374a* and *miR545*, are present within the exons of *FTX*, and this genomic structure is conserved among the eutherians. The function of the gene is firstly suggested by characterizing *Ftx*-null mouse ES cells (Chureau *et al*., 2010). *Ftx* expression influences on the neighboring genes which are transcribed with same direction to *Ftx,* like *Xist*, but not in the genes with opposite direction. And *Ftx* deficiency increased DNA methylation and reduced H3K4Me2 binding to *Xist* promoter. Therefore, *Ftx* would be positive regulator for *Xist* expression by modulating status of chromatin in close distance. A study observed that deletion of *Ftx* didn't affect to the imprinted XCI in preimplantation embryos (Soma *et al*., 2014). However, its roles in late stage of embryo development are not confirmed and only a few studies suggest the possible roles of the gene. Accordingly, exact roles of this ncRNA in imprinted or random XCI are required to be confirmed.

RLIM

RLIM is E3-ubiquitin ligase regulating LIM-homeodomain containing factors like LDB1. This gene is high-

ly conserved protein coding gene and not included in classical XIC suggested in its first identification (Rastan, 1983). Recent studies have demonstrated that this gene is another positive inducer of XCI by activating *Xist* expression (Barakat *et al*., 2011; Barakat *et al*., 2014; Jonkers *et al*., 2009; Shin *et al*., 2010). Transduction of exogenous BAC clone including this gene induces ectopic *Xist* expression in differentiating male ES cells (Jonkers *et al*., 2009). Function of the gene is conserved between mouse and human, and XCI was reduced in the differentiating *Rlim* heterozyote ES cells. These results demonstrate that Rlim regulates *Xist* expression dose-dependently. Subsequent study revealed that this gene directly activates *Xist* and XCI by confirming that Rlim bind to *Xist* promoter (Barakat *et al*., 2011). And reduced XCI in differentiating *Rlim*-heterozygous ES cells was rescued by supplemented exogenous *Rlim*. The result also demonstrates that Rlim is a trans-activator of *Xist* expression. The studies carried out using ES cells address that Rlim is involved in random XCI which is occurred in differentiation of inner cell mass (ICM) in blastocysts. However, other two studies showed differential results from the studies by investigating the function of Rlim in developing embryos (Shin *et al*., 2010; Shin *et al*., 2014). Shin and his colleagues observed that *Rlim*-null preimplantation embryos failed to induce imprinted XCI and the fetus failed to form extraembryonic tissues (Shin *et al*., 2010). The ES cells without Rlim succeeded in initiation of XCI. Add to the results, post-implantation *Rlim*-null embryos showed detectable *Xist* cloud and heterochomatin marker, H3K27me3, which are representing inactive X-chromosome marker (Shin *et al*., 2014). And also Rlim-deficient embryos and ES cells which were assessed to tetraploid complementation showed embryogenic potential. These results demonstrate that Rlim is less related to random XCI and this is opposite to previous studies (Barakat *et al*., 2011; Barakat *et al*., 2014; Jonkers *et al*., 2009). Therefore, more studies are conducted to define the exact function of Rlim in XCI.

PLURIPOTENT FACTOR

Pluripotent factors are required to maintain the pluripotency of ES cells and embryonic cells. After the first identification of pluripotent factors, OCT4, which is gate-keeper for maintaining pluripotency in ES cells and ICM, (Nichols *et al*., 1998; Niwa *et al*., 2000), various factors were reported to regulate pluripotency and reprogramming. Most representatively, deriving the pluripotent stem cell through reprogramming the differentiated cells using exogenous pluripotent factors (Takahashi and Yamanaka, 2006) demonstrates the genes are closely related to the specific cell status showing pluripotency like ICM and ES cells. As XCI is normally initiated during the differentiation of ES-cells and ICM of embryos, pluripotent factors and XCI regulators in XIC are normally share the time period for their function. The simultaneous dynamics of both groups of factors have made expect that pluripotent factors are related to XCI closely (Postlmayr and Wutz, 2017). After the first report highlighting relation of pluripotent factors to XCI (Navarro *et al*., 2008), the factors have been generally suggested to delay XCI in ES cells or embryos by repressing *Xist* expression and/or activating *Tsix* expression. And the suggestions were supported by the study that reprogramming somatic cells to pluripotent cells resulted in the reactivation of inactive X-chromosomes (Ohhata and Wutz, 2013). These tight linkages between *Xist* expression/XCI and pluripotency have suggested that pluripotent factors would participate in the XCI deeply.

OCT4, SOX2, and NANOG

The functions of these three factors which are well-known to maintain pluripotency in ES cells (Chambers *et al*., 2003; Masui *et al*., 2007; Nichols *et al*., 1998) in *Xist* expression were investigated first in 2008 (Navarro *et al*., 2008). Binding of the factors on the first intron of *Xist* was observed in undifferentiated ES cells but they were detached when the cells are differentiated. Interestingly, *Tsix* and *Xist* showed mutually opposite expression patterns during differentiation, but the timing for expression level changes of *Xist* were more sensitive rather than that of *Tsix*. Therefore, this three pluripotent factor have been suggested as negative regulators for *Xist* expression. Another study revealed that Oct4 and Sox2 also regulate *Tsix* expression by recruiting and forming complex with Ctcf and Yy1 (Donohoe *et al*., 2009) which are *trans*-regulating factors of XCI (Chao *et al*., 2002; Donohoe *et al*., 2007). Consensus sequences of Oct4 and Sox2 binding sites were observed in close to the Ctcf and Yy1 binding site in *Tsix* promoter and *Xite* which is an enhancer for *Tsix* expression (Lee, 2005). Knockdown of Oct4 and Ctcf showed drastic effect on the pairing of X-chromosome which is observed prior to one of the X-chromosome inactivation, and aberrant expression of *Xist* in female ES cells. Therefore, the results demonstrate Oct4 enhances *Tsix* expression. One study suggested indirect regulation of *Xist* and *Tsix* expression by pluripotent factors via down regulation of Rlim (Navarro *et al*., 2011) which is a *tran*-activator of *Xist* expression. However, because of low binding levels of Oct4 and Sox2 to *Xite* which is a weak enhancer for *Tsix* expression compared to the DXPas34 (Ogawa and Lee, 2003; Vigneau

et al., 2006), the functions of these pluripotent genes in *Tsix* expression were considered to be mild. And it is also possible that other factors would be associated with the *Tsix* additionally. A few studies suggested that deleting the first intron of *Xist* which would be a target region of pluripotent factors, do not influence on the inactivation and reactivation of X-chromosome (Barakat *et al*., 2011; Minkovsky *et al*., 2013). So, it is controversial whether these pluripotent factors are essential regulators for XCI repression. Although there are uncertainties about how the factors affect the *Xist* and *Tsix* expressions, previous studies in ES cells show that these factors are XCI-repressors.

REX1, MYC, and KLF4

Although one report addressed that Oct4 is top regulator of *Tsix* expression (Donohoe *et al*., 2009), other factors could be associated with *Tsix* expression additionally considering Oct4 repression influences on *Tsix* expression mildly. Therefore, relations between expression of *Tsix* expression and other pluripotent factors, such as Myc, Klf4, and Rex1 was analyzed (Navarro *et al*., 2010). The three factors bound to the 3′ region of *DXPas34* gene (Rex1) or between *DXPas34* and *Tsix* promoter (Myc and Klf4). Among them, displacement of the Rex1 from *Tsix* regulatory sites in trophoblasts showed reduced *Tsix* expression and repression. These result demonstrated that Rex1 have important roles in *Tsix* expression. In addition to the function into the *Tsix* expression, Rex1 binds to *Xist* promoter when the ligase function of the Rlim was prevented. Rex1 overexpression reduced XCI initiation in differentiating female ES cells. This means that Rex1 could be a negative regulator for XCI by repressing *Xist* expression (Gontan *et al*., 2012). Therefore, the factor would have dual functions, repressing or activating *Xist* or *Tsix*, respectively.

PRDM14

Prdm14, which is one of the subfamily genes of tandem zinc fingers and RB domain containing transcription factor, was reported to be an important regulator for XCI (Ma *et al*., 2011; Payer *et al*., 2013). This gene expressed in compacted morula, ICM, early epiblast cells, and primordial germ cells (Kurimoto *et al*., 2008; Yamaji *et al*., 2008; Yamaji *et al*., 2013) and interferences of the transcript resulted in differentiation of ES cells into extraembryonic endodermal cells (Ma *et al*., 2011). Elevation of *Xist* expression was observed when Prdm14 expression was down-regulated in ES cells but other report revealed that the *Xist* expression is not changed in ES cells by *Prdm14* deficiency (Payer *et al*., 2013). Interestingly, nevertheless the opposite results within two studies, it is considered that this factor would repress XCI. The first intron of *Xist* was target site of Prdm14 as Oct4, Sox2, and Nanog (Navarro *et al*., 2008), and the region is close to the Nanog-binding site (Ma *et al*., 2011). And promoter region of Rlim was also suggested to the target region for Prdm14 by coordination with PRC2 and its subunit, SUZ12 (Payer *et al*., 2013). And, *Prdm14*-null ES cells showed increased expression levels of Rlim compared to the wild-type female ES cells, and these results suggests that Prdm14 could repress *Xist* expression via binding to *Xist* intron or silencing *Xist* activator, Rlim. However, *Tsix*, the opposite regulator of *Xist*, was not considered to the target for Prdm14. Deletion of the gene didn't affect to the *Tsix* expression and Prdm14 was not bound to *Tsix* locus. Oppositely, *Tsix* would have effect on the localization of Prdm14 to *Xist* intron 1. These results show that Prmd14 act as a negative regulator for XCI by association with *Xist* expression rather than expression of *Tsix*.

CONCLUSION

XCI is an essential procedure for normal embryo development in eutherians. Although this phenomenon has been reported first about 50 years ago, the detailed molecular circuits are still unclear. Numerous factors are involved in XCI with accurate and complex lation each other. Two groups of factors, ncRNAs in reguXIC and pluripotent factors, could be grouped into the pos-

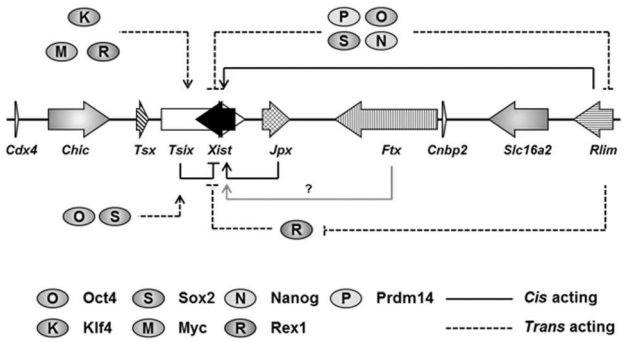

Fig. 2. Molecular networking of XCI-regulators, XIC-linked genes and pluripotent factors, in mouse XCI. *Cis* (solid lines) and *trans* (dashed lines) actions of XCI-regulators are depicted. Pluripotent factors generally act as a XCI repressors by down-regulating *Xist* expression (Oct4, Sox2, Nanog, Rex1, and Prdm14) or up-regulating *Tsix* (Oct4, Sox2, Klf4, Myc, and Rex1) expressions. XIC-linked genes are generally *cis* regulators for *XIST* expression. Rlim works as *cis* activator for *Xist* expression and also, *trans* repressor targeting Rex1 by ligating ubiquitin to the protein.

itive and negative regulators for XCI, respectively (Fig. 2). Overall process to activate or repress XCI could be linked to activation/repression of *Xist* and *Tsix*. The balanced expression of two ncRNAs could maintain the active status of X-chromosome. However, although associations of individual molecules with XCI have been reported, their interdependent networking is still unclear. And as ncRNAs are appeared recently during mammalian evolution, XCI could be severely variable among the eutherians (Okamoto *et al*., 2011). Therefore, relation between XCI regulators in diverse species should be studied further to understand this epigenetic event comprehensively including evolutionary aspect.

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