Diverse developmental strategies of X chromosome dosage compensation in eutherian mammals

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ABSTRACT In eutherian mammals, dosage compensation arose to balance X-linked gene expression between sexes and relatively to autosomal gene expression in the evolution of sex chromosomes. Dosage compensation occurs in early mammalian development and comprises X chromosome upregulation and inactivation that are tightly coordinated epigenetic processes. Despite a uniform principle of dosage compensation, mechanisms of X chromosome inactivation and upregulation demonstrate a significant variability depending on sex, developmental stage, cell type, individual, and mammalian species. The review focuses on relationships between X chromosome inactivation and upregulation in mammalian early development.

KEY WORDS: dosage compensation, mammal, X chromosome upregulation, X chromosome inactivation, Xist

Mammalian dosage compensation system

Early embryonic development of eutherian mammals is closely linked with dosage compensation of X chromosome genes. Mammalian X and Y chromosomes differ significantly in gene content and play a key role in sex determination. Y chromosome determines male development. It contains less than 100 genes and is a depleted variant of ancestral X chromosome. Conversely, X chromosome increased gene number during evolution by autosomal translocations and comprises more than 1100 genes (Deng et al., 2014). In most mammals, males have X and Y chromosomes whereas females carry two X chromosomes. Thus, male X chromosome does not seem to be compensated for gene dosage relatively to two female X chromosomes and two copies of autosomes. However, two processes occur in mammals to correct the imbalance. X chromosome inactivation (XCI) results in transcriptional silencing of one of the two X chromosomes in females, balancing dose of X-linked genes between sexes (Lyon 1961). X chromosome upregulation (XCU) doubles X-linked genes expression on the active X chromosome in both males and females, equalizing X-linked genes expression and biallelic expression of autosomal genes (Dementyeva et al., 2009; Deng et al., 2014; Nguyen and Disteche 2006). Therefore, in mammals, males have one active upregulated X chromosome (Xa) while females carry one active upregulated X chromosome (Xa) and one inactive X chromosome (Xi) (Fig. 1).

XCI is a well coordinated chromosome-wide epigenetic process controlled by a long non-coding RNA of the Xist gene in eutherians. Xist RNA is a nuclear transcript being expressed exclusively on the inactivated X chromosome. After XCI initiation, Xist RNA spreads along the X chromosome due to its capacity to bind nuclear proteins, is maintained in Xi chromatin, represses gene transcription, and forms facultative heterochromatin and Xi chromosome territory (Moindrot and Brockdorff 2016; Pinter 2016; Przanowski et al., 2018). However, in somatic tissues, gene silencing and Xi chromatin can be maintained in the absence of Xist RNA.

XCU has been studied to a lesser extent but seems to be a highly concerted process. XCU is rapidly established in a chromosome-wide manner in male and female early development and is quickly
Dosage compensation system in eutherian mammals. In females (XX), one X chromosome is upregulated and the other is inactive. In males (XY), the only X chromosome is upregulated. This provides equal expression of X-linked genes between sexes and equal expression between X-linked and autosomal genes. X, X chromosome; Y, Y chromosome; A, autosomal set. The upregulated X chromosome is shown in white and the inactive X chromosome is indicated in grey.

Dosage compensation between X-chromosome and autosomal genes was first postulated by Susumu Ohno in the 1960s (Ohno 1967). However, XCU was confirmed for all ontogenesis stages – from early embryonic development to adult somatic tissues (Deng et al., 2011, 2013; Lin et al., 2007). Single cell RNA sequencing of human embryos first detected XCU at the blastocyst stage (Moreira de Mello et al., 2017). A loss of XCU as well as XCI occurs during global genome reprogramming in primordial germ cells of both sexes in mice and humans (Sangrithi et al., 2017; Moreira de Mello et al., 2017). In early female germ cells, Xi is reactivated while Xa remains upregulated. As a result, X-linked genes are overexpressed relatively to autosomal genes and X:A ratio exceeds 1. Later, XCU appears to be switched off because X:A ratio returns to 1 in females but becomes below 1 in males. The skewed X-linked gene dosage found in both sexes may promote sexual dimorphism in germ line development, which is necessary for correct gametogenesis and may account for infertility of individuals with sex chromosome aneuploidies.

XCU is based on increasing transcription level and changes in mRNA stability. Transcription can be elevated by RNA PolII phosphorylation and histone modifications such as MOF-mediated H4K16 acetylation and high level of trimethylated H3K4 that promote open chromatin structure (Deng et al., 2013; Yildirim et al., 2011). In addition, transcript half-life was shown to be longer for X-linked genes (Deng et al., 2013; Faucillion and Larsson 2015; Yildirim et al., 2011) and to be shorter for autosomal genes (Yin et al., 2009). However, mechanisms regulating mRNA stability are still unknown. X-linked genes may use one or several mechanisms to be upregulated. For example, in mouse embryonic stem cells (ESCs), transcription level of 69 genes depended on MOF histone-methyltransferase but only 60% of the genes demonstrated increased mRNA stability (Deng et al., 2013). Thus, transcription upregulation and increasing mRNA stability may be independent XCU mechanisms.

XCU affects genes of both conservative and added regions of X chromosome but to various extents (Deng et al., 2013). In all tissues, ubiquitously expressed housekeeping X-linked genes are upregulated. Dosage of X-linked genes encoding subunits forming protein complexes together with autosomal products is expected to be regulated more strictly (Pessia et al., 2012). In addition, downregulation of autosomal genes may also occur to equalize their expression level relatively to that of X-linked genes (Julien et al., 2012). Tissue-specific X-linked genes responsible for gametogenesis and being expressed predominantly in testis...
and ovaries are repressed and, therefore, do not undergo XCI in somatic tissues (Deng et al., 2014; Pessia et al., 2012). XCI also does not affect X-linked genes that escape XCI and are expressed biallelically (Dementyeva et al., 2009; Deng et al., 2014). Expression of some X-linked genes remains unbalanced. Their expression level is thought not to be important for cell or the unbalanced expression may have functional significance, e.g. it contributes to sex-specific features.

**Imprinted X chromosome inactivation in rodents**

Imprinted X chromosome inactivation (iXCI) takes place in rodent preimplantation embryo and extraembryonic tissues. iXCI was described in mice (Takagi and Sasaki 1975), rats (Wake et al., 1976), and common voles (Dementyeva et al., 2010; Shevchenko et al., 2011). During iXCI paternally inherited X chromosome (Xp) is inactivated. In mice, imprinting protecting maternally inherited X chromosome (Xm) from XCI is supposed to be established in oogenesis. Due to the maternal imprinting mouse embryos carrying two Xm (XmXmXp or XmXmY) cannot activate extra Xm and die because of defects in extraembryonic tissues (Shao and Takagi 1990; Tada et al., 2000). Antisense RNA of the Tsix gene which is not expressed on Xp was proposed to repress Xist on Xm protecting it from inactivation during iXCI in mice (Lee 2000).

However, Xist was shown to be inhibited on Xm by Tsix RNA in differentiated trophoderm cells rather than in preimplantation mouse embryo (Mclary et al., 2014). A recent study links initial Xm imprint with a high level of trimethylated H3K27 (H3K27me3) in its Xist locus. The modification is established during oocyte growth and is retained in mouse preimplantation development (Inoue et al., 2017). Loss of H3K27me3 leads to initiation of Xist expression on Xm and subsequent Xm inactivation in preimplantation embryos.

Correct iXCI initiation in mice is also controlled by protein of an X-linked gene, Rnf12 (Shin et al., 2010). The protein is a RLIM ubiquitin ligase regulating activity of various transcription factors and co-factors. Rnf12 knockout in oocytes where a huge amount of RLIM is normally present interferes with iXCI in female embryos due to inhibition of Xist RNA cloud formation on Xp, which causes embryo death. Thus, different mechanisms may be involved in XCI imprinting during early mouse development.

After the first division of mouse zygote, at the 2-cell stage, Xp and Xm genes are transcriptionally active with no pre-inactivation signs on Xp (Borenstein et al., 2017b; Deng et al., 2014; Okamoto and Heard 2006) (Fig. 2). iXCI begins at the 4-cell stage when Xp is coated with Xist RNA. Xp is gradually inactivated during preimplantation development as facultative heterochromatin is formed.

Early-silenced genes are located in X chromosome regions that are first to bind Xist RNA. Intermediate and late silencing occurs in the regions that are located next to and outside first Xist RNA 'entry' sites, respectively. The inactivation process is completed by the blastocyst stage when about 85% of Xp genes undergo silencing (Borenstein et al., 2017b).

In mouse preimplantation development, expression of X-linked genes can remain unbalanced (Borenstein et al., 2017b). X:A ratio significantly exceeds 1 in single blastomeres beginning from the 4-cell stage, implying abundance of X chromosome transcripts. This may be seen for the fact that active Xm was already upregulated whereas Xp was not completely inactivated. Overexpression of X-linked genes relatively to autosomal genes is observed up to the blastocyst stage when X:A ratio approaches to 1.

In the absence of Xist expression iXCI is not triggered and gene transcription is not repressed (Borenstein et al., 2017b; Namekawa et al., 2010). Absence of Xist expression during mouse iXCI causes global changes in transcriptome of preimplantation embryo cells (Borenstein et al., 2017b). On the one hand, abnormal overexpression of several pluripotency genes that maintain blastomeres in a poorly differentiated state is observed. On the other hand, aberrant expression of genes involved in differentiation and extraembryonic tissues formation is revealed. The transcriptome alterations result in development abnormalities detected from the blastocyst stage and finally cause embryo death.

Trophoblast stem (TS) and extraembryonic endoderm stem (XEN) cells giving rise to extraembryonic tissues (placenta and yolk sac) are derived from preimplantation rodent (mouse and vole) embryos and are actively used to study iXCI (Calabrese et al., 2012; Merzouk et al., 2014; Prudhomme et al., 2015; Shevchenko et al., 2009, 2018; Vaskova et al., 2014). It was shown that 13–15% of X-linked genes are not inactivated on Xi and expressed from both X chromosomes in mouse TS and XEN cells (Calabrese et al., 2012; Merzouk et al., 2014). However, the patterns of genes escaping XCI in mouse TS and XEN cells have some specific traits. Similar data on incomplete iXCI and peculiarities of genes escaping XCI were obtained when studied X-linked gene expression in vole placenta and XEN cells (Dementyeva et al., 2010).

During differentiation of mouse TS cells into trophoblast derivatives transcriptional silencing of Xp becomes more relaxed. This is confirmed by increased reactivation frequency of endogenous loci and transgenic constructions on Xp (Corbel et al., 2013; Hemberger et al., 2001). Xp reactivation can be increased by inhibition of methyltransferases of PRC2 complex that seems to maintain iXCI in differentiated cells of trophoderm via H3K27 trimethylation (Kalantry et al., 2006; Wang et al., 2001). It was found that Xp in mouse TS cells could lose an inactive state and become completely reactivated. However, no obvious changes in cell phenotype like differentiation, malignization or reprogramming were observed (Prudhomme et al., 2015). TS cell clones carrying two active X chromosomes are capable to secondary non-random XCI. The same X chromosome is chosen to be inactivated in the cells of a certain clone. In most cases, Xp is repeatedly inactivated. However, Xm sometimes is inactivated de novo. In visceral endoderm and yolk sac representing differentiated derivatives of XEN cells, incomplete iXCI is more stable and is maintained more strictly (Merzouk et al., 2014). Moreover, iXCI maintenance in XEN cells and their derivatives is independent of PRC2 histone-methyltransferases. The data confirm differences in regulation of imprinted Xp silencing between extraembryonic lineages of diverse origin.

In mice, inactive Xp recruits the same histone modifications as Xi does in cells of somatic tissues (Chaumeil et al., 2004; Okamoto and Heard 2006). During iXCI Xp is associated with Xist RNA and enriched with PRC1 and PRC2 complexes as well as ubiquitinilated H2A and H3K27me3 that are typical of inactive chromatin and established by the complexes. At the same time, Xp is depleted in histone modifications characteristic of active chromatin. In XEN cells, besides chromosome domains with Xist-dependent modifications, Xp also contains blocks of inactive chromatin similar to constitutive heterochromatin of autosomal centromeric and telomeric regions (Shevchenko et al., 2009). The inactive Xp in iXCI has long been believed to be hypomethylated.
However, a genome wide 5-methylcytosine distribution assay revealed that during iXCI in mouse extraembryonic cells, DNA methylation is recruited to X-linked promoters and CpG islands at the level comparable with that during XCI in somatic tissues (Senner et al., 2012).

As in mice, vole inactive Xp demonstrates histone modification pattern similar to Xi in somatic tissues. In vole TS cells and blastocysts, Xi has a unique chromatin structure comprising $\text{Xist}$ RNA as well as trimethylated H3K9 and H4K20, HP1$\gamma$ (CBX1), and HP1$\gamma$ (CBX3) attributable to constitutive heterochromatin (Vaskova et al., 2014). Xi chromatin during iXCI in voles also contains the SETDB1 histone-methyltransferase and KAP1 protein (Shevchenko et al., 2018). Polycomb proteins and H3K27me3 are observed on Xi only in differentiated trophoblast cells that arise after implantation when placenta is formed (Vaskova et al., 2014). In vole TS cells, $\text{Xist}$ repression in the absence of PRC2 and H3K27me3 does not cause changes in chromatin modifications and global X-linked gene reactivation (Shevchenko et al., 2018). Thus, although iXCI in vole TS cells is not complete (Dementyeva et al., 2010), it is more stable (Shevchenko et al., 2018) than that in the mouse TS cells, illustrating interspecific differences in iXCI between rodents.

Random X chromosome inactivation in rodents

As shown by immunofluorescence (Mak et al., 2004; Okamoto et al., 2004) and then confirmed by single cell RNA sequencing (Borensztein et al., 2017a; Mohammed et al., 2017), starting from the middle blastocyst stage, Xp loses inactive chromatin marks and becomes reactivated in epiblast cells of the inner cell mass. The OCT4 interacting ZFHX3 transcription factor and several zinc-finger proteins rather than pluripotency factors showed correlation (positive or negative) with the Xp reactivation (Mohammed et al., 2017). Early-reactivated genes were enriched with MYC transcription factor binding sites compared to other X-linked genes (Borensztein et al., 2017a). More slowly reactivated genes were highly enriched with H3K27me3 and their reactivation strongly correlated with the presence of NANOG and loss of $\text{Xist}$ expression. After reactivation, two active X chromosomes are maintained for a short time until epiblast cells initiate random XCI (rXCI) during implantation (Fig. 2). In the next inactivation cycle, Xp and Xm have equal chances to be silenced (Okamoto and Heard 2006). In mice, rXCI is stably inherited in somatic cell generations and is retained in ontogenesis. Vole and rat somatic cells also demonstrate stable rXCI (Dementyeva et al., 2010; Shevchenko et al., 2009; Vaskova et al., 2015).

X chromosome counting and choice in rXCI of rodents

First step in mouse rXCI is counting X chromosome number relatively to autosomal sets. rXCI is triggered if cell contains more than one X chromosome per diploid autosomal set. Thus, only one X chromosome remains active in diploid cells while the others X chromosomes are inactivated (Lee 2011). Factors involved in X chromosome counting may be associated with triggering monoallelic $\text{Xist}$ expression which is a key event in XCI initiation. Nuclear non-coding RNA of the Jpx gene located upstream to the $\text{Xist}$ promoter is one of the candidates (Chureau et al., 2002). Jpx (also known as $\text{Enox}$) was found in other rodents, e.g. rats and common voles (Shevchenko et al., 2011). Jpx RNA is a dosage-dependent $\text{Xist}$ activator (Sun et al., 2013). Both a depletion of Jpx transcription by short hairpin RNAs and deletion of one of the two Jpx alleles interfere with $\text{Xist}$ RNA activation and rXCI initiation in mouse female ESCs (Tian et al., 2010, Sun et al., 2013). On the contrary, additional Jpx copies even when located to autosomes activate $\text{Xist}$ expression in male ESCs (Sun et al., 2013). Further studies showed that Jpx RNA was able to displace CTCF, transcription factor and chromatin insulator, from the $\text{Xist}$ promoter. $\text{Xist}$ activation appears to depend on balance between Jpx RNA and CTCF, which may be used to count number of X chromosomes and autosomal sets in cells. Key transcription factors of pluripotent state are likely to be other players involved in counting process as they were found to participate in direct $\text{Xist}$ repression and Tsix activation (Navarro et al., 2008, 2010). For instance, depletion in OCT4, transcription factor responsible for pluripotency maintenance, causes $\text{Xist}$ activation on both X chromosomes in mouse ESCs (Donohoe et al., 2009). Increased level of the Rnf12/RLIM ubiquitin ligase also can upregulate $\text{Xist}$ during differentiation of cultured female pluripotent cells (Jonkers et al., 2009). Rnf12/RLIM may affect stability of transcription factors and their binding to $\text{Xist}$/Tsix regulatory elements in dosage dependent manner. However, epiblasts of postimplantation embryos show RLIM-independent rXCI (Shin et al., 2014; Wang et al., 2017). Thus, X chromosome counting mechanism is still poorly understood.

In mice, Xi is suggested to be chosen via transient X chromosome pairing that occurs in the $\text{Xist}$ adjacent regions immediately before rXCI (Lee 2011; Masui et al., 2011). The pairing appears to make asymmetry necessary for monoallelic $\text{Xist}$ expression triggering. Before rXCI initiation, $\text{Xist}$ expression on both X chromosomes is repressed with a high level transcription of antisense Tsix RNA regulated by the Xite enhancer (Ogawa and Lee 2003). Tsix is believed to be randomly repressed on one of the two X chromosomes during X chromosome pairing affecting the Xite locus, leading to $\text{Xist}$ upregulation and rXCI initiation (Lee 2011; Masui et al., 2011). However, another study showed that trans-activator RLIM, cis-acting elements surrounding $\text{Xist}$ like Jpx and those involved in pairing events ensure $\text{Xist}$ monoallelic expression while X-X pairing itself is not necessary for rXCI to take place (Barakat et al., 2014). In rats and voles, Tsix is also transcribed (Shevchenko et al., 2011). Its transcription is initiated in a conservative region and terminates upstream to the $\text{Xist}$ transcription start site, which has a functional significance to repress $\text{Xist}$ promoter via inactive chromatin formation as shown in mice (Navarro et al., 2005; Sado et al., 2005). However, in voles, the region homologous to Xite was deleted by a chromosomal rearrangement and intergenic transcription characteristic of the element was not found. Therefore, rXCI regulation in rodents may be at least partially taxon-specific (Shevchenko et al., 2011).

$\text{Xist}$ RNA spreading, transcriptional silencing initiation and stabilization

After $\text{Xist}$ activation, $\text{Xist}$ RNA spreads along the X chromosome and causes X-linked gene transcription silencing which is $\text{Xist}$-dependent at early stages of rXCI (Wutz et al., 2002; Wutz and Jaenisch 2000). $\text{Xist}$ RNA acts via recruiting proteins. To date 80 proteins are shown to interact directly or indirectly with $\text{Xist}$ RNA and their functional role in XCI was confirmed using different methods (Przanowski et al., 2018). The proteins bind to $\text{Xist}$ RNA in the regions of conservative microsatellite repeats denoted with Latin letters – from A to F. Some of the $\text{Xist}$-interacting proteins are discussed in this sub-section and listed in Table 1.
At least three proteins, the YY1 transcription factor (Jeon and Lee 2011), hnRNPU nuclear matrix protein (also known as SAF-A) (Hasegawa et al., 2010), and CIIZ1 containing zinc finger domain (Sunwoo et al., 2017), are involved in Xist RNA spreading and accumulation on Xi. Knockdown of the genes encoding the proteins results in loss of Xist RNA clouds on Xi. Xist RNA localization on Xi is also disrupted when CIIZ1 is overexpressed. YY1 is shown to interact with C-repeats localized in the Xist exon 1 (Jeon and Lee 2011; Sarma et al., 2010). hnRNPU has DNA-binding (SAF) and RNA-binding (RGG) domains interacting with Xi DNA and Xist RNA in the central parts of exons 1 and 7 (Hasegawa et al., 2010; Yamada et al., 2015). CIIZ1 binds to E-repeats in exon 7 (Sunwoo et al., 2017). 3D-SIM super-resolution microscopy demonstrated that Xist RNA was located mainly in perichromatin space of Xi co-localizing with nuclear matrix proteins (Cerase et al., 2014; Hasegawa et al., 2010; Smeets et al., 2014).

A-repeats of Xist RNA are known as a domain involved in transcriptional silencing (Wutz et al., 2002). A-repeats were shown to interact with several RNA-binding proteins, namely RBM15, WTP1, SPEN, ATRX, and JARID2 (reviewed in Pinter 2016). The domain also binds HDAC3 histone deacetylase, Polycomb repressive complex 2 (PRC2) with histone methyltransferase activity, as well as NCoR2 (or SMRT) which are components of a co-repressive complex. Mechanisms of transcription silencing initiation are unknown. However, WTP1 RNA-methyltransferase as well as RBM15 and SPEN proteins that are able to link long non-coding RNA with chromatin and transcription regulators were found to play essential role in X-linked gene repression (Moindrot et al., 2015). HDAC3 and NCoR2 are also involved (McHugh et al., 2015). SPEN is thought to be an intermediary protein that links Xist RNA, the NCoR2 co-repressor complex, and HDAC3. Expression of evolutionary young transcriptionally active retrotransposones of the LINE-1 family was found on Xi during mouse ESC differentiation (Chow et al., 2010). Transcribed LINEs were supposed to facilitate spreading of the inactive state increasing chromatin accessibility for repressing factors.

After silencing initiation, inactive state of Xi genes is maintained through covalent histone modifications and becomes Xist-independent (Wutz et al., 2002; Wutz and Jaenisch 2000). Histone modifications are established by chromatin repressive complexes PRC1 and PRC2 that also can bind Xist RNA (Pintacuda et al., 2017; Sarma et al., 2014; Zhao et al., 2008). B-repeats of Xist RNA recruit the hnRNPK RNA-binding protein interacting with the PGC3/5-PRC1 complex (Pintacuda et al., 2017). PRC2 interacts with Xist RNA in the region of A-repeats through ATRX (Sarma et al., 2014). However, A-repeats are not the only Xist RNA domain capable to bind PRC2 (Wutz et al., 2002; Zhao et al., 2008). PRC1 and PRC2 establish Xist-dependent Xi chromatin modifications – monoubiquitinated H2AK119 and H3K27me3. Other proteins and chromatin modifications further appear on Xi but their recruitment does not depend on Xist RNA. MacroH2A, the Ash2l trihorax protein, CDY1, DNA hypermethylating of gene promoter regions, the DNMT3b de novo DNA methyltransferase, as well as SMCHD1 and LRF1 (or HBX1) proteins responsible for chromatin compaction are also detected on Xi (reviewed in Moindrot and Brockdorff 2016). Thus, X chromosome inactive state stabilizes and the stage of rXCI maintenance begins.

X chromosome territory is organized concurrently with transcriptional repression. Repeated DNA sequences form inner core of the chromosome territory while inactivated genes are located outside (Chaumeil et al., 2006; Clemson et al., 2006). Correct localization of inactive genes is controlled by A-repeats of Xist RNA (Chaumeil et al., 2006). The Xi chromosome territory is coated with Xist RNA and depleted in RNA PolII. In somatic cells, genes escaping XCI including Xist are localized on the edge of the chromosome territory or outside. High resolution chromatin conformation capture

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**TABLE 1**

**PROTEINS BINDING TO XIST RNA DURING rXCI IN MICE**

| Protein name | Known function(s) and/or functional domain(s) | Binding region within Xist RNA | XCI step |
|--------------|-----------------------------------------------|-------------------------------|---------|
| CIIZ1        | DNA binding zinc finger protein                | E-repeats, exon 7             | Xist RNA spreading and accumulation on Xi |
| hnRNPU (SAF-A) | Heterogeneous Nuclear Ribonucleoprotein U | Binding nucleic acids and participation in the formation of ribonucleoprotein complexes | central parts of exons 1 and 7 | Xist RNA spreading and accumulation on Xi |
| YY1          | Transcription factor belonging to GLI-Kruppel class of zinc finger proteins | C-repeats, exon 1 | Xist RNA spreading and accumulation on Xi |
| WTAP         | A subunit of the RNA N6-methyltransferase complex | A-repeats, exon 1 | Xist-dependent transcriptional silencing |
| SPEN         | Interactions with different transcriptional repressors, RNA binding | A-repeats, exon 1 | Xist-dependent transcriptional silencing |
| RRM15        | Split-end (SPEN) protein family member, participation in RNA methylation | A-repeats, exon 1 | Xist-dependent transcriptional silencing |
| HDAC3        | Histone Deacetylase 3 | A-repeats, exon 1 | Xist-dependent transcriptional silencing |
| NCoR2        | Interactions with different transcriptional repressors, RNA binding | A-repeats, exon 1 | Xist-dependent transcriptional silencing |
| JARID2       | Known as a transcriptional repressor which is able to interact with PRC2 complex | A-repeats, exon 1 | Xist-dependent transcriptional silencing |
| ATRX         | Transcriptional silencing | A-repeats, exon 1 | Xist-dependent transcriptional silencing |
| PRCC         | Chromatin modifier with histone methyltransferase activity | A-repeats, exon 1 | Xist-dependent Xi chromatin modifications |
| PRCC1        | Chromatin modifier with ubiquitin ligase activity | A-repeats, exon 1 | Xist-dependent Xi chromatin modifications |
| PRCC2        | Chromatin modifier with ubiquitin ligase activity | A-repeats, exon 1 | Xist-dependent Xi chromatin modifications |

All references are given in the text and in Przanowski et al., 2018.
technique demonstrated that Xa was organized in numerous topologically associated domains (Nora et al., 2012). In contrast to Xa, Xi condensed chromatin was divided into two giant loops (up to 77 Mb each) that were organized in two macrostructures called superdomains (Deng et al., 2015).

rXCI affects majority of X-linked genes and is stably maintained in cell generations. In rodents, rXCI is generally more complete and stable compared to iXCI (Berleton et al., 2011; Dementyeva et al., 2010). In mice, just about 3% of X-linked genes escape rXCI (Berleton et al., 2011) and seem to be protected from repressive chromatin modifications associated with transcriptional silencing.

**Modeling of rXCI and Xi reactivation in mice and rats using pluripotent stem cells**

ESCs derived from blastocyst inner cell mass as well as induced pluripotent stem cells (iPSCs) generated from somatic cells via overexpression of the OCT4, SOX2, KLF4, and c-MYC transcription factors are model systems to study rXCI ex vivo in mice (Barakat and Gribnau 2010). Mouse ESCs and iPSCs maintain two active X chromosomes. One of the X chromosomes passes all the rXCI stages upon ESC and iPSC differentiation with the dynamics similar to that in the cell of postimplantation embryos (Pinter 2016). Nevertheless, in differentiated derivatives of ESCs and iPSCs, Xi inactive state is maintained as in embryonic cells but not as in adult somatic cells.

Technology of somatic cell reprogramming to the pluripotent state allowed studying X chromosome reactivation process in mice (Pasque et al., 2014). X chromosome reactivation is a late event during reprogramming and is triggered only after establishing pluripotent state. This is in accordance with the fact that the OCT4, NANOG, and SOX2 pluripotency factors act as Xist repressors. Epigenetic changes during X chromosome reactivation mainly occur with the dynamics opposite to that during XCI with some exception, e.g. DNA methylation. Under reprogramming, Xi acquires some traits attributable to early embryonic development. For example, PRC2 complex which appears on Xi at embryonic stages of rXCI and is lost in adult somatic cells, is detected on Xi after the mesenchymal-to-epithelial transition step of reprogramming and maintained until pluripotency gene activation. Key events to initiate X chromosome reactivation are loss of Xist expression and DNA demethylation. Xist repression and biallelic Tsix expression in XX iPSCs strongly correlate with NANOG upregulation. However, X-linked gene reactivation occurs only in a subset of NANOG positive cells that also express DPPA4 and PECAM1 factors. DNA hypermethylation which is the latest event on Xi in rXCI is removed during X-reactivation just at the final stage of reprogramming to the pluripotent state. Successful pluripotency acquisition and X chromosome reactivation in iPSCs require expression of PRDM14 known as a driver of active DNA demethylation (Payer et al., 2013). It is worth noting that pluripotency factors and PRDM14 are important for X chromosome reactivation not only in vitro but also in vivo in primordial germ cells.

Presence of two Xa is also an obligatory property of pluripotent state in rat ESCs and iPSCs (Vaskova et al., 2015). When generated rat iPSCs X chromosome inactivated in original somatic cells is reactivated. X chromosome loses modifications characteristic of inactive state and restores active chromatin marks and gene expression. Loss of pluripotency during rat ESC and iPSC differentiation is accompanied by rXCI similar in dynamics of epigenetic events to that in mice. However, detailed studies of rXCI mechanisms in rat pluripotent stem cells need to be performed.

**X chromosome inactivation in other eutherian mammals**

Xi in eutherian somatic tissues has a number of common features that appear to correspond to general fundamental mechanisms underlying XCI (Moindrot and Brockdorff 2016; Pinter 2016; Przhanowski et al., 2018). At the cytological level, Xi is detected as a dense chromatin spot (called Barr body) in female interphase nuclei. Xi replicates in late S-phase, is coated with Xist RNA and depleted in acetylated H3 and H4 histones associated with active transcription. RNA PolII is almost completely excluded from Xi chromosome territory in interphase nuclei. In humans and cattle, two types of facultative heterochromatin were identified on Xi (Chadwick and Willard 2004; Coppola et al., 2008). Some Xi domains contain XistRNA and Xist-dependent chromatin modifications such as ubiquitinilated H2A and H3K27me3. The others are characterized by HP1, H3K9me3, and H4K20me3 attributable to constitutive heterochromatin. However, in elephants as in mice, Xi is not enriched with H3K9me3 and H4K20me3 (Chauimeil et al., 2011).

About 15% of human X-linked genes escape XCI (Berleton et al., 2011; Carrel and Willard 2005). Other 10% of X-linked genes have a heterogeneous expression: the genes are inactivated in most cases but can be expressed on Xi in some cells or individuals. Xi reactivation especially for X-linked genes with heterogeneous expression may be observed with age. The reactivation coincides with decreasing in Xist expression and may result in autoimmune, oncological, and age-related diseases depending on number and set of reactivated genes (Dunford et al., 2017; Forsdyke 2009; Ostan et al., 2016; Silva et al., 2008; Spatz et al., 2004). Some diseases may be due to changes in expression of genes escaping Xi that are sensitive to Xist RNA level.

Xist and its spliced transcripts were found in many eutherians including evolutionary ancient species (Chureau et al., 2002; Elisaphenko et al., 2008). Xist nucleotide sequence, exon-intron structure, and size of processed transcript have evolved significantly. Nevertheless, the A-F minisatellite repeats that act as Xist RNA functional domains in mice were identified in all the eutherian species studied despite divergence in the evolution. Thus, Xist is likely to be a key gene during XCI in eutherian mammals.

However, mechanisms of XCI initiation in early embryogenesis may differ among mammals (Fig. 2). In contrast to mice, Xistexpression is not imprinted in human and rabbit embryos (Okamoto et al., 2011). Moreover, in humans, rabbits, horses, and mules choice of X chromosome for inactivation is random in both embryo proper and extraembryonic tissues (Okamoto et al., 2011; Wang et al., 2012). XiC is triggered later in development compared to mice, so time point of XCI initiation varies between species (Okamoto et al., 2011).

In rabbit female embryos, Xist RNA accumulation occurs at the early blastocyst stage (Okamoto et al., 2011). Surprisingly, about one half of blastomeres shows biallelic Xist expression and inactivation of an X-linked gene, Hprt1. By the late blastocyst stage, majority of blastocyst cells have only one Xi expressing Xist. How initial biallelic Xist expression is settled down is still unclear. As no differences in cell death were observed between male and female rabbit blastocysts, one may suggest that XCI is reversed on one of the two X chromosomes or cells carrying Xa and Xi have selective
X chromosome upregulation and inactivation

advantage compared to cells with two Xi.

In human preimplantation development, XIST transcript is detected from the 8-cell to blastocyst stages on the only X chromosome in males and both X chromosomes in females (Okamoto et al., 2011) (Fig. 2). The fact that XIST is activated in both males and females suggests that XIST expression is not sensitive to X chromosome number, at least at initial stages. In comparison with somatic tissues, XIST RNA clouds in human embryos seem diffuse. Another non-coding RNA is accumulated simultaneously with XIST expression on active X chromosomes in ESCs and early embryos (Vallot et al., 2017). The RNA is transcribed from the XACT gene which is located several megabases from the XIST locus. XACT transcript can disturb XIST RNA association with X chromosome and its capacity to initiate XCI (Vallot et al., 2015, 2017). This may lead to XIST RNA diffuse localization. XACT is not conserved in mammals. It was not found in mice and appears to function only in primates. Biallelic expression is characteristic not only of XIST and XACT but also of other X-linked genes (Patel et al., 2017). Studies of rhesus macaques demonstrated as well XIST expression in blastocysts of both sexes and biallelic expression of Xist and other X-linked genes in females (Tachibana et al., 2012).

It was suggested that a process of X-linked gene dosage compensation different from classical XCI had to occur in early development of humans and other primates. RNA sequencing of single blastomeres of female preimplantation embryos evidences for decreasing in gene transcription on both X chromosomes, which was termed transcriptional damping (Fig. 2) (Petropoulos et al., 2016; Sahakyan and Plath 2016). Possible mechanisms of the phenomenon may be based on XIST and XACT RNA antagonism. XIST RNA interacting with WTA15, RBM15, SPEN2, etc. decreases gene transcription and initiates their inactivation. At the same time, XACT RNA eliminates the effect not allowing XIST RNA diffuse localization. XACT RNA is not conserved in human ESCs and early development. As XCI takes place quite late in human embryonic development (probably after implantation) and choice of X chromosome for inactivation is random at once, X chromosome reactivation and subsequent XCI are not required. It is worth noting that the absence of XCI in trophectoderm of human blastocyst may imply that dosage compensation is not essential at this stage.
In eutherians, Xist expression regulation appears to be species-specific, which may underlie interspecific differences in XCI initiation time, mechanisms of X chromosome counting and choice. Pluripotency factors repressing Xist in mice allow its biallelic expression in rabbits and humans (Okamoto et al., 2011). Moreover, antisense Tsix transcription regulating random monoallelic Xist expression in mice is poorly retained or even is absent in other mammals (Lee 2011). Although TSIX/Tsix was found in humans and cattle, it is transcribed from Xi simultaneously with XIST/Xist in fetus and placenta (Farazmand et al., 2004; Migeon et al., 2002). This means that TSIX/Tsix function is not preserved in humans and other mammals. Moreover, new non-coding RNAs might arise to regulate Xist expression like XACT RNA in primates. Another two non-coding RNAs, Ftx and Jpx (Enox), regulating Xist expression in mice are preserved and expressed in humans and cattle (Chureau et al., 2002) and may function at early stages of XCI. However, in humans, X chromosome counting depends mainly on autosomal factors but not on expression of X-linked sequences like Rnf12 and Jpx as in mice. Presence of two Xa in human triploid cells (69,XXX and 69,XXY) suggests that three copies of certain autosomes are able to maintain two X chromosome in the active state via encoding a dosage-sensitive XIST repressor (Migeon 2017). The repressor is likely to be located to chromosomes 1 and 19.

Pluripotent stem cells (PSCs) – ESCs and iPSCs – have been generated for many mammalian species (Ezashi et al., 2016). Nevertheless, besides rodents, X chromosome epigenetic status, XCI, and XCI were studied in human PSCs only. Human PSCs (46,XX) have a heterogeneous X chromosome epigenetic status which is not stable and can be changed during cultivation (Anguera et al., 2012; Silva et al., 2008). A few human PSC lines are able to trigger inactivation of one of the two X chromosomes upon differentiation like mouse and rat PSCs. Most 46,XX PSC lines demonstrate inactivation of one of the two X chromosomes in the pluripotent state. However, Xi inactive state degrades rapidly or undergoes erosion during cultivating human PSCs. The erosion on Xi (Xe) is characterized by irreversible loss of XIST expression and H3K27me3, depletion in DNA methylation, and gene reactivation (Anguera et al., 2012; Silva et al., 2008; Vallot et al., 2015). Xe epigenetic state remains irreversible, is transmitted into differentiated derivatives and is maintained under reprogramming of the derivatives to the pluripotent state. The difference in Xe state is obvious on the transcriptional level. In PSCs, genes are expressed along the entire Xe but transcription level from Xe is much lower than that from Xa. In differentiated derivatives, Xa transcripts are predominately detected and just several genes are expressed on Xe (Vallot et al., 2015). Cells carrying two Xa, Xa and Xe, or Xa and Xe may be often present in the same PSC culture, especially at early passages (Patel et al., 2017). When cultivated percentage of cells with Xe is increasing and can reach 100%. In comparison with PSCs carrying two Xa or Xa and Xe, PSCs bearing Xe and losing capacity to express XIST are shown to have an elevated expression level of X-linked oncogenes and other genes associated with cancer (Anguera et al., 2012; Silva et al., 2008). The erosion may result from de novo expression of XACT RNA on X. In PSCs, XACT RNA normally spreads along Xa and seems to be localized at the same loci on Xi as XIST RNA (Vallot et al., 2015). During erosion, XACT RNA is believed to dislodge XIST RNA from Xi, which causes loss of XIST-dependent repressive chromatin modifications, gene reactivation, and complete XIST repression.

Influence of human PSC cultivation conditions on X chromosome status has been postulated. Some of the conditions are thought to promote Xi reactivation and maintenance of two Xa (Hanna et al., 2010; Hasegawa et al., 2014). However, it is still unclear if X chromosome reactivation was really detected because the authors could observe XCI erosion also accompanied with XIST repression, loss of XIST-dependent repressive chromatin modifications, and Xi gene reactivation. It is obvious that unstable X chromosome status and its possible negative consequences prevent human PSCs and their differentiated derivatives from using in biomedical studies and clinical regenerative medicine. To solve the problem, approaches to control X chromosome status in PSCs and to make PSCs similar to blastocyst cells are being developed. Researchers are trying to generate and cultivate human PSCs in so called naïve pluripotency state that corresponds to cells of preimplantation blastocysts. Some protocols provide generating and maintenance of ESCs corresponding to human preimplantation embryos in gene expression (Huang et al., 2014). One of the methods, 5iLAF, based on using inhibitors of the MEK, B-RAF, GSK3b, SRC, and RHO kinases as well as the LIF, Activin A, and FGF2 growth factors restores biallelic XIST expression in ESCs. In differentiated derivatives of the ESCs, XIST-dependent XCI is reproduced (Sahakyan et al., 2017). One may expect that naïve pluripotent ESCs derived by the protocol will allow verifying transcriptional damping of X-linked genes and studying XCI mechanisms in humans. Arecent study of mouse ESCs and embryos showed that exit from naïve pluripotent state at the onset of differentiation caused Xist accumulation accompanied by transient chromatin repression and partial X-linked gene silencing on male and both female X chromosomes (Sousa et al., 2018). The result makes the XCI story more complicated and suggests that some interspecific differences in rXCI between human and rodents might arise from manipulations with naïve pluripotency gene network caused, for instance, by culture conditions. The facts that counting and choice between the two X chromosomes are not necessary for XCI initiation in mice as well as XCI initiation and spreading also occur in males make us realize XCI mechanisms are still poorly understood and many questions on the process regulation need to be clarified.

Conclusion

In mammals, dosage compensation takes place in early embryonic development. It is closely linked with pluripotent cells and their differentiation and represents an important model to study epigenetic transcription regulation. However, mechanisms of dosage compensation of mammalian sex chromosomes demonstrate high plasticity and rapidly adapt to evolutionary changes in early development. In spite of significant progress that has been made in our understanding of mammalian X-linked gene dosage compensation, numerous aspects of the phenomenon remain unclear and need to be further investigated. Forthcoming results allow clarifying reasons and mechanisms underlying human diseases caused by imbalance of X-linked gene expression and developing new approaches to X-linked disease therapy.

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