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Diverse developmental strategies of X chromosome dosage compensation in eutherian mammals

ALEXANDER I. SHEVCHENKO*[,1,2,3,4], ELENA V. DEMENTYEVA[1,2,3], IRINA S. ZAKHAROVA[1,2,3] and SUREN M. ZAKIAN[1,2,3,4]

[1]Federal Research Centre "Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences", [2]Institute of Chemical Biology and Fundamental Medicine of the Siberian Branch of the Russian Academy of Sciences, [3]E.N. Meshalkin National Medical Research Centre, Ministry of Healthcare of the Russian Federation and [4]Novosibirsk State University, Novosibirsk, Russia

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

Abbreviations used in this paper: ESC, embryonic stem cell; iPSC, induced pluripotent stem cell; iXCI, imprinted X chromosome inactivation affects predominately paternal X chromosome; PSC, pluripotent stem cell; rXCI, random X chromosome inactivation represses paternal and maternal X chromosomes with equal probability; TS cell, trophoblast stem cell; Xa, active X chromosome; XCI, X chromosome inactivation; XCU, X chromosome upregulation; Xe, X chromosome with eroded XCI; XEN, extraembryonic endoderm stem cell; Xi, inactive X-chromosome; Xm, X chromosome of maternal origin; Xp, X chromosome of paternal origin.

***Address correspondence to:** Alexander I. Shevchenko. Federal Research Centre "Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences", Prospekt Lavrentyeva, 10, Novosibirsk 630090, Russia. Tel: +7(383)3634937. E-mail: epigene@bionet.nsc.ru - https://orcid.org/0000-0001-6535-1627.

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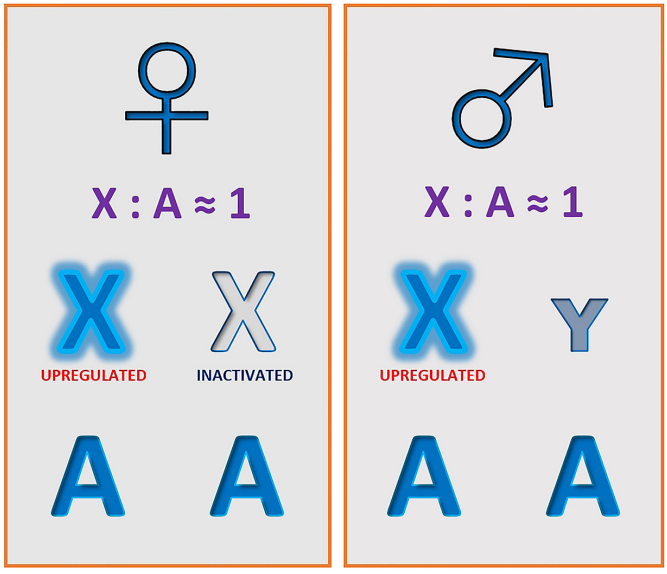

Fig. 1. Dosage compensation system in eutherian mammals. *In females (XX), one X chromosome is upregulated and the other is inactivated. In males (XY), the only X chromosome is upregulated. This provides equal expression of X-linked genes between sexes and equal expression between X-linked and autosomal genes. X, X chromosome; Y, Y chromosome; A, autosomal set. The upregulated X chromosome is highlighted in blue; the inactive X chromosome is shown in white and the Y chromosome is indicated in grey.*

switched off in gametogenesis despite the fact that different genes may use various mechanisms to increase transcription level (Borensztein *et al.,* 2017b; Nguyen and Disteche 2006; Sangrithi *et al.,* 2017).

Disruptions in dosage compensation revealed, mainly, due to XCI studies lead to dramatic effect on organism. Defects in XCI during embryogenesis result in fetus death (Borensztein *et al.,* 2017b; Lee 2000; Marahrens *et al.,* 1997). Imbalance of gene expression caused by sex chromosome aneuploidies leads to a number of genetic syndromes (Nagaoka *et al.,* 2012). Xi reactivation in female cells may cause oncologic (Dunford *et al.,* 2017; Spatz *et al.,* 2004), autoimmune (Forsdyke 2009), and age-related diseases (Ostan *et al.,* 2016).

Balance of dosage compensation mechanisms varies between developmental stages and mammalian species. Moreover, X-linked genes are differently involved in dosage compensation. In the review, relationships between XCU and XCI and their dependence on sex, developmental stage, cell type, tissue, and mammalian species will be discussed.

X chromosome upregulation

Dosage compensation between X-chromosome and autosomal genes was first postulated by Susumu Ohno in the 1960s (Ohno 1967). However, XCU at chromosomal level was confirmed only in the last decade and studied in detail using transcriptomic data obtained from microarray (Lin *et al.,* 2007; Nguyen and Disteche 2006), RNA sequencing (Deng *et al.,* 2011, 2013; Julien *et al.,* 2012; Lin *et al.,* 2011; Xiong *et al.,* 2010; Yildirim *et al.,* 2011), and ribosome profiling assays (Faucillion and Larsson, 2015). XCU was revealed by calculating X:A ratio obtained by dividing median gene expression levels of X-linked genes by that of autosomal genes. X:A close to 1 means equal expression of X-linked and autosomal genes, implying that XCU takes place. XCU was found in mouse, rat, and human somatic tissues, early embryos, and embryonic stem cells. Nonetheless, in several studies, X:A ratio was found close to 0.5, rejecting XCU (Julien *et al.,* 2012; Xiong *et al.,* 2010). Re-examination of the inconsistent result demonstrated that Xa comprised a large portion of transcriptionally silenced genes decreased median level of X-linked gene expression (Deng *et al.,* 2011; Kharchenko *et al.,* 2011). Exclusion of numerous tissue-specific genes with extremely low and high expression levels as the genes were not relevant for the evaluation of dosage compensation restored X:A ratio up to 1. However, XCU was not confirmed at the translation level using proteome analysis (Chen and Zhang 2015).

As XCI, XCU initiates in early embryogenesis. In mice, according to single cell RNA sequencing data, XCU was observed from the 4-cell stage (Borensztein *et al.,* 2017b). Doubling transcription on mouse Xa 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 XCU in somatic tissues (Deng *et al.,* 2014; Pessia *et al.,* 2012). XCU 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 inactivate 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 trophectoderm cells rather than in preimplantation mouse embryo (Maclary *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 (Borensztein *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 (Borensztein *et al.,* 2017b).

In mouse preimplantation development, expression of X-linked genes can remain unbalanced (Borensztein *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 accounted 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 (Borensztein *et al.,* 2017b; Namekawa *et al.,* 2010). Absence of *Xist* expression during mouse iXCI causes global changes in transcriptome of preimplantation embryo cells (Borensztein *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 placentas 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 trophectoderm 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 vole 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 *Xist* RNA as well as trimethylated H3K9 and H4K20, HP1β (CBX1), and HP1γ (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, *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 *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 *Xist* expression which is a key event in XCI initiation. Nuclear non-coding RNA of the *Jpx* gene located upstream to the *Xist* promoter is one of the candidates (Chureau *et al.,* 2002). *Jpx* (also known as *Enox*) was found in other rodents, e.g. rats and common voles (Shevchenko *et al.,* 2011). *Jpx* RNA is a dosage-dependent *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 *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 *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 *Xist* promoter. *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 *Xist* repression and *Tsix* activation (Navarro *et al.,* 2008, 2010). For instance, depletion in OCT4, transcription factor responsible for pluripotency maintenance, causes *Xist* activation on both X chromosomes in mouse ESCs (Donohoe *et al.,* 2009). Increased level of the *Rnf12*/RLIM ubiquitin ligase also can upregulate *Xist* during differentiation of cultured female pluripotent cells (Jonkers *et al.,* 2009). *Rnf12*/RLIM may affect stability of transcription factors and their binding to *Xist*/*Tsix* regulatory elements in dosage dependent manner. However, epiblast cells 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 *Xist* adjacent regions immediately before rXCI (Lee 2011; Masui *et al.,* 2011). The pairing appears to make asymmetry necessary for monoallelic *Xist* expression triggering. Before rXCI initiation, *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 *Xist* upregulation and rXCI initiation (Lee 2011; Masui *et al.,* 2011). However, another study showed that *trans*-activator RLIM, *cis*-acting elements surrounding *Xist* like *Jpx* and those involved in pairing events ensure *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 *Xist* transcription start site, which has a functional significance to repress *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).

Xist RNA spreading, transcriptional silencing initiation and stabilization

After *Xist* activation, *Xist* RNA spreads along the X chromosome and causes X-linked gene transcription silencing which is *Xist*-dependent at early stages of rXCI (Wutz *et al.,* 2002; Wutz and Jaenisch 2000). *Xist* RNA acts via recruiting proteins. To date 80 proteins are shown to interact directly or indirectly with *Xist* RNA and their functional role in XCI was confirmed using different methods (Przanowski *et al.,* 2018). The proteins bind to *Xist* RNA in the regions of conservative microsatellite repeats denoted with Latin letters – from A to F. Some of the *Xist*-interacting proteins are discussed in this sub-section and listed in Table 1.

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
CIZ1, *CDKN1A Interacting Zinc Finger Protein 1*	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 ribo-nucleoprotein complexes	central parts of exons 1 and 7	*Xist* RNA spreading and accumulation on Xi
YY1, *YY1 Transcription Factor*	Transcription factor belonging to GLI-Kruppel class of zinc finger proteins	C-repeats, exon 1	*Xist* RNA spreading and accumulation on Xi
WTAP, *WT1 Associated Protein*	A subunit of the RNA N6-methyltransferase complex	A-repeats, exon 1	*Xist*-dependent transcriptional silencing
SPEN, *Split-end Family Transcriptional Repressor*	Interactions with different transcriptional repressors, RNA binding	A-repeats, exon 1	*Xist*-dependent transcriptional silencing
RBM15, *RNA Binding Motif Protein 15*	Split-end (SPEN) protein family member, participation in RNA methylation	A-repeats, exon 1	*Xist*-dependent transcriptional silencing
HDAC3, *Histone Deacetylase 3*	Histone deacetylase activity, transcription repression	A-repeats, exon 1	*Xist*-dependent transcriptional silencing
NCoR2 (SMRT), *Nuclear Receptor Corepressor 2*	Transcriptional silencing	A-repeats, exon 1	*Xist*-dependent transcriptional silencing
JARID2, *Jumonji and AT-Rich Interaction Domain Containing 2*	Known as a transcriptional repressor which is able to interact with PRC2 complex	A-repeats, exon 1	*Xist*-dependent transcriptional silencing
ATRX, *Alpha Thalassemia/ Mental Retardation Syndrome X-Linked*	Chromatin remodeler	A-repeats, exon 1	*Xist*-dependent Xi chromatin modifications
PRC2, *Polycomb repressive complex 2*	Chromatin modifier with histone methyltranferase activity	A-repeats, exon 1; exon 7	*Xist*-dependent Xi chromatin modifications
PRC1, *Polycomb repressive complex 1*	Chromatin modifier with ubiquitin ligase activity	B-repeats, exon 1	*Xist*-dependent Xi chromatin modifications

All references are given in the text and in Przanowski *et al.,* 2018.

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 CIZ1 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 CIZ1 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). CIZ1 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, WTAP, SPEN, ATRX, and JARID2 (rewieved 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, WTAP 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 intermediator 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 PCGF3/5-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 – monoubiquitinilated H2AK119 and H3K27me3. Other proteins and chromatin modifications further appear on Xi but their recruitment does not depend on *Xist* RNA. MacroH2A, the ASH2l trithorax protein, CDYl, DNA hypermethylation of gene promoter regions, the DNMT3b *de novo* DNA methyltransferase, as well as SMCHD1 and LRF1 (or HBiX1) proteins responsible for chromatin compaction are also detected on Xi (rewieved in Moindrot and Brockdorff 2016). Thus, X chromosome inactive state stabilizes and the stage of rXCI maintenance begins.

Xi 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

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 (Berletch *et al.,* 2011; Dementyeva *et al.,* 2010). In mice, just about 3% of X-linked genes escape rXCI (Berletch *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; Przanowski *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 *Xist* RNA 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 (Chaumeil *et al.,* 2011).

About 15% of human X-linked genes escape XCI (Berletch *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 XCI that are sensible 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, *Xist* expression 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). XCI 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

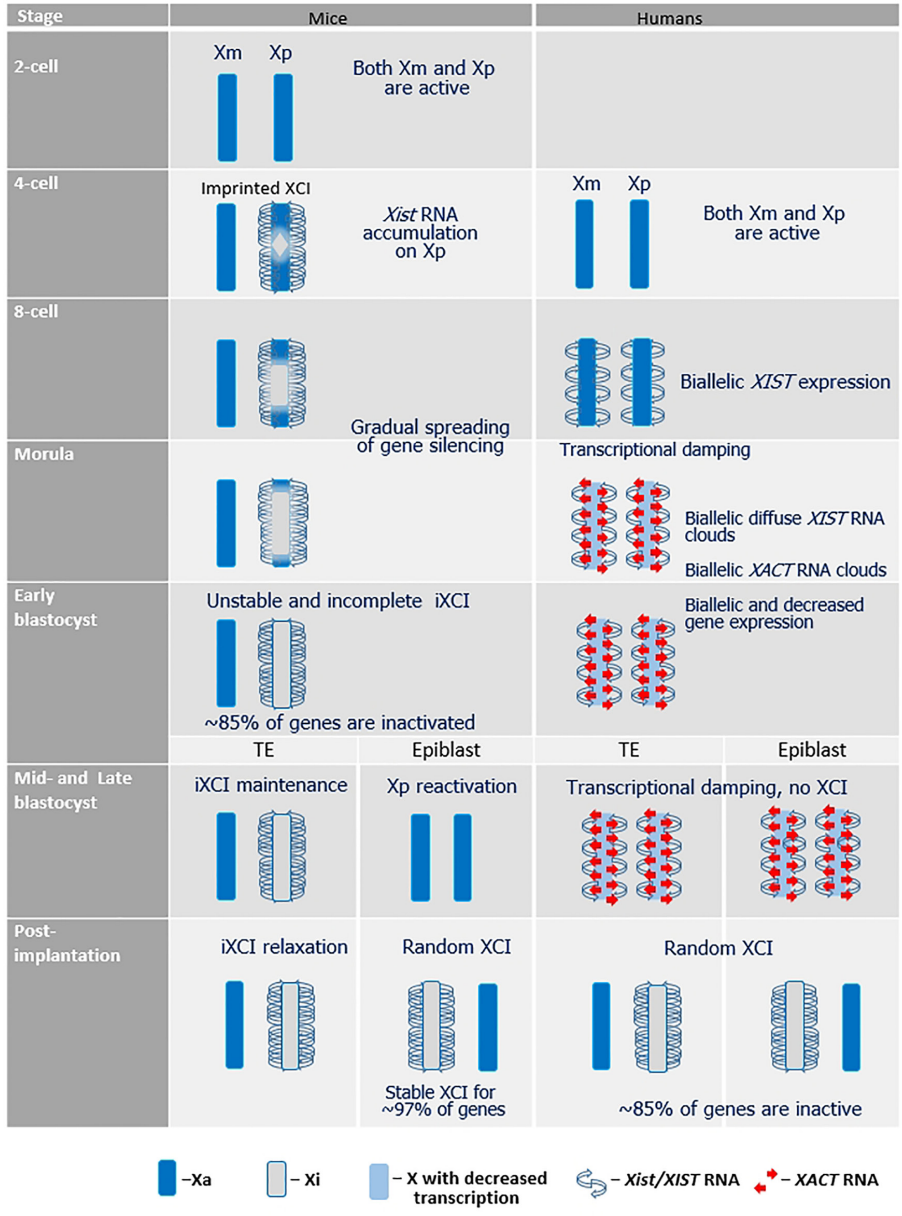

Fig. 2. Diverse strategies of X chromosome inactivation in mice and humans. *In mice, XCI initiates at the 4-cell stage. XCI is imprinted and* Xist *is expressed exclusively on Xp.* Xist *RNA gradually spreads along Xp and induces gene silencing. The process is completed by the blastocyst stage. In epiblast of late blastocyst, Xp is reactivated and subsequent XCI is random. In trophectoderm, XCI retains imprinted but degree of gene silencing is reducing with time. In humans,* XIST *expression initiates later, at the 8-cell stage, on both X chromosomes. X-linked genes are biallelically expressed but their expression level is reduced. The transcriptional damping may be due to interaction of* XIST *RNA and its antagonist,* XACT *RNA. XCI is established only at postimplantation stages and it is at once random. Xm and Xp, maternal and paternal X chromosomes; Xa and Xi, active and inactive X chromosomes; XCI, X chromosome inactivation; iXCI, imprinted X chromosome inactivation; TE, trophectoderm.*

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 WTAP15, RBM15, SPEN2, etc. decreases gene transcription and initiates their inactivation. At the same time, *XACT* RNA eliminates the effect not allowing XCI to be stabilized and to become irreversible. However, it is still unclear why transcriptional damping is not detected in male embryos where *XIST* and *XACT* RNA are also co-localized on Xa. Another study argues the idea of transcriptional damping. In early embryogenesis, decreasing in biallelic expression and subsequent increasing in monoallelic expression were observed, which was in accordance with classical conception of rXCI (Moreira de Mello *et al.*, 2017). Moreover, active X chromosomes were upregulated 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 XCU 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 Xi, 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 Xi, 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 Xi. 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. A recent 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 plupipotency 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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