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X-Chromosome Inactivation during Preimplantation Development and in Pluripotent Stem Cells

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Pluripotent stem cells · Preimplantation embryo · X-chromosome inactivation · X-chromosome reactivation · *Xist*

Abstract
X dosage compensation between XX female and XY male mammalian cells is achieved by a process known as X-chromosome inactivation (XCI). XCI initiates early during preimplantation development in female cells, and it is subsequently stably maintained in somatic cells. However, XCI is a reversible process that occurs in vivo in the inner cell mass of the blastocyst, in primordial germ cells or in spermatids during reprogramming. Erasure of transcriptional gene silencing can occur though a mechanism named X-chromosome reactivation (XCR). XCI and XCR have been substantially deciphered in the mouse, whereas they still remain debated in the human. In this review, we summarized the recent advances in the knowledge of X-linked gene dosage compensation during mouse and human preimplantation development and in pluripotent stem cells.

X-chromosome inactivation (XCI) is a multistep compensatory mechanism through which one of the 2 female X-chromosomes is randomly inactivated to equalize X-linked gene expression between the mammalian sexes [Disteche, 2016]. Random XCI is established during preimplantation development and then maintained in all female somatic cells of the new organism. This process, first proposed by Mary Lyon about 60 years ago [Lyon, 1961, 1962], consists of global chromatin condensation. It is mediated by epigenetic modifications which induce transcriptional silencing of most genes on the inactive X-chromosome (Xi), with the exception of few "escapee genes" that remain active with cell and tissue specificity [Tukiainen et al., 2017]. Thus, females are mosaics with respect to allelic X-linked gene expression.

The initiation of XCI is controlled by the X-inactivation center (Xic), a complex X-linked locus which, in the mouse (Fig. 1A), contains a variety of *cis*- and *trans*-acting players (long noncoding sequences and protein-coding genes). Within Xic, *Xist* (X inactive specific transcript), an untranslated spliced 17-kb-long noncoding RNA (lncRNA), whose role was described at the beginning of the 1990s [Borsani et al., 1991; Brown et al., 1991, 1992; Clemson et al., 1998], was historically considered the first actor mediating the silencing during the XCI

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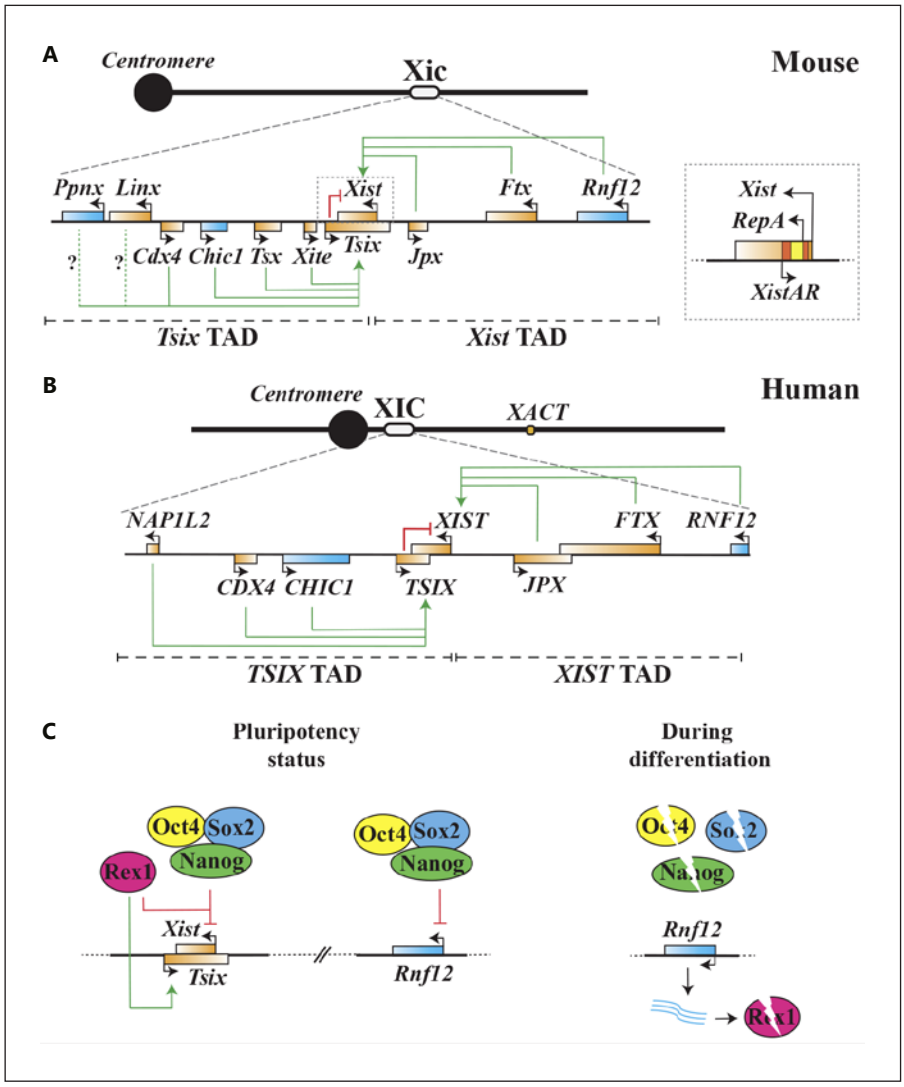

Fig. 1. Mouse (**A**) and human (**B**) X-inactivation centers (Xic/XIC), located on the X chromosomes, contain noncoding (orange) and protein-coding (blue) genes, partitioned in 2 topologically associated domains (TAD), that integrate negative (*Tsix* TAD) and positive (*Xist* TAD) regulators of *Xist* expression. **A** In the gray-framed square, *Xist*, *RepA*, and *XistAR* are reported. The distance among genes is not in scale. **C** Molecular link between pluripotency factors and *Xist* repression. Oct4, Sox2, Nanog, and Rex1 bind to *Xist* intron 1 and *Rnf12* promoter repressing their transcription. They also contribute to *Tsix* activation. During differentiation, Rnf12 targets pluripotency protein Rex1, inducing its degradation to initiate X-chromosome reactivation and promoting *Xist* expression.

process. In the mouse, this lncRNA is transcribed at low level from both active X (Xa) chromosomes prior to the initiation of XCI; then, it is upregulated and expressed from the presumptive inactive X (Xi) through a multicomponent silencing process [Brown et al., 1992; Clemson et al., 1998] that leads to gradual chromosome coating and silencing. *Xist*, promoting the recruitment of chromatin remodelers (such as polycomb repressive complex 1 and 2), histone deacetylases, histone variants, and the DNA methylation machinery [Lee, 2012; McHugh et al., 2015; Minajigi et al., 2015; Almeida et al., 2017], modifies the chromatin organization of the X-chromosome and its positioning within the nucleus [Mira-Bontenbal and Gribnau, 2016; da Rocha and Heard, 2017]. Embedded within *Xist* exon 1, an antisense lncRNA, called *Xist*-activating antisense RNA (*XistAR*), is co-expressed with *Xist* only by the inactive X-chromosome. Although its function is not fully understood, *XistAR* may drive or enhance *Xist* expression rather than mediate its elongation [Sarkar et al., 2015]. Like *XistAR*, a repeat sequence, termed "A" repeat (*RepA*), contained within the first *Xist* exon, codes for a 1.6-kb lncRNA transcript, in the same orientation as *Xist*. *RepA* RNA interacts with polycomb repressive complex 2 (PRC2) proteins, and the PRC2–RepA complex locally trimethylates H3K27 at the 5′ end of *Xist*, creating the heterochromatic patch essential for *Xist* transactivation [Sun et al., 2006]. Thus, RepA is an activator of *Xist* expression and is necessary for *Xist* upregulation [Zhao et al., 2008; Maclary et al., 2013].

Xist is negatively regulated by *Tsix*, a spliced 40-kb-long lncRNA, transcribed in an antisense direction through the *Xist* locus. It operates as *cis*-acting repressor of *Xist* upregulation [Stavropoulos et al., 2001] by inducing repressive chromatin modifications, i.e., H3K9me3 and CpG methylation at the *Xist* promoter [Morey et al., 2004; Navarro et al., 2005, 2006, 2009]. In turn, *Tsix* is regulated by *Xite* [Ogawa and Lee, 2003], a proximal noncoding element that interacts with *Tsix*'s promoter [Tsai et al., 2008] and sustains its expression on the future Xa [Ogawa and Lee, 2003]. In addition to *Xist*, *Tsix*, and *Xite*, a number of *cis*-acting regulatory elements are also located in the Xic locus, such as the neighboring *Jpx* [Tian et al., 2010], *Linx* [Nora et al., 2012], and *Ftx* [Chureau et al., 2011] genes, as well as the protein-coding genes *Rnf12* [Barakat et al., 2011], *Chic1*, *Xpr* [Augui et al., 2007, 2011], *Ppnx*, and *Nap1L2* [Chureau et al., 2002]. These elements act cooperatively for the induction of monoallelic *Xist* expression from the Xi (Fig. 1A).

The human XIC region (Fig. 1B) shows both similarities and differences when compared to that of the mouse. For example, the region between *XIST* and *JPX* is about 90 kb in humans, compared to the 9 kb in mice [Chureau et al., 2002]. *XIST*, located at the center of the XIC, partially overlaps with the repressive antisense gene *TSIX*. This latter one is not transcribed through the entire *XIST* locus as it is in mice and displays little sequence conservation between the 2 species. The genes *JPX*, *FTX*, and *RNF12* are also present in human XIC. In addition, a human-specific lncRNA, named *XACT* (X-active coating transcript, 252 kb) [Vallot et al., 2013], participates in the compensatory mechanism occurring during the early stages of development [Petropoulos et al., 2016] (see below).

XCI from Zygote to Blastocyst

XCI occurs in mouse and human preimplantation embryos with several differences, suggesting high plasticity of XCI regulation across species mediated by species-specific lncRNAs.

In the mouse, 2 subsequent forms of X inactivation are operative: imprinted and random (Fig. 2). The female mouse zygote inherits the maternal X (X^m) in an active state (Xa), whereas it is not completely defined whether the paternal X (X^p) is inherited active [Talon et al., 2019] or, alternatively, in a pre-inactivated state. Although random XCI is female-specific, X silencing begins also in the mouse male germline [Lifschytz and Lindsley, 1972], although through a different mechanism type. In spermatocytes, during the first meiotic prophase, chromosomes undergo "meiotic sex chromosome inactivation" with the formation of the "sex body." At the end of meiosis, the sex chromosomes do not wholly reactivate, and about 85% of genes on the X-chromosome remain transcriptionally suppressed in postmeiotic cells [Namekawa et al., 2006]. X^p-linked genes are reactivated at the zygote stage, with the exception of some repetitive elements [Lee and Bartolomei, 2013] that might induce the preferential X^p inactivation later in the female early embryo [Cooper, 1971; Lyon, 1999; Huynh and Lee, 2003].

The first wave of XCI occurs at the 2/4-cell stages, rapidly after zygotic genome activation (ZGA). Histone deacetylation and H2AK119 ubiquitination are the earliest chromatin changes occurring during XCI. Then, *Xist,* acting in *cis*, selectively coats the X^p (imprinted XCI; Xi^p), accompanied by the accumulation of repressive H3K27me3 histone modifications, the PRC2 remodeling complexes, containing Ezh2 and Eed enzymes, the loss of activating H3K4 methylation and H3K9 acetylation, the increase of H3K9me3, the inclusion of non-canonical histones (e.g., macro-H2A), and extensive DNA methylation (Fig. 2). All together, these modifications lead to transcriptional silencing, due to strong chromatin condensation into a perinuclear structure [Jeon et al., 2012]. Xi^p is maintained during preimplantation development in morula blastomeres and then in the trophectoderm, following blastocyst formation (Fig. 2). Upon implantation, within 24 hours, mouse epiblast (Epi) cells undergo random XCI (Xi^p/Xa^m or Xa^p/Xi^m) (Fig. 2), and the embryo will develop as a mosaic containing cells with either Xi^p or Xi^m. The relative ratio of *Xist/Tsix* expression controlling the initiation of random XCI and the transcriptional upregulation of *Xist*, regulated by the pluripotency factors [Gribnau and Grootegoed, 2012] (Fig. 1C), represents the molecular switch that triggers XCI. At 6.5 days post coitum (E6.5), almost all Epi cells have undergone XCI [Rastan et al., 1980; Rastan, 1982], leading to monoallelic expression of most X-linked genes.

Different from the mouse, in human preimplantation embryos, the X^p does not undergo imprinted inactivation; however, due to their limited availability, different sources and culture conditions, the precise mechanism of dosage compensation in our species remains still debated [Saiba et al., 2018]. Recently, 2 differing models (Fig. 2), based on single-cell RNA sequencing (scRNA-seq), have been proposed: (1) X dampening, Xd [Petropolous et al., 2016] or (2) X inactivation, Xi [Moreira de Mello et al., 2017]. The female zygote inherits active X^m and X^p

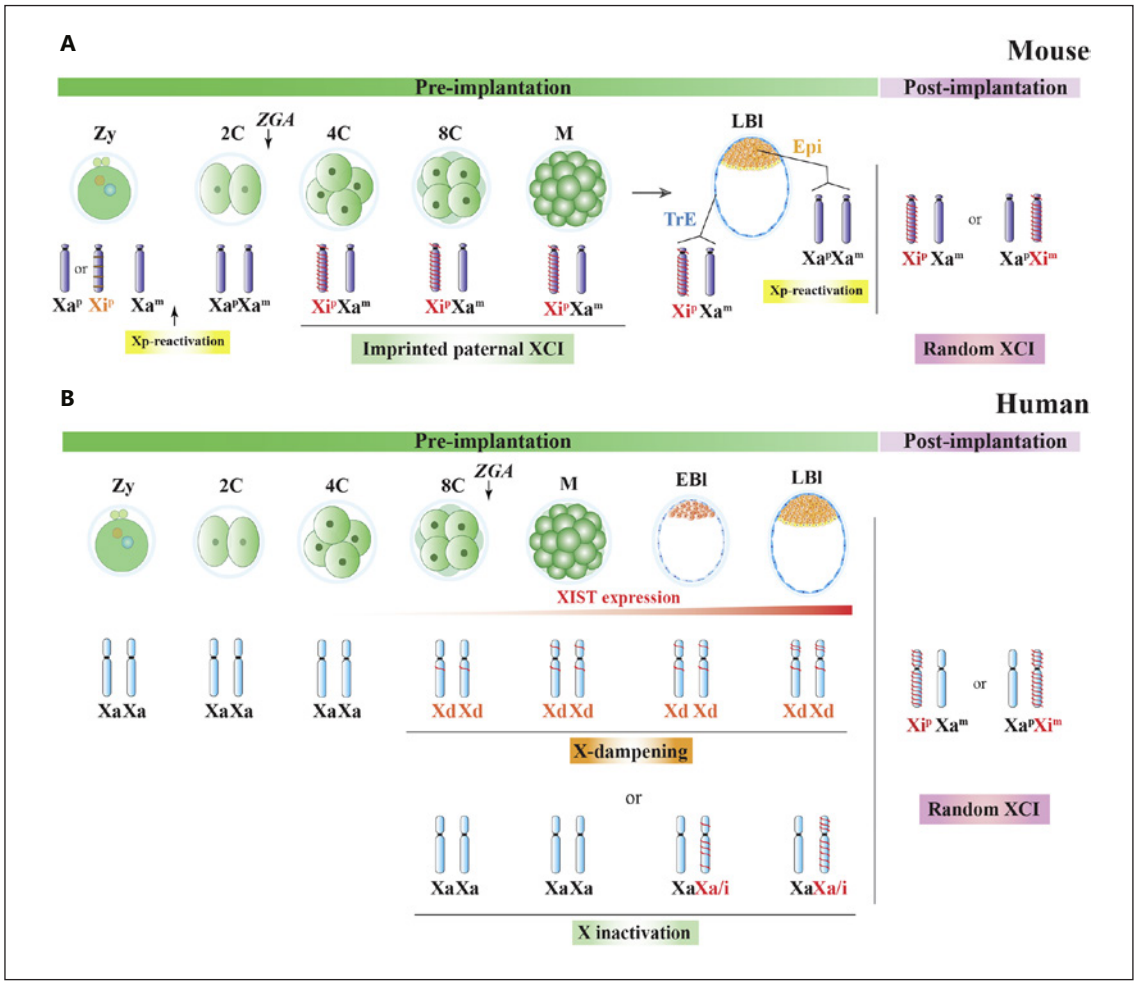

Fig. 2. Schematic representation of X chromosome inactivation (XCI) dynamics during mouse (**A**) and human (**B**) early development. **A** In the mouse, following zygotic genome activation (ZGA), only the paternal X chromosome (X^P) undergoes *Xist*-mediated silencing (Xi^P). At the late blastocyst (LBl) stage, Xi^P is maintained in the trophectoderm (TrE), whereas in epiblast progenitor cells (Epi) the inactive X^P is reactivated. Upon implantation, Epi cells undergo random XCI. **B** XCI models in human. In the dampening model, following the biallelic *Xist* expression starting at the 8-cell stage, the biallelic expression of X-linked genes is reduced until the LBl stage. On the contrary, according to the X inactivation model, monoallelic *Xist* expression leads to random XCI, completed following implantation. Zy, zygote; M, morula; EBl, early blastocyst.

[Pasque and Plath, 2015]; they biallelically express their genes till the 8-cell stage of development. According to the dampening model, soon after ZGA biallelic *XIST* expression starts, and the levels of its lncRNA progressively increase from the 8-cell to the blastocyst stage. In turn, the biallelically expressed X-linked genes become gradually downregulated till the late blastocyst embryos reaching dosage compensation through Xd/Xd [Petropolous et al., 2016].

Using the same scRNA-seq data set, but excluding the genes located in the pseudoautosomal regions from the analysis and adopting more stringent evaluation parameters, Moreira de Mello et al. [2017] recorded concomitant decrease of biallelic and increase of monoallelic expression of the majority of X-linked genes during preimplantation development. *XIST* expression and its accumulation on Xa begins at the 8-cell stage rapidly after ZGA. *XIST* and the lncRNA *XACT* co-accumulate, and this latter seems to control the association of *XIST* to the putative Xi in *cis*, possibly to antagonize or temper its silencing ability [Vallot et al., 2017]. Nevertheless, it is still debated whether X-linked genes are monoallelically or biallelically expressed, reflecting the possible dampening expression of a still undetermined process.

All cells composing the preimplantation embryo display the same pattern of X inactivation, retained also in the postimplantation embryo and in the somatic cells of the future adult organism. However, recently, Moreira de Mello et al. [2017] suggested that, upon implantation, definitive dosage compensation is reached by the complete inactivation of one Xd, which becomes Xi, whereas the other Xd undergoes upregulation, reaching Xa state.

In summary, despite different mechanisms, in both humans and mice, XCI is determined by 3 different sequential phases: (1) initiation, (2) establishment, and (3) maintenance of the Xi. In the initiation phase, activators and inhibitors of XCI, localized in XIC, finely regulate *Xist* expression. In the establishment phase, *Xist*, acting in *cis*, coats the entire future Xi, resulting in the loss of active histone marks and gain of inactive histone marks, contributing to the silencing process, together with the recruitment of enzymes that catalyze chromatin remodeling. Once XCI is complete, the Xi is stably maintained and clonally propagated through cell divisions.

XCI in Mouse and Human Embryonic Stem Cells in vitro

Embryonic stem cells (ESCs) are pluripotent stem cells (PSCs), isolated from the inner cell mass (ICM) of mouse blastocysts in 1981 [Martin, 1981; Evans and Kaufman, 1981] and of human blastocysts in 1998 [Thomson et al., 1998]. PSCs were also obtained from the mouse epiblast (Epi stem cells, EpiSCs) [Tesar et al., 2007; Brons et al., 2007]. Pluripotency, broadly defined as the capacity to give rise to several different cell types, is a transient and highly dynamic state typical of both ICM and epiblast [Weinberger et al., 2016] that can be successfully maintained in vitro under artificially induced self-renewal culture conditions [Nichols and Smith, 2012].

ESCs and EpiSCs differ in their degree of pluripotency, corresponding to the in vivo early and late phases of pluripotency, respectively [Nichols and Smith, 2009]. Mouse ESCs show a "naïve" pluripotency, reflecting the molecular and cellular properties of the ICM (E3.5) or preimplantation epiblast (E4.5) [Boroviak and Nichols, 2017], whereas mouse EpiSCs, display a "primed" pluripotency, as that of postimplantation epiblast (E6.5–7.0). Naïve mouse ESCs and primed mouse EpiSCs show high expression of the pluripotency factors Oct4 and Sox2, whereas they differ in Nanog and pluripotency-associated transcription factor (Klf2, Klf4, Prdm14, Sall4, Tfcp2l1, Esrrb, and Tbx3) expressions, which are drastically lower in primed cells [Nichols and Smith, 2009]. In addition, female mouse naïve ESCs and primed EpiSCs exhibit different X-chromosome states, strictly correlated to their differential cell potency. Naïve ESCs have 2 Xa chromosomes, reflecting the molecular and functional feature of the naïve pluripotent embryonic Epi cells [Silva et al., 2009]. Instead, primed EpiSCs have an Xa and an Xi, mediated by *Xist* expression.

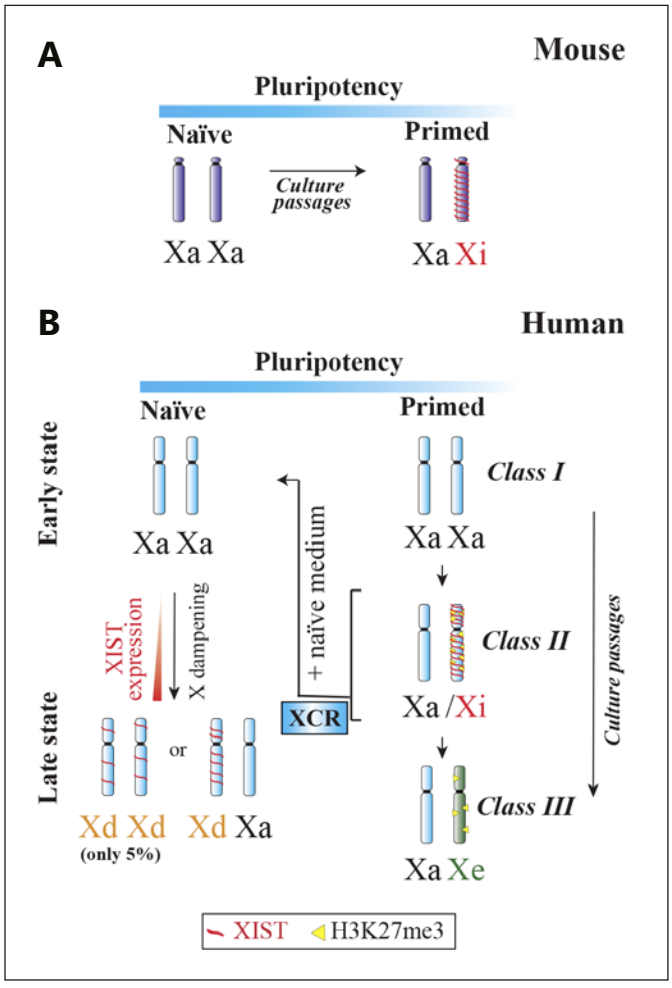

Fig. 3. Schematic representation of X chromosome inactivation in mouse and human naïve and primed embryonic stem cells (ESCs). **A** Female mouse naïve pluripotent stem cells show 2 active X chromosomes (Xa), reflecting the embryonic feature of the naïve pluripotent epiblast cells. Primed pluripotent stem cells have an Xa and an inactive X (Xi) chromosome, silenced by *Xist*. **B** Human blastocyst-derived ESCs are in a Class I primed state. During culture, they gradually progress from Class I to Class III where an eroded X (Xe) is present. When cultured in a naïve medium, Class II ESCs can undergo X chromosome reactivation (XCR) (early naïve state). Then, biallelic *Xist* expression gradually dampens gene activity in both X chromosomes (dampened X, Xd) (late naïve state).

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During the in vitro establishment of EpiSCs lines, the changes of X-chromosome state observed in vivo are recapitulated, ending with randomly selected Xa and a *Xist* expressing Xi (Xa^{Xist-}/Xi^{Xist+}) (Fig. 3).

Unlike mouse ESCs, which can be easily maintained in a naïve ground state of pluripotency in vitro [Brook and Gardner, 1997; Nichols and Smith, 2009], human ESCs, although derived from the ICM, exhibit primed pluripotency, corresponding to that of mouse EpiSCs [Tesar et al., 2007; Rossant, 2015]. XCI in human ESCs does not recapitulate the molecular events active in preimplantation Epi cells in vivo leading to Xd^{XIST+}/Xd^{XIST+}. Instead, 3 distinct X-chromosome states are present in primed human ESCs, categorized into separated classes: (1) Class I: cells with both Xa chromosomes and with low or undetectable expression of *XIST* RNA (Xa^{XIST-}/Xa^{XIST-}) (as in naïve mouse ESCs); (2) Class II: cells in which *XIST* RNA expression and deposition of H3K27me3 lead to the random inactivation of one of the 2 X-chromosomes (Xi) (Xa^{XIST-}/Xi^{XIST+}); (3) Class III: cells in which *XIST* is downregulated and H3K27me3 depleted on the inactive X. Some Xi-linked genes undergo partial reactivation, generating an X eroded (Xe) chromosome (Xa^{XIST-}/Xe^{XIST-}) (Xe stands for an eroded Xi state) [Silva et al., 2008]. A gradual progression through the 3 classes (from Class I to Class III) occurs during prolonged culture of human ESCs in vitro (Fig. 3).

Regulation of *Xist* Transcription by the Core Pluripotency Transcriptional Gene Network

The establishment and the maintenance of pluripotency, both in vivo and in vitro, relies on strong cooperation of transcription and epigenetic factors, exerting a central role in the maintenance of ESC identity, activating self-renewal genes, and repressing lineage commitment genes [Young, 2011]. *Xist* lncRNA expression is regulated by several factors, which modulate its transcription in time and space. Among these, Navarro et al. [2008] demonstrated that, in both male and female undifferentiated pluripotent ESCs, Oct-4, Nanog, Sox2 (the central functional core of the pluripotency gene regulatory network) [Ng and Surani, 2011; Young, 2011; Niwa, 2014], and Rex1 co-bind to *Xist* intron 1. Also, Rex1 acts through the activation of *Tsix* [Navarro et al., 2010] (Fig. 1C). Their binding is sharply reduced in differentiating ESCs and almost undetectable in fully differentiated cells [Navarro et al., 2008] (Fig. 1C). *Nanog* or *Oct4* depletion leads to inappropriate *Xist* upregulation in male mouse ESCs or biallelic *Xist* upregulation in differentiating female mouse ESCs, suggesting their repressive role in *Xist* transcription and confirming the intimate relationship existing between the pluripotency gene regulatory network and *Xist* [Navarro et al., 2008; Donohoe et al., 2009]. Specifically, in male $Nanog^{-/-}$ ESCs, a moderate increase in *Xist* expression was detected, being an early consequence of Nanog deletion, but independent of *Tsix* downregulation. Also, in these cells, Oct4 and Sox2 remained bound to the *Xist* promoter, potentially preventing its complete re-activation. In male ESCs, *Oct4* silencing triggers the drastic loss of Oct4 itself, but also of Nanog and Sox2 from *Xist* intron 1, with the consequent rapid increase of *Xist* expression, but before any measurable downregulation of *Tsix,* a phenomenon also described in differentiating female ESCs [Navarro et al., 2008]. In addition, overexpression of *Rex1* leads to a marked reduction of *Xist* upregulation during ESC differentiation [Gontan et al., 2012].

Therefore, in undifferentiated ESCs, the triad Nanog, Oct4, Sox2 together with Rex1 acts synergistically to repress *Xist* transcription independently of *Tsix* [Navarro et al., 2008] (Fig. 1C).

X-Chromosome Reactivation during Development and Reprogramming

In female cells, X-chromosome reactivation (XCR) represents the opposite phenomenon to XCI, through which the Xi is reversed to an Xa form. This process leads to the erasure of the epigenetic memory, and it is achieved through 3 phases: (1) initiation, (2) progression, and (3) completion. These phases entail progressive transcriptional gene activation, changes in chromatin and epigenetic states, and in genome topology [Pasque and Plath, 2015; Talon et al., 2019].

During postimplantation development, once randomly established, the epigenetic memory determining X-linked gene silencing is stably inherited through cellular generations. However, in vivo, Xi is reactivated in different cell types like mouse Epi cells [Mak et al., 2004; Borensztein et al., 2017], mouse [Sugimoto and Abe, 2007; Chuva de Sousa Lopes et al., 2008; Mallol et al., 2019] and human [Von Meyenn and Reik, 2015; Vértesy et al., 2018] primordial germ cells (PGCs) and spermatids, during their differentiation into spermatozoa [Ernst et al., 2019].

XCR also occurs in vitro in several experimental conditions, including the transition from primed to naïve state of human ESCs or during somatic cell reprogramming into induced PSCs (iPSCs).

Mouse Epiblast Cells

X^P reactivation is effective within few hours in mouse Epi cells [Borensztein et al., 2017]. Although XCR correlates with Epi differentiation, reactivation of some genes starts in the blastocyst, before the explicit commitment of primitive endoderm and Epi precursor cells. The reactivation of some X^P-linked genes occurs in some ICM cells before *Xist* expression downregulation and H3K27me3 loss, suggesting that *Xist* silencing is not necessary for all X^P-linked genes to be reactivated [Williams et al., 2011; Borensztein et al., 2017]. This early XCR causes fluctuation and heterogeneous Xi status in cells between E3.5–4.0, rather than a constant maintenance of X^P silencing in the future primitive endoderm.

Later, the progressive biallelic gene reactivation is lineage-specific and restricted to the pre-Epi cells from the mid-stage blastocyst onwards and strongly correlates with silencing of *Xist*, the expression of the antisense *Tsix*, the complete loss of the epigenetic memory, and the expression of the Nanog pluripotency protein [Mak et al., 2004; Borensztein et al., 2017].

Mouse and Human PGCs

In female mouse embryos, PGCs display XCR, which initiates when, at E7.0, *Blimp1*-expressing Epi cells, destined to become PGC and displaying random XCI, start their migration to the genital ridges. *Xist* repression begins accompanied by a progressive drop of H3K27me3 levels. In this initial phase, few genes are biallelically expressed [Chuva de Sousa Lopes et al., 2008]. Between E7.5 and E9.5, the fraction of PGCs with prominent H3K27me3 accumulation on the Xi drastically declines concomitantly with 2 waves of DNA demethylation [de Napoles et al., 2007]. The first wave of global DNA demethylation occurs at E8.0, whereas the second wave, at E9.0–9.5, covers those X-linked genes previously protected from demethylation erasure [Hargan-Calvopina et al., 2016], leading to biallelical expression of most X-linked genes between E10.5 and E12.

PRDM14, a site-specific DNA-binding protein, is important for XCR in PGCs, as it contributes to the very low global DNA methylation characteristic of these cells, by repressing DNA methyltransferases and recruiting TET DNA demethylases [Okashita et al., 2014]. In addition, very recently, it has been demonstrated that it regulates the removal of H3K27me3 from the Xi chromosome along the PGC migration path [Mallol et al., 2019]. However, at E14.5, XCR is not yet complete, suggesting that XCR in PGCs is slower than in Epi cells [Sugimoto and Abe, 2007].

In human PGCs (hPGCs), XCR is a process still partially unknown. Global analysis of X-chromosome expression and allelic investigation of selected genes, known to escape XCI, suggested that the X-chromosome is already reactivated in 4–5.5-week embryos [Guo et al., 2015; Li et al., 2017]. However, more recently, it has been shown that about 30% of hPGCs at 4–9 weeks of development still exhibit incomplete XCR, as suggested by the presence of faint perinuclear spots of H3K27me3, a marker of XCI. XCR appears to be more related to the transcriptional signature of the cells rather than to the fetal age [Vértesy et al., 2018]. These observations suggest that, in hPGCs, XCR is heterogeneous and asynchronous, starting from 4 weeks of development onward [Guo et al., 2015; Tang et al., 2015].

In hPGCs, *XIST* is expressed regardless of the XCR status [Gkountela et al., 2015; Vértesy et al., 2018]. Its expression is not associated with H3K27me3 gathering [Tang et al., 2015], and it is unknown whether it accumulates on the X-chromosomes. Thus, similar to preimplantation development, X-chromosome expression in the female human germline does not depend on the presence of *XIST*, but rather on its ability to trigger chromosome silencing, although with a still elusive mechanism.

Mouse Spermatids

During spermiogenesis, postmeiotic cells undergo XCR. By combining bulk and single-cell RNA-seq approaches, Ernst et al. [2019] showed that, during spermatid differentiation into spermatozoa, the X-chromosome undergoes an extensive postmeiotic chromatin remodeling. X-linked genes that were strongly repressed by H3K9me3 in spermatocytes acquire an active chromatin state and are gradually reactivated, generating a spermatid-specific X-linked gene expression. The early reactivation event involves members of the *Ssxb* multi-copy gene family (*Ssxb1*, *Ssxb2*, and *Ssxb3*), *Rhox11*, *Mageb5*, and *Slxl1* genes, which might have an active role in postmeiotic XCR [Ernst et al., 2019].

Human Embryonic Stem Cells

The conversion from primed human ESCs to a naïve-like state, induced by specific naïve 5iLAF [Theunissen et al., 2014, 2016] or t2iLGö [Takashima et al., 2014] growth media, entails XCR. This transition involves progressive *XIST* silencing, as well as *XACT* reactivation [Vallot et al., 2015; Theunissen et al., 2016], associated with the reduction of repressive H3K27me3 and H3K9me3 histone marks [Gafni et al., 2013; Theunissen et al., 2014; Ware, 2017]. These events generate an intermediate *XIST*-neg-

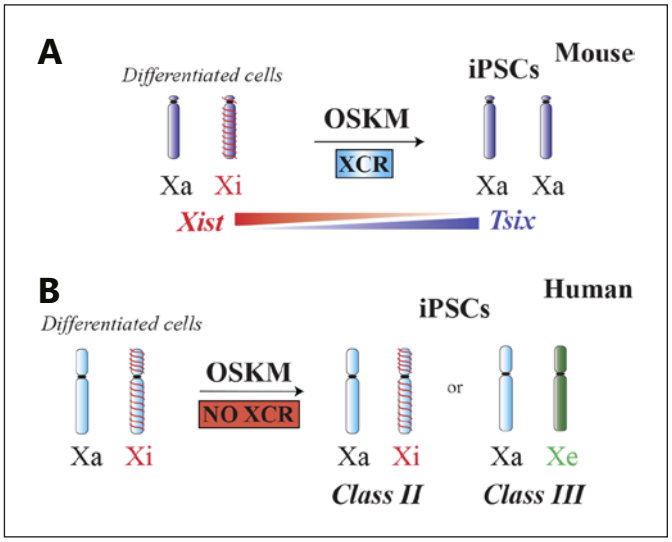

Fig. 4. X-chromosome reactivation (XCR) during somatic cell reprogramming. **A** In the mouse, after reprogramming with OKSM factors, induced pluripotent stem cells (iPSCs) have 2 Xa chromosomes. **B** After reprogramming, human iPSCs do not undergo XCR. An inactive X (Xi) or, following partial reactivation, an eroded X (Xe) are present.

ative status named "early naïve state." In this phase, the inactive X-chromosome is reactivated, giving rise to XaXa cells. RNA-FISH and RNA-seq analyses revealed that XCR occurs within 4 passages in naïve media and is completed primarily during the conversion from primed to naïve state of pluripotency [Collier et al., 2017; Sahakyan et al., 2017].

However, progressively during culture, *XIST* expression is reactivated, generating *XIST*-positive cells, where XCI does not occur. Instead, compared to the early naïve *XIST*-negative cells, *XIST*-positive cells progressively evolve to a "late naïve state" of pluripotency, showing reduction of X-linked gene expression through the dampening of X-linked gene expression from both X-chromosomes (Fig. 3).

Recently, the different accumulation of *XIST* on X-chromosomes between blastocysts and naïve human ESCs raised the question of whether naïve human ESCs truly reflect the X-chromosome dampening of preimplantation embryos. Indeed, in blastocysts, *XIST* accumulation on both X-chromosomes is observed in about 80–85% of cells [Okamoto et al., 2011; Petropoulos et al., 2016], whereas it is observed in only 5% of naïve human ESCs. In preimplantation embryos, the initiation of *XIST* expression and X-chromosome dampening through its coating occur simultaneously, suggesting direct *XIST* involvement in the X dampening phenomenon. Although X dampening also occurs in naïve human ESCs, the majority of them harbor the *XIST* coat on only 1 X-chromosome (Fig. 3).

Recent studies suggested that both male and female human primed ESCs displayed an upregulated state of X-chromosome genes [Lin et al., 2011; Moreira de Mello et al., 2017]. Based on these observations, more recently, Kaur et al. [2020] proposed that primed human ESCs harbor an Xi and an upregulated Xa, named X2a. Upon transition to the early naïve state, Xi-linked genes are reactivated, generating an Xa/i chromosome, whereas X2a-linked genes are progressively downregulated, leading to an X2a/a chromosome. The Xi-to-Xa and X2a-to-Xa transitions get completed in late naïve cells, finally giving rise to XaXa cells. In this hypothesis, the conversion from primed to naïve state induces an erasure of X-chromosome upregulation in female naïve human ESCs, leading to a reduction in X-linked gene expression, instead of the dampening phenomenon on 2 active X-chromosomes [Kaur et al., 2020].

Somatic Reprogramming to Induced Pluripotent Stem Cells

In vitro reprogramming of somatic cells into iPSCs entails profound changes in genome organization, DNA methylation, histone acetylation and methylation, and gene expression [reviewed in Apostolou and Hochedlinger, 2013] and, among these, XCR is mandatory for the faithful reprogramming of the founder cells to pluripotency. In mouse cells, XCR is a progressive and slow event [Stadtfeld et al., 2008; Payer et al., 2013] that takes about 1 week to occur [Janiszewski et al., 2019], and it is strongly linked to the sequential hierarchical activation of pluripotency-associated genes (*Esrrb*, *Sall4*, and *Lin28*) [Buganim et al., 2012; Pasque et al., 2014]. The expression of the reprogramming factors Oct4, Sox2, Klf4, and cMyc (OSKM) in female fibroblasts is not sufficient for *Xist* repression, suggesting an active role of the other pluripotency-associated factors. Immediately after the exposure to the OSKM reprogramming factors [Takahashi and Yamanaka, 2006], an upregulation of cadherin-1 (*CDH1*) occurs, starting the XCR process. This event is followed by the enrichment of the PRC2 protein EZH2 on the Xi, after the mesenchymal-to-epithelial transition, but before the activation of the endogenous pluripotency genes. Allele-resolution RNA-seq analysis recently demonstrated that these early chromatin remodeling events induce reactivation of clusters of Xi-linked genes by day 8 of re-

programming [Janiszewski et al., 2019]. Then, only after reactivation of *Nanog*, *Xist* starts to be repressed, *Tsix* is progressively activated and CpG islands demethylated on the Xi [Payer et al., 2013; Pasque et al., 2014]. The reactivation of *Dppa4* and PECAM1, together with several chromatin changes, marks reprogramming progression until complete XCR [Pasque and Plath, 2015].

Whether or not XCR also occurs during human iPSC reprogramming is still highly debated. A number of studies have shown that Xi reactivation is mainly based on the drop of *XIST* expression and H3K27me3 accumulation, followed by the initiation of biallelic X-linked gene expression [Marchetto et al., 2011; Tomoda et al., 2012; Barakat et al., 2015; Kim et al., 2015]. Others have reported that XCI remains stable, with the maintenance of the Xi present in the somatic starting cells [Tchieu et al., 2010; Amenduni et al., 2011; Ananiev et al., 2011; Cheung et al., 2011; Pomp et al., 2011; Mekhoubad et al., 2012]. During long-term human iPSC culture, XCI undergoes instability, as reported for human ESCs, with the erosion of the Xi (XaXi, Class II) towards the Xe state (XaXe) (Class III) [Tchieu et al., 2010; Bruck and Benvenisty, 2011; Mekhoubad et al., 2012; Nazor et al., 2012; Bar and Benvenisty, 2019] (Fig. 4). The X-chromosome erosion is characterized by loss of *XIST* expression and of H3K27me3 marks, DNA methylation of the X-linked promoter, and reactivation of the human-specific and pluripotency-specific lncRNA XACT [Vallot et al., 2013, 2015].

Concluding Remarks

The cellular and molecular complexity behind the mechanism that leads to the inactivation of one of the 2 X-chromosomes in female mammalian cells or to its reactivation has been gradually unraveled in human and mouse early embryos. More recently, the understanding of the 2 phenomena has been deepened thanks to the availability of ESCs and iPSCs, the former representing an in vitro model of the ICM and of the epiblast, the latter an important tool for the understanding of de-differentiation and its associated gene regulation.

Although largely deciphered in the mouse, both XCI and XCR processes are not completely understood in humans, and thus further investigations are needed. The profound diversity of the mechanisms that govern X-chromosome expression in mouse and human species elicits the interest on its investigation also in other species, to understand how X-chromosome gene dosage compensation is regulated in mammals.

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Conception and design, manuscript writing: P.R. and S.G. Critical revision and final approval of the manuscript: P.R., M.Z., and S.G.

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