Solving the “X” in Embryos and Stem Cells

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X-chromosome inactivation (XCI) is a complex epigenetic process that ensures that most X-linked genes are expressed equally for both sexes. Female eutherian mammals inactivate randomly the maternal or paternal inherited X-chromosome early in embryogenesis, whereas the extra-embryonic tissues experience an imprinting XCI that results in the inactivation of the paternal X-chromosome in mice. Although the phenomenon was initially described 40 years ago, many aspects remain obscure. In the last 2 years, some trademark publications have shed new light on the ongoing debate regarding the timing and mechanism of imprinted or random XCI. It has been observed that XCI is not accomplished at the blastocyst stage in bovines, rabbits, and humans, contrasting with the situation reported in mice, the standard model. All the species present 2 active X-chromosomes (Xa) in the early epiblast of the blastocyst, the cellular source for embryonic stem cells (ESCs). In this perspective, it would make sense to expect an absence of XCI in undifferentiated ESCs, but human ESCs are highly heterogeneous for this parameter and the presence of 2 Xa has been proposed as a true hallmark of ground-state pluripotency and a quality marker for female ESCs. Similarly, XCI reversal in female induced pluripotent stem cells is a key reprogramming event on the path to achieve the naïve pluripotency, and key pluripotency regulators can interact directly or indirectly with *Xist*. Finally, the presence of 2 Xa may lead to a sex-specific transcriptional regulation resulting in sexual dimorphism in reprogramming and differentiation.

Introduction

IN EUTHERIAN MAMMALS, X-chromosome inactivation (XCI) is required to ensure an equal transcriptional level of most X-linked genes for both sexes in adult tissues [1]. The process compensates the double dosage of X-linked genes in females, originated by the divergence of the sex chromosome complement between males (XY) and females (XX), by the transcriptional silencing of the second X-chromosome in females. The inactivation occurs randomly in the female maternal or paternal inherited X-chromosome early in embryogenesis [1] and affects most of the genes of the chromosome with some, ranging from 5% to 25% depending on the study and cell type [2–4], escaping from inactivation.

The complete inactivation of a full chromosome is a unique epigenetic phenomenon whose regulation differs from classical transcriptional regulation and requires the action of specific long noncoding RNAs. The molecular mechanism for XCI involves a complex network of proteins and specific noncoding RNAs that has been described in diverse reviews [5–9]. As a simple notion to understand the studies of XCI in embryos and stem cells, a summarized view of the process may be the following: XCI is initiated by the expression of the long noncoding RNA *Xist*, which coats the *cis* X-chromosome-recruiting proteins such as the histone variant macroH2A, *ASHL2*, *SAFA*, and polycomb group

proteins from the repressive complex 2, that coated the X-chromosome by diverse epigenetic marks. Among them, the most characteristic is the trimethylation of histone H3 at lysine 27 (H3K27me3), a repressive mark that impedes transcription by excluding the binding of RNA-pol II [10]. H4K20me1 also marks the inactive X-chromosome (Xi) [11]. *Xist* expression is regulated by other noncoding RNAs, 2 activating *Xist* (*RepA* and *Jpx*) and *Tsix*, its major regulator that antagonizes *Xist* induction *in cis*, and it is positively regulated by *Xite*, another noncoding RNA [7]. Finally, *Rnf12* is an X-linked gene encoding for an E3 ubiquitin ligase that activates *Xist* expression [12].

Methods for Analyzing XCI Status

Having this basic scheme in mind, 2 main methods can be used to analyze the status of XCI. First, cytology-based methods such as fluorescence in situ hybridization (FISH) and immunohistochemistry can determine the presence of active X-chromosome (Xa) or Xi in each cell. The Xi has been characterized by being coated by *Xist* and/or H3K27me3 and/or excluding RNA-pol II. In contrast, unlike the Xi, the Xa does not show either *Xist* or a punctuate signal of H3K27me3 or H4K20me1 and it is positive for RNA-pol II and FISH signals for X-linked transcripts that are inactivated during XCI. Those X-linked genes escaping from XCI are

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present in both Xa and Xi, thereby allowing the identification of both X-chromosomes. The second method consists of the transcriptional analysis of X-linked genes by reverse transcriptase–polymerase chain reaction, microarray, or sequencing between male and female cells. By this approach, XCI completion can be determined by the equal expression of X-linked genes between sexes.

Both methods present benefits and drawbacks. With respect to cytology, the simple association of an epigenetic mark with chromatin, rather than genetic analysis, should be carefully evaluated, since physical association does not necessarily equate with functional significance [13–17]. For instance, *Xist* coating, used as unique indicator of XCI in early studies, may be uncoupled from XCI. As it will be discussed below, *Xist* expression may not necessarily indicate that XCI has been accomplished [14–17], and the absence of *Xist* may not rule out XCI [17,18]. In addition, the transcriptional analysis of few genes by FISH may not be conclusive, as the silencing does not occur concomitantly for all X-linked genes in the whole chromosome [16,17,19]. However, cytology-based methods provide information at a single-cell level that is essential to understand the dynamics of the process and constitutes the main flaw of the transcriptional analysis. Data from transcriptional analyses reflect the population average, thereby losing the information about differences between cells. Conversely, they provide a high-throughput data that not only numerically quantify the XCI status of the population, but also uncover its effect on the transcription of autosomal genes. Further, when polymorphisms in the parental alleles are available, these approaches allow to quantify the expression of each chromosome and to detect possible skews in XCI toward one of the chromosomes. Taking these aspects into consideration, both methods are complementary to understand the phenomenon.

XCI in Preimplantation Embryos

Preimplantation embryo development constitutes a fascinating scenario for the study of epigenetics. During that very limited time and in few cell divisions, the genome experiences a complete epigenetic reprogramming involving dramatic epigenetic changes, including in some cases the inactivation or reactivation of 1 X-chromosome. Most of the knowledge about XCI during this period comes from studies focused on the mouse model, with surprisingly few lines of evidence in other species until very recently. In the female mouse embryo, both X chromosomes are fully active after the major activation of the embryonic genome, occurring at the 2-cell stage [19]. Then, *Xist* is expressed exclusively from the paternal X-chromosome (Xp) [20] that consequently undergoes imprinted inactivation from the cleavage states up to the blastocyst stage. The required imprint mark that either protects the maternal X-chromosome from inactivation or predestines the Xp for inactivation during these first cleavage divisions remains elusive [9]. It has been suggested that the inactivation of the Xp during cleavage stages may not require *Xist*, because transgenic embryos lacking the paternal *Xist* inactivate some X-linked genes—*Ddx3x*, *Ube1x*, *Zfx*, *Pdha1*, and *Rnf12*—in the Xp [16]. However, a later study concluded that *Xist* is in fact required for initiation of XCI of coding genes, but it is not necessary to silence repetitive elements [17]. The authors observed that these intergenic

noncoding elements are silenced in the Xp by the 2-cell stage even in the absence of *Xist* and proposed that they may arrive in the zygote in a preinactivated condition, providing a mark to predestine the Xp for inactivation [17].

The timing of inactivation is a matter of controversy. The imprinted X-inactivation is a gradual process that does not simultaneously occur in all the blastomeres of the mouse embryo that show biallelic or monoallelic expression patterns for distinct X-linked genes [16]. It has been reported that some X-linked genes silence very early, at the 4–8-cell stage (*Rnf12*, *Kif4*, *Chic1*, and *Atp7a*), whereas others are inactivated before the morula stage (*G6pdx*, *Fmr1*, and *Rps4x*), at the blastocyst stage (*Lamp2*, *Scl25a5*, *Atrx*, *Gla*, *Fgd1*, and *Pdha1*), after the blastocyst stage (*Huwe1*), or even escape from XCI (*Atp6ap2*, *Utx*, *Mecp2*, and *Jarid1c*) in mice [19]. However, other studies suggest that some of these genes may be inactivated later [16], and that dosage compensation occurs several divisions after the formation of *Xist* and *Cot1* silent compartments, initiating in the morula stage and not reaching a significant extent until the blastocyst stage [17]. At the mouse blastocyst stage, a reversal of the inactive state of the Xp occurs specifically in the cells of the inner cell mass (ICM), with a loss of epigenetic marks such as histone modifications or polycomb proteins [21,22]. Consequently, the mouse expanded or hatching blastocysts present 2 Xa in the ICM blastomeres, while the trophoctoderm (TE) retains the inactive Xp. A global transcriptional study observed that the expression of most X-linked genes did not differ between male and female mouse blastocysts [23]. This result is in agreement with FISH data, as transcriptional data represent the average of the different cell types of the blastocyst, which contains 3 times more TE (XaXi) than ICM (XaXa) blastomeres. After blastocyst expansion and implantation (days 3.5–5.5) XCI is re-established in epiblast cells during gastrulation, giving rise to the embryo-proper random X inactivation [22,24]. In contrast, the TE and its derived extra-embryonic tissues retain the paternal XCI established during the cleavage stages [25].

Compared to the mouse model previously described, little is known about XCI during the preimplantation period in other species (Fig. 1). Although morphologically similar, the blastocysts from distinct species differ greatly in their epigenetic status. Mouse early development constitutes an epigenetic exception compared with rabbits, ungulates, or humans in terms of embryonic genome activation, expression of TE/ICM differentiation markers, gene-imprinting dynamics [26], and embryonic stem cell (ESC) derivation [27]. In this sense, XCI may require a specific global epigenetic landscape that occurs at the blastocyst stage in mouse, but may occur later in other species. Further, there are large differences between species in the sequences of the noncoding RNAs regulating XCI, which may result in diverse XCI accomplishment strategies (reviewed in [6]); notably, human *TSIX* does not overlap with *XIST*, whereas mouse *Tsix* completely covers the *Xist* sequence. Another crucial difference is that the imprinted XCI seems to be unique for mice. Initially, human extra-embryonic tissues were reported to show imprinted XCI [28–30], but this notion has been refuted [31,32], and it has been reported that *Xist* is never imprinted during human preimplantation development [14].

In female human embryos, 1 study showed that *XIST* foci begin to form at the 8-cell stage, when embryonic genome

FIG. 1. XCI evolution during early development. After EGA both X-chromosomes are active. In mice, they undergo imprinted XCI that is maintained in the blastocyst trophoctoderm and extra-embryonic lineages, whereas in the blastocyst early epiblast (*middle column*) there is a reversal of the inactive state followed by a random XCI in the late epiblast (*right column*). Rabbit (e.4), bovine (e.7), and human (e.7) blastocysts (*middle column*) present 2 Xa in most blastomeres and random XCI occurs around gastrulation (*right column*), being accomplished in the late blastocyst in rabbits (e.5) and in the elongated embryo in bovines (e.14). EGA, embryonic genome activation; XCI, X-chromosome inactivation; Xa, active X-chromosome; Xi, inactive X-chromosome.

activation occurs, and expand forming a cloud in around half of the morula blastomeres and most of the blastocysts cells, whereas in males only pinpoint-like signals were detected in morula and blastocysts [33]. They also observed that XCI was fully accomplished in human blastocysts, based on the presence of 1 *XIST*-coated X-chromosome with the repressive marks H3K27me3 and macroH2A and no FISH signal for the X-linked gene *CHIC1* [33]. In contrast, *HPRT*, an X-linked gene that does not escape from inactivation, was reported to be upregulated in female human blastocysts [34], and a recent study has concluded that XCI is not accomplished in human blastocysts and observed a different pattern of *XIST* and H3K27me3 during early development [14]. Authors of this study detected 1 or 2 *XIST* RNA domains in male or female human embryos, respectively, in both morula and blastocyst stages (in both TE and ICM) [14]. However, despite the presence of *XIST*, 3 X-linked transcripts (*ATRX*, *FGD1*, and *HUWE1*) known to be silent in the Xi in somatic tissues were detected in both X-chromosomes of the female blastocyst and H3K27me3 enrichment was absent in the *XIST*-coated chromosomes. These findings suggest that in human *XIST*, upregulation occurs on all X-chromosomes regardless of parental origin or sex, but does not result in chromosome-wide XCI [14].

The absence of XCI in the blastocyst stage has been also observed in 2 other mammalian models. Bovine preimplantation development mimics that of humans in terms of timing of embryonic genome activation and development to

blastocysts, and it has been widely used as a model to improve human in vitro culture conditions [35]. Transcriptional studies initially observed upregulation of X-linked genes such as *HPRT*, *G6PD*, *HPRT*, and *XIAP* in female bovine blastocysts [36–40], despite *XIST* being strongly expressed in females and residually in males. Later, a global transcription analysis between male and female bovine blastocysts showed that XCI is far from being accomplished at this stage: most of the X-linked transcripts present in the array (88.5%) were upregulated in females, and most of them (70%) exhibited a fold change lower than 1.66, which suggests either a reduced expression in the duplicated X-chromosomes or an initiation of XCI in some blastomeres [15]. After the blastocyst stage, ungulate embryo development is characterized by a period of elongation before initiation of implantation and concomitant with gastrulation forming the so-called elongated embryos. In these embryos, the expression level of 5 out of 7 X-linked transcripts upregulated in female bovine blastocysts was effectively equalized among sexes, suggesting that a substantial XCI occurs between hatching and gastrulation in bovines [41]. A similar phenomenon occurs in rabbit embryo development; the rabbit blastocyst experiences a large cellular proliferation that multiplies the cell number by 10 and gives rise to a blastocyst-like structure containing an embryonic disc. Rabbit embryos show punctuate *Xist* RNA signals on all X-chromosomes from the 8-cell stage in male and female embryos, which begin to accumulate in a few cells by the morula stage only in female

embryos. By the blastocyst stage, 90% of the TE cells had at least 1 *Xist* RNA domain in rabbits, but similarly to humans and bovines, *Hprt1* was still biallelically expressed in almost half of the cells. However, after the initiation of gastrulation, the blastomeres of the rabbit embryo containing the embryonic disk show 1 *Xist* RNA domain and monoallelic expression of *Hprt1* [14]. As a summary, it seems that the imprinting XCI occurring in the cleavage stages and persisting in the TE and extra-embryonic tissues in mouse does not occur in the other species. Consequently, these other species—including humans—present 2 Xa in most of the cells of the blastocyst despite *XIST* coating, and random XCI does not take place until gastrulation. Nevertheless, ICM cells, which give rise to the embryo proper and are the precursors of ESCs, contain 2 Xa in all the species.

XCI in Embryo-Derived Stem Cells

The XCI status in stem cells may depend on the initial XCI status in the cells from which they are derived. Stem cells can be classified into 3 major types based on the cell type of origin: (1) ESCs derived from blastomeres of the early epiblast of the blastocyst's ICM; (2) epiblast stem cells (EpiSCs) obtained from blastomeres of the epiblast after implantation; and (3) induced pluripotent stem cells (iPSC) resulting from the artificial reprogramming of differentiated somatic cells. ESCs are derived from a cell type that, as discussed above, contains 2 Xa, and thus it makes sense to expect an absence of XCI before differentiation. This situation occurs in female mouse ESCs (mESCs) [42,43], which exhibit both X-chromosomes active in the pluripotent state and initiate random XCI upon differentiation launched by *Xist* upregulation and followed by H3K27me3 mark [44]. Nevertheless, the status of XCI in female human ESCs (hESCs) is largely inconsistent among studies [45]. When mESCs and hESCs are compared, large differences in morphology, maintenance requirements, transcriptome, and epigenome are evident. It has been proposed that hESCs are not developmentally equivalent to mESCs, but may correspond to a later stage similar to mouse EpiSCs (mEpiSCs). Based on the distinct properties of stem cells derived from embryonic tissues, they can be classified in 2 phases of pluripotency named as naïve and primed [27]. Naïve-type cells (mESCs), unlike the primed type (hESCs and mEpiSCs), are able to form germ line chimeras; express *Klf2*, *Llf4*, *Rex1*, *NrOb1*, and *Fgf4*; are *Lif*/*Stat3* dependent; and differentiate to the primed type in response to *Fgf*/*Erk* activation [27].

One of the distinct features proposed for the primed type is the presence of 1 Xi, which occurs in the case of mEpiSCs that are derived from XaXi cells [46], but it is controversial in the case of hESCs. Initially, hESCs were reported to have both X-chromosomes active [47], but later it was found that undifferentiated hESCs could exhibit XCI [48] and a meta-analysis observed that 50% of all established female hESC cell lines expressed *XIST* and/or show H3K27me3 mark in 1 X-chromosome [49]. More recently, the status of XCI has been reported to vary greatly not only between different hESCs lines but also between subcultures of a single hESC line [50]. hESCs exhibiting different XCI status may be present even in the same culture [11,18,51,52]. In this perspective, 3 distinct states of XCI inactivation have been described for hESCs [18] (Fig. 2). The class I cells resemble

mESCs; they possess 2 Xa with no *XIST* clouds or H3K27me3 mark and inactivate one of them upon differentiation. The class II cells contain only 1 Xa with the other showing *XIST* clouds and H3K27me3 in both undifferentiated and differentiated state. Finally, the class III cells do not express *XIST* and do not exhibit H3K27me3 mark but contain 1 Xi [52].

The existence of different classes of hESCs based on XCI status suggests that XCI in hESCs is driven by in vitro culture rather than been an original feature [52]. In agreement, different environmental stressors have been reported to favor XCI. hESCs derived in physiological oxygen tension (5%) retain the pre-X-inactivation state of the human blastocyst, whereas those derived in 20% oxygen undergo XCI that could not be reverted by later culture in 5% oxygen and experience an increase in spontaneous differentiation [53]. Similarly, cryopreservation was observed to convert class I and II hESCs to class III [52] and stressed culture conditions lead to abnormal nuclear morphology and are associated to the loss of *XIST* expression [51]. Thereby, the presence of 2 Xa has been proposed as a hallmark of *bona fide* hESCs, indicating a ground stage of pluripotency. Further evidence supporting this hypothesis comes from studies reprogramming stem cells to the naïve state. Recently, naïve hESCs [54] have been derived from hESCs transiently transfected with Oct4 and *Klf4* or *Klf4* and *Klf2* and then growth in a combination of ERK1/2 inhibitor PD0325901, the GSK3 inhibitor CHIR99021, LIF, and *Klf4*/*Flf2* regulator forskolin. These naïve hESCs show the defining features of mESCs and regarding XCI they can be classified as class I, as they lack *XIST* clouds and inactive one of the X-chromosomes randomly upon differentiation [54]. A recently published study has confirmed XCI reversal in the same conditions [55]. Similarly, the reprogramming of mEpiSCs to an mESC-like state able to form chimeras by exogenous expression of *Klf4*, and the presence of LIF and small molecule inhibitors of Mep/Erk mitogen-activated protein kinase signaling and glycogen synthase kinase entails the reactivation of the Xi [46]. In the same trend, mouse trophoblast stem cells (mTSCs), derived from the blastocyst trophoblast and being XaXi, can be converted to ES-like cells (TSCiPSCs) by overexpression of Oct4, Sox2, *Llf4*, and c-myc [56] or by Oct4 alone [57]. TSC-iPSCs are able to form chimeras [56,57] and present 2 Xa [57].

Reprogramming of the Xi

Reversal of XCI can be accomplished during reprogramming of somatic cells into iPSCs. Reactivation of the Xi is a late event in the reprogramming process and has been proposed as a critical marker of pluripotency and the return to the naïve pluripotent state [54,58]. Mouse iPSCs (miPSCs), like mESCs, carry 2 Xa that are randomly inactivated upon differentiation [58]. However, some female human iPSCs (hiPSCs) maintain XCI during reprogramming [55,59,60], whereas others undergo an extensive X-chromosome reprogramming exhibiting biallelic expression of *Tsix* and other X-linked genes, and loss of H3K27me3 [61,62]. A recent report showed a partial XCI reversal in hiPSCs derived from differentiated hESCs based on microarray fold changes for X-linked genes between hiPSCs and the original hESCs line, which the authors assumed being XaXi [63]. However, it is not clear whether there is a partial XCI reversal on each cell or the fold changes resulted from a mixed population of

FIG. 2. Stem cells can be divided into 3 classes according to XCI status. Class I presents both X-chromosomes active and it is characteristic of early epiblast blastomeres and mESCs. hESCs and hiPSCs exhibit all 3 classes. Class II cells present 1 Xi marked with *XIST* and H3K27me3 and have been converted to class I following different reprogramming strategies. However, class III, characterized by an Xi without *XIST* or H3K27me3 had not been converted to class I or II. mESC, mouse embryonic stem cells; hESC, human embryonic stem cells; iPSC, induced pluripotent stem cells; miPSC, mouse iPSC; hiPSC, human iPSC; TSCs, trophoblast stem cells; mEpiSCs, mouse epiblast stem cells.

hiPSCs exhibiting different degrees of XCI, as hiPSCs can exhibit the 3 distinct states of XCI present in hESCs and be divided in the same 3 different classes depending on XCI status [64]. Because class I may represent the gold standard for hESCs and hiPSCs [52], several attempts have been made to convert classes II and III to class I. As previously mentioned, the reprogramming of conventional hiPSCs to naïve-like hiPSCs [54,55], mEpiSCs to mES-like cells [46], or mTSCs to mTSC-iPSCs [57] was accompanied by XCI reversal. Similarly, sodium butyrate, an inhibitor of histone deacetylases

that has been reported to decrease cellular differentiation of hESCs, also reverts partially class II to class I [65]. A study more specifically focusing on XCI found that a combination of sodium butyrate and DZNep—a molecule that depletes the polycomb protein EZH2—removes H3K27me3 from the genome-reverted class II hESCs to class I, but could not restore class III [52].

Molecular studies have provided evidence for a direct link between several pluripotency markers and XCI regulation (Fig. 3). The most relevant finding is that Oct4, Nanog, and

FIG. 3. Molecular links between pluripotency-related factors and XCI regulators. *Solid arrows* indicate upregulation, whereas *dashed arrows* evidence repression.

Sox2 bind *Xist* intron 1 in both male and female undifferentiated mESCs cells, resulting in downregulation of *Xist* expression independently of *Tsix* expression [66]. However, this is not the sole cause of *Xist* regulation, as deletion of *Xist* intron 1 does not result in upregulation of *Xist* in female mESCs [67]. Unlike *Oct4* and *Sox2*, *Nanog* upregulation coincides with *Xist* repression during preimplantation development, and *Xist* downregulation does not occur in miPSCs until *Nanog* is expressed [68], so it has been proposed that *Nanog* may be responsible for XCI reversal and *Oct4* and *Sox2* for the maintenance of both Xa in mice [5]. *Tsix*, an *Xist* repressor, has been also reported to be regulated by pluripotency markers in mice. Thus, *Oct4* binds to the *Tsix* and *Xite* loci and *Oct4* knockdown results in biallelic *Xist* expression [69], whereas *Sox2* directly binds *Xite*, interacts with the *Tsix* transactivator YY1, and indirectly networks with *Tsix* [69]. *Rex1*, *Klf4*, and *c-myc*—the last 2 being key markers of the naïve state [70]—bind to *Tsix* 5' regulatory regions and appear to be important for *Tsix* elongation in mice [66]. However, the role of *Tsix* as a general *Xist* repressor is controversial [5], because *Tsix* mutation does not result in X inactivation in undifferentiated mESCs [71]. Finally, it has been also proposed that in mice, *Oct4*, *Sox2*, and *Nanog* may act in XCI by repressing the *Xist* activator RNF12 binding its 5' region [6,72].

XCI, an X-Factor for Pluripotency?

Combining the facts discussed above, there are 2 convincing reasons to recognize the presence of 2 Xa as a marker of pristine pluripotency. First, XCI seems to be coupled with pluripotency/differentiation, being reversed following the acquisition of pluripotency and re-established when the loss of pluripotency occurs. Second, although initially XCI was considered a secondary effect of pluripotency [21,73], key pluripotency-associated transcriptional regulators are directly responsible for XCI reversal and maintenance of Xa [5]. In this perspective, the X-chromosome epigenetic instability found in hESCs and hiPSCs may have implications for their use in regenerative medicine [6]. Interestingly, it has been questioned whether class III hESCs are pluripotent, because they poorly differentiate as embryoid bodies [18] and trophoblasts [11], but they were able to differentiate in vitro and form teratomas [52]. At a molecular level, increased *XIST* transcription was correlated with lower OCT4 in undiffer-

entiated hESCs [11], but no correlation between OCT4 protein levels measured by immunofluorescence intensity and the presence or absence of an H3K27me3 focal dot was found in a mixed population of class I and II [52]. No difference in the expression of the key pluripotency markers Oct4, Nanog, and Sox2 was observed between the different classes of female hESCs or male hESCs [64].

From another point of view, the presence of 2 Xa can be also considered as an active character in pluripotency rather than just a mere consequence. In the absence of XCI, sex chromosome dosage can determine the transcriptional level not only of X-linked genes, but also of autosomal genes. Preimplantation embryos constitute an ideal model to study this phenomenon, because they exhibit a relatively low epigenetic variability compared with different lines or passages of stem cells. Using the bovine blastocyst as a model to compare the presence of 2 Xa with just 1 (XaY), sex chromosome composition was found to impose an extensive transcriptional regulation upon autosomal genes, affecting one-third of the transcripts expressed [15]. Interestingly, gene ontology of the differentially expressed transcripts suggested a higher global transcriptional activity in female embryos [15], in accordance with the lower expression of de novo DNA methyltransferases and lower methylation status reported for XaXa bovine embryos [74,75] or mESCs [76] compared with XaY. Mouse sex reversal models based on the deletion of the sex-determination gene *Sry* or its autosomal insertion and different combinations of X- and Y-chromosome constitution also concluded that heterochromatic gene silencing differs depending on X-chromosome complement rather than phenotypic sex [77]. This study also observed a large transcriptional effect depending on X-chromosome composition, with over 1,000 autosomal genes differing between XY and XX males or XX and XY females [77].

A plausible explanation for this sex chromosome-led transcriptional regulation is the presence of genes encoding for chromatin modifiers in the X-chromosome. Among them, there are 2 genes related with histone 3 methylation: *KDM5C* (H3K4 demethylase also known as *JARID1C*) and *UTX* (H3K27 demethylase, also known as *KDM6A*). These chromatin marks are especially relevant in the genome regulation during reprogramming and differentiation. H3K4me3 and H3K27me3 are associated with contrasting chromatin states: while the first favors transcriptional activation, the second is linked with repression. Studies in mESCs have suggested that developmental regulatory genes often show bivalent H3K4me3/H3K27me3 domains, suggesting that H3K27me3 prevents transcription from H3K4me3-bound promoters [78,79]. Consistently, derivation of mESCs is accompanied by losses and gains of H3K4me3/H3K27me3 co-enriched promoters of genes with developmental and differentiation functions [80]. This way, the bivalent domains would keep developmental genes poised for activation during differentiation. Similarly, H3K27me3 has been proposed to regulate the differentiation between ICM and TE in mouse blastocysts [80], as the majority of H3K4/K27me3 co-enriched promoters were distinct between the 2 lineages, and genes expressed in ICM such as *Sox2*, *Lifr*, and *Nanog* were hypo-trimethylated on H3K27 compared with TE, whereas genes expressed in TE (*Cdx2*, *Eomes*, and *Tbpa*) showed a clear enrichment in H3K27me3 in the ICM compared with TE [80]. MicroRNAs may also play a role in sexual dimorphism. MiR-302 family

has been reported to be enriched in male mESCs compared with females [81]. Target genes for this miRNA family are *Arid4a* and *Arid4b*, which encode E2F-dependent transcription repressors involved in recruitment of mSin3A-Histone deacetylase [82]. In agreement, male mESCs progressively loose *Arid4b* protein throughout early differentiation, whereas an accumulation was observed in females [81].

The epigenetic consequences of the presence of 2 Xa may affect derivation and differentiation of stem cells, leading to the distinct differentiation abilities between the 3 classes of hESCs and hiPSCs previously mentioned [11,18] or to sexually dimorphic features between XaXa and XaY stem cells. There is scant information about the latter, as a variety of XX and XY hESC lines are not generally available in most laboratories and most mESCs commonly used for transgenesis are males due to the superior interest in generating male chimeras. A recent study has reported a statistically significant skew in the sex ratio of hESCs lines toward females (29:9, 76% females) that was originated in the derivation process, as an equal representation of male and female embryos was initially plated [83]. The same authors reviewed the sex of the available hESCs lines worldwide and observed that the trend was also statistically significant for hESCs derived after preimplantation genetic diagnosis (99:49, 63% females), but was reduced when any hESCs were analyzed (220:177, 55.4% females). However, the result for this meta-analysis may be misleading, as it is difficult to determine whether these cell lines obtained from diverse laboratories under different conditions were also obtained from a 1:1 mix of male and female embryos.

Concluding Remarks

Forty years after the pioneering publication [1], XCI remains a bustling area of knowledge; initial ideas have been recently refuted, some mechanisms remain controversial, and novel implications in stem cell reprogramming and differentiation are being uncovered. Contrary to the initial thoughts, XCI mechanisms and timing during preimplantation greatly differ between species, with the most studied model—the mouse—being an exception compared with rabbits, ungulates, and humans. In contrast to the mouse model, which exhibits an imprinted XCI before the blastocyst stage and then a reversal of the XCI in the ICM, XCI occurs after the blastocyst in other species, coinciding with gastrulation. Being derived from XaXa cells, female hESCs have been proposed to be initially XaXa and then undergo inactivation due to suboptimal culture conditions. Further, XCI is reversed following the acquisition of pluripotency and re-established during differentiation, and key pluripotency regulators interact directly or indirectly with *Xist*. This coupling between XCI and pluripotency has led to consider the presence of 2 Xa as a hallmark of true pluripotency in female hESCs or iPSCs. Finally, the presence of 2 Xa may result in an extensive transcriptional regulation affecting epigenetic pathways that, in turn, may result in differences in reprogramming or differentiation between sexes or the distinct classes of hESCs or hiPSCs. The study of XCI and sexual dimorphism in stem cells could explain part of the heterogeneity observed between different cell lines and may change the concept of reprogramming and differentiation in a sex-specific manner.

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