

X chromosome inactivation in human and mouse pluripotent stem cells

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Abstract Since the groundbreaking hypothesis of X chromosome inactivation (XCI) proposed by Mary Lyon over 50 years ago, a great amount of knowledge has been gained regarding this essential dosage compensation mechanism in female cells. For the mammalian system, most of the mechanistic studies of XCI have so far been investigated in the mouse model system, but recently, a number of interesting XCI studies have been extended to human pluripotent stem cells, including both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Emerging data indicate that XCI in hESCs and hiPSCs is much more complicated than that of their mouse counterparts. XCI in human pluripotent stem cells is not as stable and is subject to environmental influences and epigenetic regulation in vitro. This mini-review highlights the key differences in XCI between mouse and human stem cells with a greater emphasis placed on the understanding of the epigenetic regulation of XCI in human stem cells.

Introduction

X chromosome inactivation (XCI) is the process by which one of the two X chromosomes in female mammalian development is inactivated through a series of epigenetic modifications. This process allows for dosage compensation of the X-linked genes in female cells as proposed elegantly by Mary Lyon over 50 years ago (Lyon 1961,

1962). Since then, XCI has become a beautiful model system of molecular, cell, and developmental biology. One of the milestone discoveries in XCI is the isolation and cloning of the X-inactive specific transcript (*XIST*) gene, which is transcribed by the inactivated X chromosome (Xi) (Brown et al. 1991). As reviewed by many other excellent papers in this issue, we have gained insight into the mechanistic action of *XIST* and its associated epigenetic regulators including small non-coding RNAs, specific histone modifications, and DNA methylation in the initiation, choice, and maintenance of XCI. Much of the knowledge regarding the mechanism of XCI in mammals has largely been derived from the mouse model through the study of both mouse embryonic stem cells (mESCs) and embryos. Developmental studies of XCI in humans lag behind that of the murine model due to the obvious difficulty of working with human embryogenesis. Nevertheless, the advent of hESCs and hiPSCs provides an alternative avenue to understanding human XCI regulation in vitro (Dhara and Benvenisty 2004; Takahashi et al. 2007; Tchieu et al. 2010). Unexpectedly, several recent studies of hESCs and hiPSCs have indicated that XCI exhibits variations and switches from one state to another in culture, suggesting that there are significant differences in XCI regulation between human and mouse stem cells (Dvash and Fan 2009; Makhoul and Rougeulle 2011).

Similarities and differences in XCI between human and mouse embryogenesis and embryonic stem cells (ESCs)

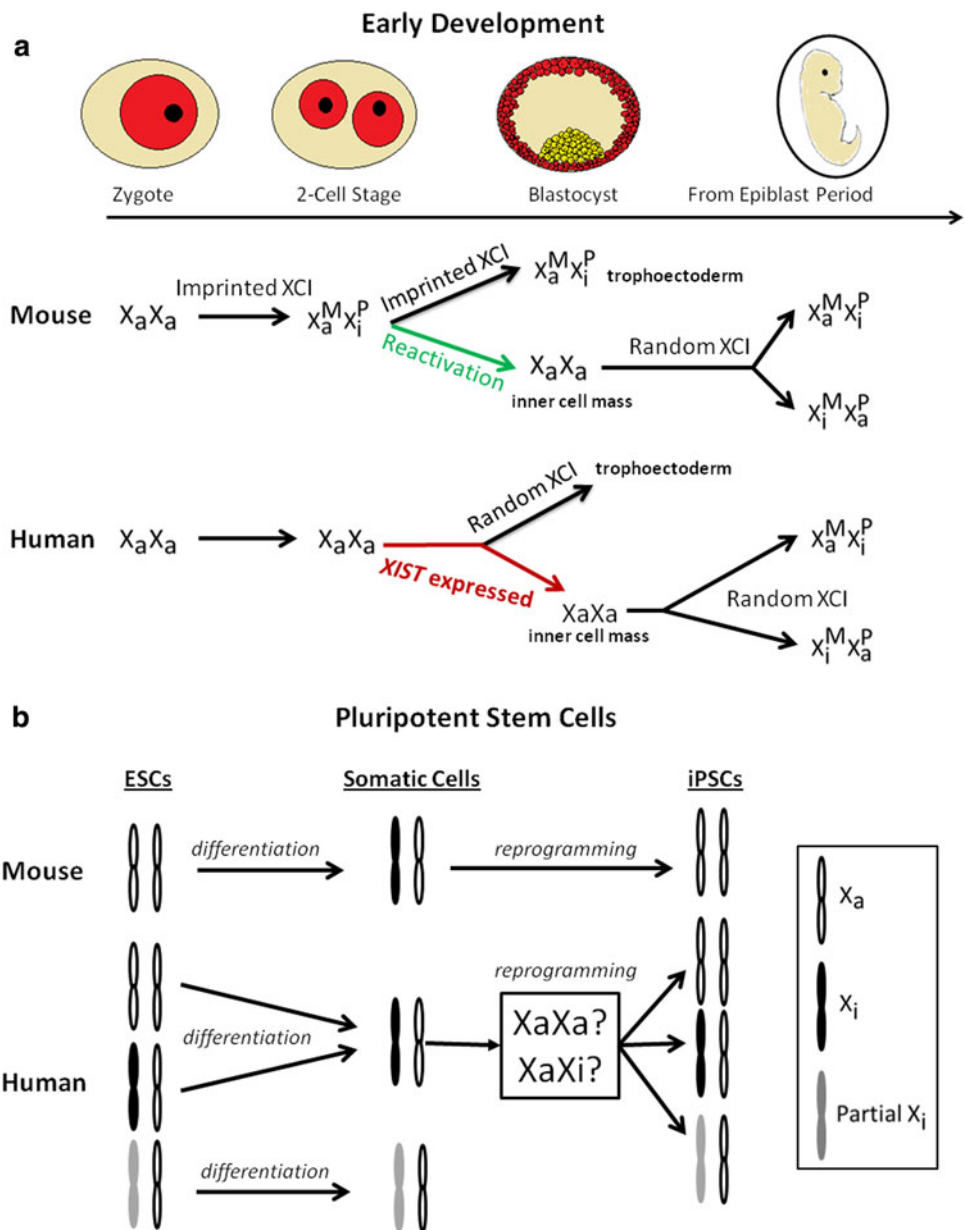
During mouse embryogenesis, the zygote contains two active X chromosomes (XaXa); however, upon the first cleavages, XCI establishes in two discrete events. At the

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two-cell stage, the first event of XCI initiates when the imprinted paternal X chromosome is exclusively inactivated (Okamoto et al. 2004; Huynh and Lee 2005) (Fig. 1). During blastocyst formation, cells of the inner cell mass (ICM) reactivate their paternal X chromosomes, while the trophoctoderm and primitive endoderm preserve their imprinted XCI (Okamoto et al. 2004; for review, see Heard and Disteché 2006). The second stage of XCI occurs upon differentiation when epiblast cells randomly inactivate one of their X chromosomes (Heard and Disteché 2006). Therefore, each somatic cell will express either a maternal or paternal X chromosome (Heard and Disteché 2006; Pablo and Philip 2009) (Fig. 1).

Whereas XCI has been extensively studied in the mouse model, XCI in early human development is still inconclusive and more difficult to study. It is still unclear whether the biphasic nature of XCI establishment observed in mouse embryos also takes place in human preimplantation embryos. The presence of several key differences between murine and primate development may contribute to the variation of XCI in mammalian species. For example, the mouse embryo transitions from maternal to zygotic gene expression during the two-cell stage, earlier than does the human embryo, which shifts gene expression from maternal to zygotic at the four- to eight-cell stage. The primate embryo also devotes its first 2 weeks after implantation to

Fig. 1 Comparison of mouse and human XCI during embryogenesis and upon stem cell differentiation and reprogramming. **a** A schematic drawing of XCI status in the early stages of mouse and human development. XCI establishes in two discrete events in mouse, while only random XCI appears to occur in human embryonic development. *XIST* expression in human preimplantation embryos can be detected, but no XCI takes place yet in ICM. **b** XCI status in mouse and human pluripotent stem cells, upon differentiation, and after reprogramming. XCI in hESCs and hiPSCs show more variation with three distinct classes: pre-XCI, full XCI, and partial XCI. XCI status after the reprogramming of human somatic cells into hiPSCs also presents three states. It is still debated if XCI in human somatic cells are subjected to reactivation in human iPSCs as the case of mouse iPSCs upon cell reprogramming



develop an extraembryonic membrane (Enders and Schlafke 1981; Enders et al. 1986; Luckett 1978), while the mouse embryo has already passed midgestation and has initiated germ layer formation by the end of the second week. In extraembryonic tissues such as the placenta, mouse placental cells exclusively exhibit imprinted paternal X inactivation whereas human placental cells present random XCI (Moreira de Mello et al. 2010). A very recent study also suggests that human embryonic cells initiate XCI later than their mouse counterpart although *XIST* expression can already be detected during the morula stage in human preimplantation embryos (Okamoto et al. 2011) (Fig. 1). This finding is in contrast to the situation in hESCs because *XIST* is not expressed in pre-XCI hESCs in culture as will be discussed later in this review. It would be of interest to address whether the pre-XCI state in hESCs corresponds to a state in which *XIST* is not yet expressed in preimplantation embryos, or alternatively a result of silencing the biallelic *XIST* expression of human ICM in the derivation process of hESCs. In any case, the differences in the initiation and maintenance of XCI during embryonic development in humans, mice, and rabbits suggest that the mouse is not a sufficient model to understand XCI in human development. The differences in the initiation and maintenance of XCI during development in humans, mice, and rabbits suggest that the mouse is not a sufficient model to understand XCI in human development.

The study of XCI in the mouse model has been greatly advanced by the discovery that female mESCs, derived from ICM, exhibit XaXa in the pluripotent state, but initiate random XCI upon in vitro differentiation. The in vitro system of recapitulating XCI through mESCs differentiation has prompted a number of mechanistic studies of XCI at the molecular and cellular levels. As seen with mouse embryos, once XCI initiates in differentiated mESCs, the *Xist* gene is transcribed at the X inactivation center (Xic) of the X chromosome that was randomly selected to be inactivated. Only the X chromosome that is predestined to undergo XCI will express the *Xist* gene. The resulting RNA transcripts that are transcribed from the *Xist* gene will coat the X chromosome (Sado et al. 2005; Wutz 2007). Occurring simultaneously with *Xist* expression, Polymerase II is excluded on the Xi and followed by a series of repressive chromatin marks (Payer and Lee 2008).

A series of histone modifications induce the soon to be Xi into a heterochromatin structure, thereby silencing the genes localized on the chromosome (Wutz 2007; Wutz and Gribnau 2007). Meanwhile, the X chromosome whose expression remains active (Xa) expresses the *Tisx* gene, which prevents the expression of its respective *Xist* gene, thus preventing its own inactivation (Navarro et al. 2005; Sado et al. 2005).

Because human ESCs are also derived from the ICM, it was initially postulated that hESCs are the counterpart to

mESCs in the study of human XCI (Dhara and Benvenisty 2004). However, subsequent studies found that hESC cultures do not exhibit a consistent XCI status. The status of XCI in hESCs has displayed three distinct classes: class I hESCs resemble mESCs in that they do not yet have an Xi, but when exposed to conditions favorable for differentiation, the hESCs undergo XCI; class II hESCs display an Xi and *XIST* expression although the cells remain in the undifferentiated and pluripotent state; class III hESCs exhibit partial XCI although *XIST* expression and other markers of XCI are absent (Dvash and Fan 2009; Makhoulouf and Rougeulle 2011). Culture conditions and the length of passaging appear to influence the class in which XCI belongs (Hall et al. 2008; Shen et al. 2008; Silva et al. 2008). In our recent work, we minimized in vitro effects by studying XCI in hESCs of very early passages (P5-10) (Dvash et al. 2010). Nevertheless, we still found that XCI status had already displayed three distinct states (Dvash et al. 2010). Furthermore, even within subcultures of several female hESC lines, there is variation in XCI (Shen et al. 2008). We also demonstrated that class III female hESCs can lose *Xist* expression and exhibit partial reactivation of the originally silenced X chromosome (Shen et al. 2008).

Differences in XCI between mESCs and hESCs may be attributed to the innate property of cells. Global gene expression studies have suggested that hESCs are similar to mouse epiblast stem cells (EpiSCs) in terms of developmental stage. XCI is fully established and highly stable in mouse EpiSCs (Guo et al. 2009). In fact, genome-wide gene expression analysis confirms that hESCs are more similar to mouse EpiSCs than mESCs (Guo et al. 2009). This had led to the concept of deriving hESCs with a naïve mESC-like state of pluripotency, which has the potential of exhibiting XaXa with a pre-XCI pattern. Indeed, Hanna et al. (2010) was able to convert hESCs with XCI into a meta-stable state of hESCs that display XaXa and a mESC-like morphology. This was achieved by ectopic activation of signaling pathways involved in pluripotency and enforcing a culturing condition suitable for mESC growth in hESCs (Hanna et al. 2010). These results lead to the hypothesis that hESCs are initially in a naïve pluripotent and pre-XCI state; however, normal culture conditions are unable to preserve this pre-XCI state (Hanna et al. 2010).

Consistent with this notion that the naïve pre-XCI state is difficult to be preserved in normal culture conditions, Lengner et al. (2010) found that the level of O₂ during the derivation of hESCs influences the status of XCI. When hESC derivation is done under physiological concentration of oxygen (5% O₂ instead of 20% O₂), hESC lines derived and cultured in this hypoxic condition displayed a naïve pre-XCI state (Lengner et al. 2010). These pre-XCI hESCs display two active X chromosomes and randomly

inactivate one of the X chromosomes upon exposure to 20% O₂ culture conditions or upon in vitro differentiation (Lengner et al. 2010). The introduction of antioxidants into culture media to reduce the levels of reactive oxidative species from atmospheric oxygen is also able to prevent the onset of XCI (Lengner et al. 2010). The authors conclude that XCI is highly sensitive to cellular stress, including stress from atmospheric O₂ (20% O₂), which is able to irreversibly induce XCI. This result is consistent with previous postulation that the XCI status in hESCs can be altered under different culture conditions (Hall et al. 2008; Shen et al. 2008; Silva et al. 2008; Dvash et al. 2010).

Similarities and differences in XCI between human and mouse induced pluripotent stem cells (iPSCs)

iPSCs avoid much of the ethical controversy associated with the derivation of human embryonic stem cells and can also become a potential source of patient-specific cells in regenerative medicine. This has generated significant interest in the study of iPSCs. Mouse and human iPSCs (miPSCs and hiPSCs, respectively) can be derived from either mouse or human somatic cells by the forced expression of transcription factors associated with pluripotency, including Oct4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka 2006; Takahashi et al. 2007; Park et al. 2008; Hochedlinger and Plath 2009). In the mouse model system, reprogramming of somatic fibroblasts into pluripotent stem cells is tightly associated with the switch of full XCI into the pre-XCI state (Maherali et al. 2007; Stadtfeld et al. 2008). Based on this relationship, a reversal of XCI in miPSCs during the reprogramming process from the somatic state can serve as an indicator of pluripotency and the return to the naïve pluripotent state (Maherali et al. 2007) (Fig. 1). Like mESCs, the differentiation of mouse iPSCs also results in random XCI in iPSC derivatives (Maherali et al. 2007; Stadtfeld et al. 2008).

The status of XCI in hiPSCs has only just begun to be studied. One study observed that female hiPSCs retain an inactivate X chromosome (Tchieu et al. 2010). *XIST* gene detection and allele specific expression assays suggest that hiPSCs maintain XCI during reprogramming (Tchieu et al. 2010). However, several other studies have also found that a portion of hiPSCs do exhibit XaXa, and upon in vitro differentiation, random XCI is established in the progeny cells (Marchetto et al. 2010; Bruck and Benvenisty 2011) (Fig. 1b). Bruck and Benvenisty (2011) systemically classified XCI status in a number of hiPSCs lines through a meta-analysis of the expression of the genes on the X chromosome. Like the aforementioned three classes of XCI in hESCs (reviewed by Dvash and Fan 2009; Makhlof and Rougeulle 2011), the authors have also identified three

classes of XCI in hiPSCs: those displaying complete XCI, partial XCI (X chromosome is partially inactivated regardless of the presence or absence of *Xist* expression), and pre-XCI (Bruck and Benvenisty 2011). The authors emphasize that *XIST* expression assays alone are not enough to characterize XCI in hiPSCs, especially those that display partial XCI. All these studies have so far indicated that the state of XCI in established lines of hiPSCs is variable, similar to that of female hESCs (Fig. 1b).

There is an ongoing debate on whether XCI in human somatic cells is subjected to reprogramming as in mouse iPSCs. Using inducible vectors expressing OCT4, SOX2, KLF4, and C-MYC together with chemical compounds and culture conditions normally used for mESCs, Hanna et al. (2010) have also generated hiPSCs with a mESC-like morphology. These mESC-like hiPSCs appear to be in the naïve pre-XCI with two active X-chromosomes (XaXa). However, when the naïve hiPSCs were transferred to hESC-culture medium containing bFGF treatment, hiPSCs began to exhibit *XIST* expression and the XCI phenotype (Hanna et al. 2010). This study further suggests that human somatic cells can be reprogrammed into the pre-XCI state as observed in miPSCs only under optimal culture conditions. Taken all these findings together, we suggest that XCI in human somatic cells is subject to reprogramming in hiPSCs as in miPSCs. However, due to the selection pressure and metastability of pre-XCI in human pluripotent stem cells, hiPSCs also exhibit variable XCI status in different experiments and different laboratories as currently reported in the literature.

The implications of XCI variations in studying X-linked genetic disorder using human stem cells

The understanding of XCI variations in female human stem cells may not only improve knowledge regarding epigenetic regulation of XCI in human biology, but also help establish appropriate cell models to study many X-linked human diseases. For example, a neurodevelopmental autism spectrum disorder, Rett syndrome (RTT) in females, is caused primarily by heterozygous mutations in the X-linked gene encoding methyl-CpG binding protein 2 (MECP2). Due to random XCI, RTT patients exhibit mosaic expression of mutant MECP2. A few hemizygous mutations of RTT in males cause a much earlier lethal phenotype. Recently, two independent groups have succeeded in deriving RTT-hiPSCs from female patients. One group was able to produce iPSCs that are at a pre-XCI state (Marchetto et al. 2010). Because random XCI is coupled with in vitro neural differentiation of RTT iPSCs, investigators are able to obtain neurons that express either wild-type or mutant MECP2. The other group was able to obtain iPSCs that already exhibit XCI at the undifferentiated stem cell stage

(Cheung et al. 2011). Therefore, it allowed this group to compare the phenotypes of the isogenic control and RTT-hiPSCs from the same RTT patient. Both groups found interesting phenotypes of RTT neurons from RTT-iPSCs. These two studies provide an excellent example of using the proper human stem cells to study the pathogenesis of X-linked human genetic disorders by taking advantage of iPSCs technology and XCI variations in human stem cells.

It is also worth noting that the deregulation of XCI has also been associated with the altered expression of X-linked genes in pluripotent stem cells (Shen et al. 2008). Furthermore, altered X-inactivation has an impact on tumorigenesis and other genetic disorders (Agrelo and Wutz 2010). We suggest that when using female pluripotent stem cells to study human genetic disorders, one should carefully monitor the status of XCI in both pluripotent stem cells and their differentiated progenitor cells to assess the influence of a particular XCI state on the expression of X-linked genes and the pathogenesis of diseases.

Concluding remarks

The successful derivation of human and mouse ESCs and iPSCs has provided useful systems that can be used to further the understanding of XCI regulation in differentiation and reprogramming. While murine stem cells can recapitulate XCI regulation in vivo, XCI in human ESCs and iPSCs appears in multiple states: (a) a naïve pre-XCI state as seen in mouse ESCs and iPSCs; (b) the presence of full XCI; or (c) partial XCI due to epigenetic influences. Thus, for many of X-linked genetic disorders, random XCI in human pluripotent stem cells and differentiating derivatives allow investigators to isolate isogenic controls and mutant cells from female somatic cells carrying heterozygous mutations. Many important questions remain to be resolved concerning XCI in stem cells. For example, what is the molecular change in human stem cells that underlies variations of XCI states in different culture conditions or upon reprogramming? How different is XCI regulation between human stem cells and embryos? Do we have optimal experimental conditions to consistently obtain hESCs and hiPSCs in the naïve pluripotent and pre-XCI state with two active X chromosomes? We expect that X-inactivation study and stem cell research will continue to intersect with each other and yield more exciting findings in the near future.

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