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X chromosome reactivation in reprogramming and in development

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Dramatic epigenetic changes take place during mammalian differentiation from the naïve pluripotent state including the silencing of one of the two X chromosomes in female cells through X chromosome inactivation. Conversely, reprogramming of somatic cells to naive pluripotency is coupled to X chromosome reactivation (XCR). Recent studies in the mouse system have shed light on the mechanisms of XCR by uncovering the timing and steps of XCR during reprogramming to induced pluripotent stem cells (iPSCs), allowing the generation of testable hypotheses during embryogenesis. In contrast, analyses of the X chromosome in human iPSCs have revealed important differences between mouse and human reprogramming processes that can partially be explained by the establishment of distinct pluripotent states and impact disease modeling and the application of human pluripotent stem cells. Here, we review recent literature on XCR as a readout and determinant of reprogramming to pluripotency.

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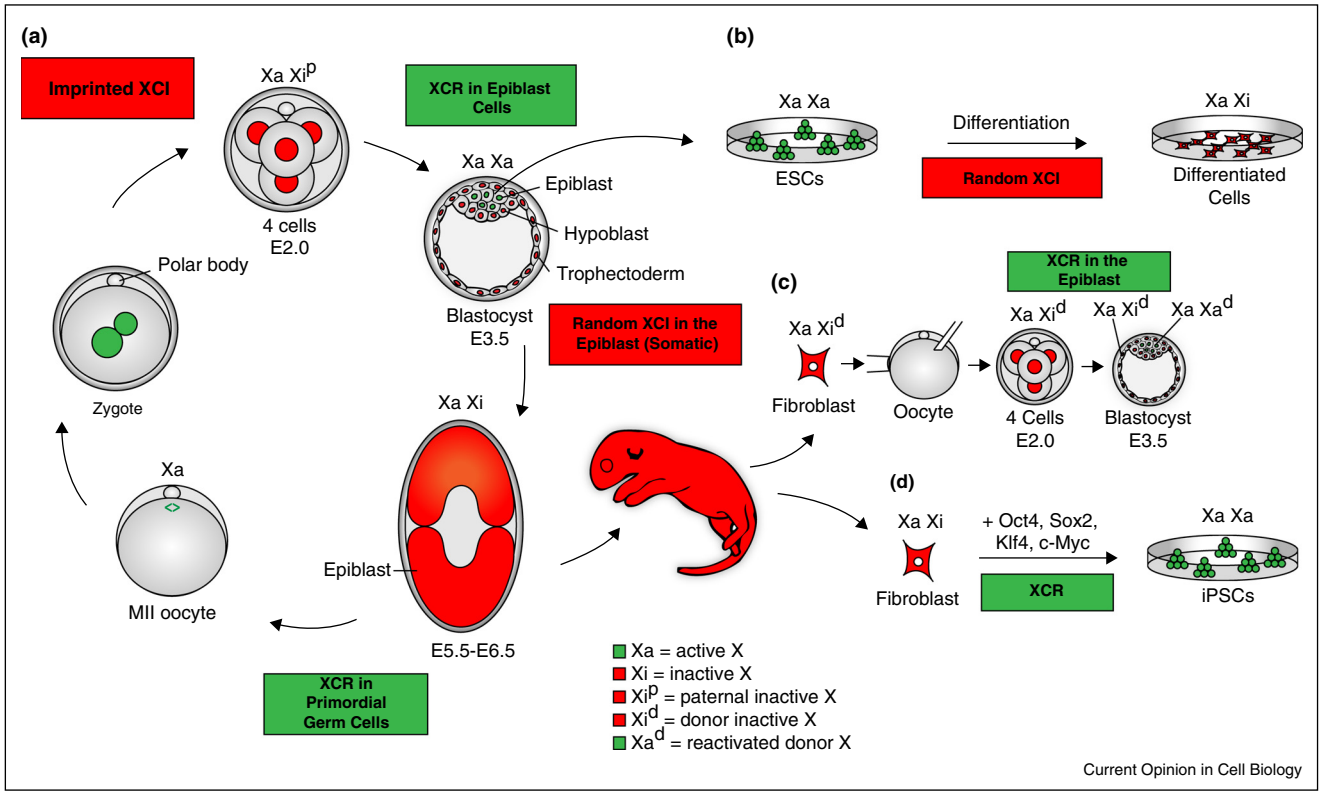
Introduction

Evolution of female mammals has selected X chromosome inactivation (XCI) as a means to mediate sex chromosome dosage compensation by reducing gene expression to one X chromosome, which is thought to be important for normal embryonic development [1–4]. XCI has been extensively used to study development and the epigenetic regulation of gene expression as well as heterochromatin formation [5–9]. In the mouse, the developmental cycle in females is coupled to two rounds of XCI and X chromosome reactivation (XCR) (Figure 1a). Inactivation of the paternal X chromosome takes place during the first days of embryogenesis, and is followed by XCR in the epiblast cells of the pre-implantation blastocyst [1–4,10–12]. XCR follows the restricted expression of pluripotency gene *Nanog*, resulting in a transient pluripotent cell population with two active X chromosomes (Xa's) in early development [12]. Random XCI initiates in epiblast cells soon after implantation [1,13,14]. As a result of random XCI in eutherian mammals, adult individuals are mosaics of cells with a paternal or maternal inactive X chromosome (Xi), which can influence the expression of X-linked diseases. XCI and XCR offer striking examples of heterochromatin formation and reversion, respectively, making these processes attractive for the study of gene regulation in the context of cell fate transitions.

Pluripotent stem cells (PSCs) are useful models to study development and disease and bear important potential for therapy due to their ability to self-renew and differentiate in any cell type of the body [15,16]. However, major differences exist between mouse and human PSCs. Mouse embryonic stem cells (mESC) are PSCs derived from the epiblast of the pre-implantation blastocyst, maintain two Xa's and undergo XCI upon differentiation in the female genotype (Figure 1b). Mouse ESCs reside in the 'naive' pluripotent state, which is characterized by broad developmental potential and dependence on leukemia inhibitory factor (LIF) or a combination of MEK/ERK (mitogen activated protein kinase/extracellular signal regulated kinases) and GSK3β (glycogen synthase kinase-3β) inhibitors (2i for two inhibitors) (reviewed in [15]). 'Primed' pluripotency describes the state of epiblast stem cells (EpiSCs), isolated from early mouse post-implantation embryos [17,18]. Primed PSCs depend on Fgf4 (fibroblast growth factor 4) and activin culture media, are constrained by an epigenetic barrier that opposes their reversion to the naive pluripotent state, and are characterized by the presence of an Xi [17–20]. It has been suggested that human ESCs (hESCs) reside in the primed state due to their resemblance to primed PSCs of the mouse [18]. At the X chromosome level, several laboratories have reported that female hESCs lines have an Xi [21–24]. Conversely, work from others has argued for the presence of two Xa's in hESCs [25,26]. Therefore, the current view is that different female hESC lines exhibit different states of XCI [21,23]. A range of small

Figure 1



Dynamics of XCI and XCR in development and reprogramming. **(a)** XCR and XCI are initiated twice during female mouse development. **(b)** Female mouse ESCs retain two Xa's. Differentiation triggers XCI in female ESCs. **(c)** XCR following somatic cell nuclear transfer. After somatic cell nuclear transfer in the mouse, the Xi of the donor cell is retained as an Xi. XCR is induced in the epiblast of the blastocyst and random XCI upon implantation. Many cloned embryos show defects in XCI regulation, including *Xist* expression from the Xa in males and in females [83,84]. **(d)** XCR can be induced by somatic cell reprogramming to iPSCs. The resulting XX iPSCs have two Xa's and undergo random XCI upon differentiation.

molecule combinations to derive hESCs in different pluripotent states, closer to the naive state of the blastocyst, have recently been reported [27•,28•,29–32]. These provide an opportunity for further studies on X chromosome status to better understand the epigenetic regulation of the X chromosome in early human embryonic development. In this review, we will address X chromosome regulation during reprogramming to iPSCs in both mouse and human.

XCI is initiated by the long noncoding RNA (lncRNA) *Xist* which itself is expressed from the X chromosome (see [4,33] for review). *Xist* acts as a scaffold to recruit proteins to the Xi and mediate XCI. The repertoire of proteins that interact with *Xist* RNA has recently been identified [5–7]. The mechanisms by which these proteins work still remain to be determined. It is already known that as a result of *Xist* RNA coating of the X chromosome and silencing of X-linked genes, several repressive chromatin marks enrich on the Xi while active chromatin marks are depleted, and these events occur in a sequential order during the initiation of XCI [34]. Therefore, once established, the Xi is extraordinarily stable due to a multi-layer of epigenetic mechanisms [35], an idea further reinforced by the plethora of proteins that are recruited to the Xi by *Xist*. These redundant epigenetic mechanisms all need to be reversed during XCR. For example, in differentiated cells, turning off *Xist* expression is not sufficient for XCR, although long-term stability is compromised [35].

In vivo, XCR is a developmentally regulated process that takes place upon the restriction of epiblast cells in pre-implantation embryos and in germ cells [36–40] (Figure 1a). One key question is how the entire Xi, and all of the chromatin marks that it carries, are reversed during XCR? To better understand the molecular processes leading to XCR it is interesting to consider reprogramming approaches *in vitro*. It is now widely recognized that the differentiated state can be reprogrammed to an earlier developmental state (reviewed in [41,42]). Importantly, reprogramming of somatic cells to pluripotency, by nuclear transfer or by reprogramming factor expression leads to XCR in mouse cells (Figure 1c,d) [43•,44]. This opens up interesting questions: How does the state of the

Xi change as somatic cells progress to pluripotency? How do the changes in Xi chromatin state relate to changes in reprogramming state?

Here, we integrate recent literature on how the epigenetic state of the Xi changes during somatic cell reprogramming to pluripotency as well as how X chromosomes influence the pluripotent state. Advances in reprogramming technologies have shed light on XCR and uncovered major differences between mouse and human systems. We discuss how these insights inform our understanding of cell fate reprogramming and pluripotency and can be used to generate hypotheses testable in development.

Induced pluripotency as a model system to study XCR

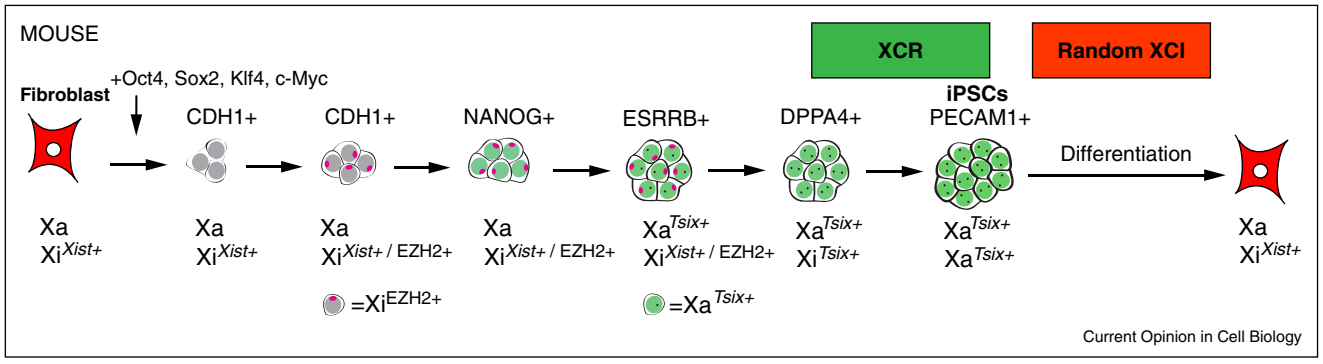
In vivo, the systems to study XCR can be technically challenging in part due to the small number of cells undergoing XCR, thereby complicating mechanistic work. Reprogramming of mouse somatic cells into iPSCs provides an exciting alternative and complementary system to induce and study XCR (Figure 1d). iPSCs can easily be obtained in tissue culture from somatic cells, using overexpression of transcription factors such as *Oct4*, *Sox2*, *Klf4*, and *cMyc* [45], and are molecularly and functionally equivalent to ESCs [43•]. As a result, female mouse iPSCs have two active X chromosomes, indicating that reprogramming of mouse somatic cells to pluripotency leads to XCR [43•]. Female iPSCs with two Xa's therefore can undergo random XCI upon further differentiation [43•].

Given that many studies have described the process of step-wise Xi assembly during differentiation [34], the examination of events leading to the reactivation of the inactive X chromosome can illuminate the processes resulting in reprogramming to pluripotency, for example, by helping to further understand how heterochromatin is reset during reprogramming. This is particularly interesting given that the reprogramming of somatic cells encounters major epigenetic barriers such that only a small set of cells are usually induced to pluripotency [45]. In addition, reprogramming events are initiated with different latencies across the cell population, leading to highly heterogeneous reprogramming cultures [46]. Hence, despite several advances, it has been difficult to identify markers of reprogramming progression [47–49]. At the same time, the epigenetic state of the Xi can easily be defined at the single cell level in cultures of cells induced to reprogram [50••]. Accordingly, recent reports have characterized the sequence of epigenetic remodeling events of XCR during iPSC generation and have begun to address the molecular mechanisms at play [50••,51]. The picture that emerges from these studies in the mouse system is that XCR is strongly linked to the sequential activation of pluripotency-associated factors.

The reactivation of genes on the Xi is tightly linked to hierarchical pluripotency gene activation [50••]. For example, the Xi is maintained when pluripotency-associated factor NANOG first reactivates late in reprogramming. Reactivation of Xi-linked genes then correlates with reactivation of the additional-associated factors DPPA4 and PECAM1, which occurs subsequent to NANOG activation. Mechanistically, *Nanog* knockdown or knockout of pluripotency-associated factor PRDM14 decreases the formation of iPSC colonies with XCR, but also reduces the absolute number of iPSC colonies in reprogramming cultures [50••,51]. Conversely, overexpression of *Klf2* and *Prdm14* induces EpiSCs to undergo XCR and acquire naive pluripotency [52]. Thus, XCR appears to require pluripotency induction during reprogramming to iPSCs [50••].

In addition, we found that the epigenetic state of the Xi is surprisingly dynamic throughout reprogramming [50••]. The chromatin changes that take place on the Xi define a novel sequence of steps for XCR and can also be used as paradigm for reprogramming progression (Figure 2). For example, the enrichment of the Polycomb Repressive Complex 2 (PRC2) protein EZH2 on the Xi, not seen in the starting mouse fibroblasts, appears after the mesenchymal-to-epithelial transition, before pluripotency gene activation, then disappears in fully reprogrammed iPSCs [50••]. This dynamic event can be compared to the sequence of XCI during development, which shows that the transient recruitment of EZH2 to the Xi during mouse iPSC generation follows the inverse sequence of development [50••,53,54]. The transient enrichment of EZH2 on the Xi during reprogramming toward XCR seems counterintuitive since PRC2 recruitment is also one of the steps of XCI [50••,53,54]. We speculate that the recruitment of EZH2 to the Xi during reprogramming is not required for XCR, but instead represents an intermediate reprogramming stage in which cells are in a dedifferentiated state that precedes pluripotency. Accordingly, overexpression of EZH2 in mouse embryonic fibroblasts is not sufficient for Xi-enrichment, indicating that coordinated changes in expression of one or more EZH2 co-factors and/or changes in chromatin structure are required for enrichment of EZH2 on the Xi. Interestingly, macroH2A, which enriches on the Xi only late during differentiation [55,56], is retained on the Xi all the way during reprogramming up until late stages [50••]. Therefore, macroH2A recruitment to the Xi deviates from the inverse sequence of development because it resists reprogramming until late reprogramming stages [50••,55,56]. Together, the study of the dynamic changes of various epigenetic marks of the Xi in relation to pluripotency markers revealed unprecedented details on the stages of somatic cell reprogramming to induced pluripotency (Figure 2), and represents a valuable foundation that can be used for future applications such as staging reprogramming cultures and the isolation of intermediates.

Figure 2



Stages of XCR in mouse iPSC reprogramming. Steps of reprogramming and change in Xi state during pluripotency induction. The sequential induction of CDH1, NANOG, ESRRB, DPPA4 and PECAM1 is indicated. Enrichment of EZH2 on the Xi is shown as a pink focus. *Tsix* expression is represented by a black dot. The epigenetic state of the Xa and Xi is summarized on the lower line.

Role of *Xist* and *Tsix* in XCR

Xist is required for initiation of XCI and is thought to also participate in maintenance of the Xi, although to a minor extent [35]. One likely requirement for XCR is therefore *Xist* repression. Expression of the reprogramming factors *Oct4*, *Sox2*, *Klf4*, and *cMyc* in female fibroblasts is not sufficient for *Xist* repression, indicating that other pluripotency-associated factors are also required for *Xist* repression [50••]. Recent studies have shown that, similar to XCR in the blastocyst *in vivo*, *Xist* is repressed after reactivation of *Nanog* during iPSC reprogramming [50••,51]. The repression of *Xist* is indeed important, but not sufficient for XCR during iPSC reprogramming [50••]. This is quite interesting and has led our group to look at DNA methylation, which is acquired late in development during XCI and acts as one of the key mechanisms of Xi maintenance [35,57]. We therefore asked the question when DNA methylation on the Xi is reversed during reprogramming to iPSC, and found that Xi demethylation occurs late in the process, only after NANOG activation [50••]. Thus, unlike the transient EZH2 Xi-accumulation that reverses the developmental steps on the Xi, DNA methylation behaves differently from the simple reversion of the developmental path to XCI (which is similar to macroH2A's behavior on the Xi). This indicates that different epigenetic marks have different propensities for reversal during reprogramming to the iPSC state. Notably, inhibition of DNA methylation during reprogramming is not sufficient to lead to precocious XCR. However, combined deletion of *Xist* and inhibition of DNA methylation accelerate XCR, indicating that both *Xist* and DNA methylation maintain the Xi in a repressed state during most of the reprogramming process until pluripotency-associated genes are activated [50••]. There have been ongoing efforts to determine whether DNA demethylation proceeds via passive and/or active mechanisms, for example involving *Tet* proteins. We found that *Tet1* and *Tet2* are dispensable for XCR and Xi demethylation, suggesting that DNA demethylation on the Xi can take place passively, though a role of *Tet3* or other proteins has not been excluded [50••]. Together, these studies show that reactivation of the pluripotency program leads to repression of *Xist* and demethylation of CpG islands on the Xi necessary for XCR. As such, XCR during reprogramming to induced pluripotency will serve as a useful model to generate hypotheses about XCR *in vivo*, such as in the germline.

In the mouse, the lncRNA *Tsix* is transcribed antisense to *Xist* and is thought to be involved in the regulation of XCI [58]. A recent study suggests that the primary function of *Tsix* is to prevent the induction of *Xist* from the active X chromosome [59]. This raises the possibility that *Tsix* may be required for *Xist* repression and therefore XCR during iPSC reprogramming. However, XCR can take place in iPSCs in the absence of *Tsix* [50••,51], and *Tsix* is also dispensable for *Xist* repression in the epiblast lineage of the blastocyst [60]. Another interesting question is how the developmental pattern of *Tsix* expression is reversed during reprogramming? Our team reported that *Tsix* activation is first seen on the Xa then on the Xi before reactivation of X-linked genes [50••] (Figure 2). Thus, the developmental sequence of *Tsix* expression appears perfectly reversed during reprogramming. This suggests that the trans-acting factors that regulate *Tsix* expression are more freely available to access the Xa than the Xi, which is coated with *Xist*. We conclude that induction of pluripotency converges on the activation of multiple pathways that trigger hallmarks of the active X chromosome in pluripotent cells and reverse XCI.

Stabilization of pluripotency by two active X chromosomes

A long-lasting question is whether two Xa's impact the pluripotent state? A recent study found that, in the mouse, two Xa's stabilize the 'naive' pluripotent state

in ESCs and establish what is coined ground-state pluripotency with a lower propensity for differentiation [61••]. It has been suggested that stabilization of pluripotency by two Xa's provides a likely explanation for the observation that female mammalian development is often slightly delayed compared to male individuals [61••]. Transcriptome analyses revealed a lower expression of pro-differentiation ERK1 and GSK3β signalling pathway targets in XX ESCs compared to XY ESCs [61••]. Consequently, exit of pluripotency is delayed in XX ESCs. Two Xa's in epiblast cells of the pre-implantation blastocyst may thus delay differentiation slightly until the decision of which X to inactivate has been made, thereby ensuring XCI for development to proceed [61••]. Thus, X chromosome status impacts the differentiation behavior of mouse PSCs.

Notably, the presence of two Xa's in female mouse ESCs also has consequences for the epigenomic landscape of pluripotent cells (Table 1). When grown in serum and LIF (serum/LIF), the genome of female ESCs is globally hypomethylated compared to male ESCs [62•,63,64]. This striking difference in DNA methylation level is directly linked to the presence of two Xa's, since loss of one of the two X chromosomes (XO ESCs) returns the female cells to male levels of DNA methylation [62•].

Inhibition of pro-differentiation signals by culturing ESCs in 2i [65,66] promotes ground-state naive pluripotency, and also induces a globally hypomethylated state [63,67,68]. Male ESCs in 2i have DNA methylation levels comparable to those of female ESCs grown in serum/LIF, while female ESCs in 2i have even lower DNA methylation [63,67–69]. Thus, two Xa's stabilize naive pluripotency even when ESCs are grown in 2i. The X-linked factor responsible for female-specific hypomethylation in PSCs remains to be identified. Major efforts have been deployed to understanding the resetting of DNA methylation during somatic cell reprogramming to iPSCs [47,70]. We speculate that XCR during somatic cell reprogramming to iPSCs could have important implications for the dynamics of DNA methylation and pluripotency, by inducing XaXa-dependent, female-specific hypomethylation in iPSCs. Therefore, it will be important to define the kinetics of DNA methylation in male and female reprogramming intermediates to gain a better understanding of how XCR impacts epigenetic reprogramming to pluripotency.

Table 1

Summary of reported DNA methylation levels of PSCs as a function of sex chromosome content and growth conditions.

		Autosomal DNA methylation — mouse	
Cells	**Growth media**	**Average DNA methylation**	**Reference**
XY ESCs	Serum/LIF	High	[62•,63,64,69]
XX ESCs	Serum/LIF	Low	[62•,64]
XO ESCs	Serum/LIF	High	[62•]
XX ESCs differentiated	Embryonic bodies	Low	[62•]
XX somatic cells	Kidney	High	[62•]
XY ESCs	2i	Low	[63,69]
XX ESCs	2i	Very low	[63]
XX iPSCs	–	?/(Minor satellites low)	[43•]
XY iPSCs	–	?	
XO iPSCs	–	?	

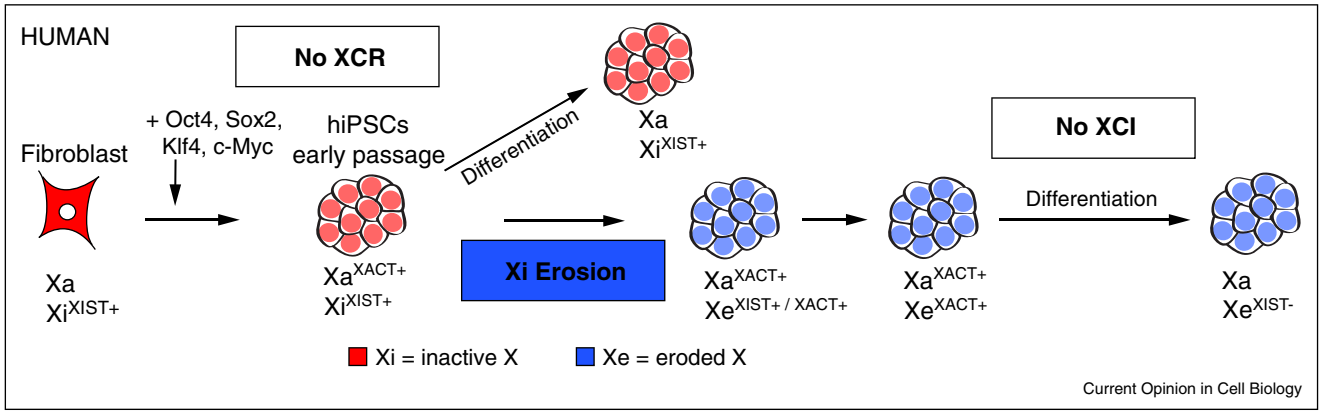
XCR in human reprogramming and in pluripotent stem cells

The question of whether the Xi undergoes XCR during human reprogramming to iPSCs is highly controversial and has been the subject of several studies [71,72,73•,74,75]. Multiple groups have reported that XCR takes place during human reprogramming as evidenced by reactivation of X-linked genes in human iPSC (hiPSC) lines [71,72,75]. By contrast, others, including our group, reported that female hiPSCs do not show reactivation of X-linked genes and retain an Xi [73•,74,76••]. In this case, the Xi showed enrichment of EZH2 on the Xi [73•], an epigenetic state like that of primed mouse PSCs which have an Xi and express *Xist* [77] (Figure 3). Therefore, our interpretation of a major difference between iPSC reprogramming in mice and in humans is the absence of XCR in the latter. We believe that the absence of XCR in hiPSCs is best explained by differences in pluripotent states between human and mouse, in agreement with the current notion that conventionally cultured human PSCs reside in the primed state whereas mouse ESCs and iPSCs are in the naive state [15].

Two studies have confirmed our finding that in hiPSCs the Xi is initially retained, and that the same chromosome is inactive in the starting somatic cell [74,76••]. However, as hiPSCs are subjected to long-term culture, the Xi is unstable and 'erodes' [73•,76••]. Erosion of XCI is characterized by loss of XIST and foci of H3-K27-trimethylation, and transcriptional reactivation, none of which are restored upon differentiation (Figure 3) [73•,76••,78]. Erosion also involves loss of X-linked promoter DNA methylation and transcriptional repression in hESC lines [76••,78] (Figure 3), and reactivation of the human-specific and pluripotency-specific lncRNA XACT [79••,80]. Erosion of XCI highlights a high incidence of epigenetic instability in hiPSCs, with different hiPSC lines showing variable degrees of erosion. The paradigm that emerges is that hiPSCs initially have an Xi which erodes upon extended passage. We reason that the instability of the Xi in human PSCs may explain reports of XCR in human reprogramming studies. It will be of great interest to test if reprogramming to the naive state in human PSCs can reset the erosion problem.

Importantly, erosion of XCI in hiPSCs impacts X-linked disease modeling, and hence could also impact our ability

Figure 3



Stages of XCR in human iPSC reprogramming. Human iPSCs resemble primed mouse PSCs and initially retain an Xi, marked by foci of EZH2 (pink focus). hiPSCs are subject to epigenetic instability in which erosion of XCI leads variable parts of the Xi to reactivate (blue). Erosion cannot be reversed by differentiation [76••,78].

to use the cells in the clinic [76••]. This is because multiple genes along the inactive X chromosome reactivate due to erosion, impacting X-linked disease modeling using hiPSCs as well as the quality of iPSCs [76••]. For example, erosion was shown to impact the modeling of Lesch-Nyhan syndrome with female iPSCs [76••]. Furthermore, in hiPSCs, loss of XIST has been highly correlated with upregulation of X-linked oncogenes, accelerated proliferation and poorer differentiation [81]. Therefore, XIST loss may result in stem cell lines of lower quality. This function of *Xist* may extend beyond PSCs as experimentally induced deletion of *Xist* in the blood compartment of mice resulted in tumor development including primary myelofibrosis, leukemia and histiocytic sarcoma which has also been argued to be due to Xi erosion and loss of silencing [82]. This illustrates the need to understand how epigenetic stability of the Xi links to maintenance of cell identity.

Concluding remarks

Not only is XCI a good tool to understand heterochromatin formation, but it is also a valuable paradigm to follow developmental states, in particular during reprogramming of somatic cells to pluripotency, as well as to monitor the epigenetic stability of human pluripotent stem cells. Differences regarding the extent of XCR in human iPSCs are best explained by differences in pluripotent states and by erosion of the Xi, the molecular underpinnings of which remain to emerge and will teach us more about pluripotency states in human development. It is still interesting why pluripotent stem cells may be prone to erosion and XIST loss. Therefore, the epigenetics of the X chromosome will be very valuable in the quest to establish high quality naive human PSCs for disease modeling and for the clinic. The epigenetic state of the Xi is also a good indicator of reprogramming progression in the mouse, and it will be of interest to define the stages of XCR during reprogramming to human naive pluripotency, and of XCI upon differentiation. Finally, findings on XCR using *in vitro* systems can help generate hypotheses that can be tested in developmental XCR events.

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- • of special interest
- •• of outstanding interest

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