Published in final edited form as:

Curr Opin Cell Biol. 2015 December ; 37: 75–83. doi:10.1016/j.ceb.2015.10.006.

X Chromosome Reactivation in Reprogramming and in Development

Vincent Pasque^{1,2} and Kathrin Plath^{1,*}

¹Department of Biological Chemistry, Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research, Jonsson Comprehensive Cancer Center, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA 90095, USA

Abstract

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.

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

*Correspondence: Kathrin Plath, UCLA School of Medicine, Department of Biological Chemistry, 615 Charles E. Young Drive South, BSRB 390D, Los Angeles, CA, 90095, Phone: 310-206-8688, kplath@mednet.ucla.edu.

²Present address: KU Leuven – University of Leuven, Stem Cell Biology and Embryology, Department of Development and Regeneration, Herestraat 49, B-3000 Leuven, Belgium.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

[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 GSK3 β (glycogen synthase kinase-3 β) 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 molecule combinations to derive hESCs in different pluripotent states, closer to the naive state of the blastocyst, have recently been reported [27–32]. 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 non coding 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 leading to 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 pluripotency 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 protein EZH2 on the Xi, absent 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]. Thereby, 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.

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 us to look at DNA methylation, which is acquired late in development during the XCI process 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 GSK3 β 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 behaviour 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 [62–64]. 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.

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 [71–75]. 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 modelling, and hence could also impact our ability 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 modelling using hiPSCs as well as the quality of iPSCs [76]. For example, erosion was shown to impact the modelling 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.

Acknowledgments

We thank Paula Petrella for illustrations and editing. VP was supported by the CIRM Training Grant TG2-01169. Research in the Plath lab is supported by the Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell

Research at UCLA, the Jonsson Comprehensive Cancer Center at UCLA, NIH (P01 GM099134), and CIRM (RB4-06133).

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Monk M, Harper MI. Sequential X chromosome inactivation coupled with cellular differentiation in early mouse embryos. *Nature*. 1979; 281:311–313. [PubMed: 551278]
2. Penny GD, Kay GF, Sheardown SA, Rastan S, Brockdorff N. Requirement for Xist in X chromosome inactivation. *Nature*. 1996; 379:131–137. [PubMed: 8538762]
3. Marahrens Y, Panning B, Dausman J, Strauss W, Jaenisch R. Xist-deficient mice are defective in dosage compensation but not spermatogenesis. *Genes Dev*. 1997; 11:156–166. [PubMed: 9009199]
4. Lee JT, Bartolomei MS. X-Inactivation, Imprinting, and Long Noncoding RNAs in Health and Disease. *Cell*. 2013; 152:1308–1323. [PubMed: 23498939]
5. McHugh CA, Chen C-K, Chow A, Surka CF, Tran C, McDonel P, Pandya-Jones A, Blanco M, Burghard C, Moradian A, et al. The Xist lncRNA interacts directly with SHARP to silence transcription through HDAC3. *Nature*. 2015; 521:232–236. [PubMed: 25915022]
6. Chu C, Zhang QC, da Rocha ST, Flynn RA, Bharadwaj M, Calabrese JM, Magnuson T, Heard E, Chang HY. Systematic Discovery of Xist RNA Binding Proteins. *Cell*. 2015; 161:404–416. [PubMed: 25843628]
7. Minajigi A, Froberg JE, Wei C, Sunwoo H, Kesner B, Colognori D, Lessing D, Payer B, Boukhali M, Haas W, et al. A comprehensive Xist interactome reveals cohesin repulsion and an RNA-directed chromosome conformation. *Science*. 2015;349. [PubMed: 26206902]
8. Monfort A, Di Minin G, Postlmayr A, Freimann R, Arieti F, Thore S, Wutz A. Identification of Spen as a Crucial Factor for Xist Function through Forward Genetic Screening in Haploid Embryonic Stem Cells. *Cell Rep*. 2015; 12:554–561. [PubMed: 26190100]
9. Moindrot B, Cerase A, Coker H, Masui O, Grijzenhout A, Pintacuda G, Schermelleh L, Nesterova TB, Brockdorff N. A Pooled shRNA Screen Identifies Rbm15, Spen, and Wtap as Factors Required for Xist RNA-Mediated Silencing. *Cell Rep*. 2015; 12:562–572. [PubMed: 26190105]
10. Okamoto I, Otte AP, Allis CD, Reinberg D, Heard E. Epigenetic dynamics of imprinted X inactivation during early mouse development. *Science*. 2004; 303:644–649. [PubMed: 14671313]
11. Mak W, Nesterova TB, de Napoles M, Appanah R, Yamanaka S, Otte AP, Brockdorff N. Reactivation of the paternal X chromosome in early mouse embryos. *Science*. 2004; 303:666–669. [PubMed: 14752160]
12. Williams LH, Kalantry S, Starmer J, Magnuson T. Transcription precedes loss of Xist coating and depletion of H3K27me3 during X-chromosome reprogramming in the mouse inner cell mass. *Development*. 2011; 138:2049–2057. [PubMed: 21471155]
13. Rastan S. Timing of X-chromosome inactivation in postimplantation mouse embryos. *Journal of embryology and experimental morphology*. 1982; 71:11–24. [PubMed: 6185603]
14. Takagi N, Sugawara O, Sasaki M. Regional and temporal changes in the pattern of X-chromosome replication during the early post-implantation development of the female mouse. *Chromosoma*. 1982; 85:275–286. [PubMed: 6180866]
15. Hackett JA, Surani MA. Regulatory Principles of Pluripotency. From the Ground State Up. *Cell Stem Cell*. 2014; 15:416–430. [PubMed: 25280218]
16. Merkle FT, Eggan K. Modeling human disease with pluripotent stem cells. from genome association to function. *Cell Stem Cell*. 2013; 12:656–668. [PubMed: 23746975]
17. Tesar PJ, Chenoweth JG, Brook FA, Evans EP, Mack DL, Gardner RL, McKay RDG. New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature*. 2007; 448:196–199. [PubMed: 17597760]

18. Brons IGM, Smithers LE, Trotter MWB, Rugg-Gunn P, Sun B, Chuva de Sousa Lopes SM, Howlett SK, Clarkson A, Ahrlund-Richter L, Pedersen RA, et al. Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature*. 2007; 448:191–195. [PubMed: 17597762]
19. Bao S, Tang F, Li X, Hayashi K, Gillich A, Lao K, Surani M. Epigenetic reversion of post-implantation epiblast to pluripotent embryonic stem cells. *Nature*. 2009; 461:1292–1295. [PubMed: 19816418]
20. Chou Y-F, Chen H-H, Eijpe M, Yabuuchi A, Chenoweth JG, Tesar P, Lu J, McKay RDG, Geijsen N. The growth factor environment defines distinct pluripotent ground states in novel blastocyst-derived stem cells. *Cell*. 2008; 135:449–461. [PubMed: 18984157]
21. Hoffman LM, Hall L, Batten JL, Young H, Pardasani D, Baetge EE, Lawrence J, Carpenter MK. X-inactivation status varies in human embryonic stem cell lines. *Stem Cells*. 2005; 23:1468–1478. [PubMed: 16123389]
22. Hall LL, Byron M, Butler J, Becker KA, Nelson A, Amit M, Itskovitz-Eldor J, Stein J, Stein G, Ware C, et al. X-inactivation reveals epigenetic anomalies in most hESC but identifies sublines that initiate as expected. *J Cell Physiol*. 2008; 216:445–452. [PubMed: 18340642]
23. Silva SS, Rowntree RK, Mekhoubad S, Lee JT. X-chromosome inactivation and epigenetic fluidity in human embryonic stem cells. *Proc Natl Acad Sci USA*. 2008; 105:4820–4825. [PubMed: 18339803]
24. Shen Y, Matsuno Y, Fouse SD, Rao N, Root S, Xu R, Pellegrini M, Riggs AD, Fan G. X-inactivation in female human embryonic stem cells is in a nonrandom pattern and prone to epigenetic alterations. *Proceedings of the National Academy of Sciences*. 2008; 105:4709–4714.
25. Lengner CJ, Gimelbrant AA, Erwin JA, Cheng AW, Guenther MG, Welstead GG, Alagappan R, Frampton GM, Xu P, Muffat J, et al. Derivation of Pre-X Inactivation Human Embryonic Stem Cells under Physiological Oxygen Concentrations. *Cell*. 2010; 141:872–883. [PubMed: 20471072]
26. Dhara SK, Benvenisty N. Gene trap as a tool for genome annotation and analysis of X chromosome inactivation in human embryonic stem cells. *Nucleic Acids Res*. 2004; 32:3995–4002. [PubMed: 15284332]
27. Theunissen TW, Powell BE, Wang H, Mitalipova M, Faddah DA, Reddy J, Fan ZP, Maetzel D, Ganz K, Shi L, et al. Systematic identification of culture conditions for induction and maintenance of naive human pluripotency. *Cell Stem Cell*. 2014; 15:471–487. This paper reports culture conditions thought to promote the pluripotency ground state in human ESCs. [PubMed: 25090446]
28. Takashima Y, Guo G, Loos R, Nichols J, Ficiz G, Krueger F, Oxley D, Santos F, Clarke J, Mansfield W, et al. Resetting Transcription Factor Control Circuitry toward Ground-State Pluripotency in Human. *Cell*. 2014; 158:1254–1269. This paper reports culture conditions thought to promote the pluripotency ground state in human ESCs. [PubMed: 25215486]
29. Hanna J, Cheng AW, Saha K, Kim J, Lengner CJ, Soldner F, Cassady JP, Muffat J, Carey BW, Jaenisch R. Human embryonic stem cells with biological and epigenetic characteristics similar to those of mouse ESCs. *Proceedings of the National Academy of Sciences*. 2010; 107:9222–9227.
30. Gafni O, Weinberger L, Mansour AA, Manor YS, Chomsky E, Ben-Yosef D, Kalma Y, Viukov S, Maza I, Zviran A, et al. Derivation of novel human ground state naive pluripotent stem cells. *Nature*. 2013; 504:282–286. [PubMed: 24172903]
31. Ware CB, Nelson AM, Mecham B, Hesson J, Zhou W, Jonlin EC, Jimenez-Caliani AJ, Deng X, Cavanaugh C, Cook S, et al. Derivation of naive human embryonic stem cells. *Proceedings of the National Academy of Sciences*. 2014; 111:4484–4489.
32. Chan Y-S, Göke J, Ng J-H, Lu X, Gonzales KAU, Tan C-P, Tng W-Q, Hong Z-Z, Lim Y-S, Ng H-H. Induction of a human pluripotent state with distinct regulatory circuitry that resembles preimplantation epiblast. *Cell Stem Cell*. 2013; 13:663–675. [PubMed: 24315441]
33. Augui S, Nora EP, Heard E. Regulation of X-chromosome inactivation by the X-inactivation centre. *Nat Rev Genet*. 2011; 12:429–442. [PubMed: 21587299]
34. Chow J, Heard E. X inactivation and the complexities of silencing a sex chromosome. *Curr Opin Cell Biol*. 2009; 21:359–366. [PubMed: 19477626]

35. Csankovszki G, Nagy A, Jaenisch R. Synergism of Xist RNA, DNA methylation, and histone hypoacetylation in maintaining X chromosome inactivation. *J Cell Biol.* 2001; 153:773–784. [PubMed: 11352938]
36. Monk M, McLaren A. X-chromosome activity in foetal germ cells of the mouse. *Journal of embryology and experimental morphology.* 1981; 63:75–84. [PubMed: 7310296]
37. Sugimoto M, Abe K. X chromosome reactivation initiates in nascent primordial germ cells in mice. *PLoS Genet.* 2007; 3:e116. [PubMed: 17676999]
38. Guo F, Yan L, Guo H, Li L, Hu B, Zhao Y, Yong J, Hu Y, Wang X, Wei Y, et al. The Transcriptome and DNA Methylome Landscapes of Human Primordial Germ Cells. *Cell.* 2015; 161:1437–1452. [PubMed: 26046443]
39. Chuva de Sousa Lopes SM, Hayashi K, Shovlin TC, Mifsud W, Azim Surani M, McLaren A. X chromosome activity in mouse XX primordial germ cells. *PLoS Genet.* 2008; 4:e30. [PubMed: 18266475]
40. de Napoles M, Nesterova T, Brockdorff N. Early loss of Xist RNA expression and inactive X chromosome associated chromatin modification in developing primordial germ cells. *PLoS ONE.* 2007; 2:e860. [PubMed: 17848991]
41. Gurdon JB, Melton DA. Nuclear reprogramming in cells. *Science.* 2008; 322:1811–1815. [PubMed: 19095934]
42. Papp B, Plath K. Epigenetics of Reprogramming to Induced Pluripotency. *Cell.* 2013; 152:1324–1343. [PubMed: 23498940]
43. Maherali N, Sridharan R, Xie W, Utikal J, Eminli S, Arnold K, Stadtfeld M, Yachechko R, Tchieu J, Jaenisch R, et al. Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. *Cell Stem Cell.* 2007; 1:55–70. This paper was the first to show global epigenetic resetting following somatic cell reprogramming to iPSCs as well as the notion that X chromosome reactivation takes place in female iPSCs. This paper opened up the possibility to use somatic cell reprogramming to iPSCs for further mechanistic studies of X chromosome regulation during reprogramming. [PubMed: 18371336]
44. Eggan K, Akutsu H, Hochedlinger K, Rideout W, Yanagimachi R, Jaenisch R. X-Chromosome inactivation in cloned mouse embryos. *Science.* 2000; 290:1578–1581. [PubMed: 11090356]
45. Takahashi K, Yamanaka S. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. *Cell.* 2006; 126:663–676. [PubMed: 16904174]
46. Hanna J, Saha K, Pando B, van Zon J, Lengner CJ, Creighton MP, van Oudenaarden A, Jaenisch R. Direct cell reprogramming is a stochastic process amenable to acceleration. *Nature.* 2009; 462:595–601. [PubMed: 19898493]
47. Polo JM, Anderssen E, Walsh RM, Schwarz BA, Nefzger CM, Lim SM, Borkent M, Apostolou E, Alaei S, Cloutier J, et al. A Molecular Roadmap of Reprogramming Somatic Cells into iPS Cells. *Cell.* 2012; 151:1617–1632. [PubMed: 23260147]
48. O'Malley J, Skylaki S, Iwabuchi KA, Chantzoura E, Ruetz T, Johnsson A, Tomlinson SR, Linnarsson S, Kaji K. High-resolution analysis with novel cell-surface markers identifies routes to iPS cells. *Nature.* 2013; 499:88–91. [PubMed: 23728301]
49. Buganim Y, Faddah DA, Cheng AW, Itskovich E, Markoulaki S, Ganz K, Klemm SL, van Oudenaarden A, Jaenisch R. Single-Cell Expression Analyses during Cellular Reprogramming Reveal an Early Stochastic and a Late Hierarchic Phase. *Cell.* 2012; 150:1209–1222. [PubMed: 22980981]
50. Pasque V, Tchieu J, Karnik R, Uyeda M, Dimashkie AS, Case D, Papp B, Bonora G, Patel S, Ho R, et al. X Chromosome Reactivation Dynamics Reveal Stages of Reprogramming to Pluripotency. *Cell.* 2014; 159:1681–1697. This study identifies sequential molecular events of XCR and reprogramming to iPSCs. [PubMed: 25525883]
51. Payer B, Rosenberg M, Yamaji M, Yabuta Y, Koyanagi-Aoi M, Hayashi K, Yamanaka S, Saitou M, Lee JT. Tsix RNA and the Germline Factor, PRDM14, Link X Reactivation and Stem Cell Reprogramming. *Molecular Cell.* 2013; 52:805–818. [PubMed: 24268575]
52. Gillich A, Bao S, Grabole N, Hayashi K, Trotter MWB, Pasque V, Magnúsdóttir E, Surani MA. Epiblast stem cell-based system reveals reprogramming synergy of germline factors. *Cell Stem Cell.* 2012; 10:425–439. [PubMed: 22482507]

53. Plath K, Fang J, Mlynarczyk-Evans SK, Cao R, Worringer KA, Wang H, la Cruz de CC, Otte AP, Panning B, Zhang Y. Role of histone H3 lysine 27 methylation in X inactivation. *Science*. 2003; 300:131–135. [PubMed: 12649488]
54. Silva J, Mak W, Zvetkova I, Appanah R, Nesterova TB, Webster Z, Peters AHFM, Jenuwein T, Otte AP, Brockdorff N. Establishment of histone h3 methylation on the inactive X chromosome requires transient recruitment of Eed-Enx1 polycomb group complexes. *Dev Cell*. 2003; 4:481–495. [PubMed: 12689588]
55. Pasque V, Radzisheuskaya A, Gillich A, Halley-Stott RP, Panamarova M, Zernicka-Goetz M, Surani MA, Silva JCR. Histone variant macroH2A marks embryonic differentiation in vivo and acts as an epigenetic barrier to induced pluripotency. *J Cell Sci*. 2012; 125:6094–6103. [PubMed: 23077180]
56. Mermoud JE, Costanzi C, Pehrson JR, Brockdorff N. Histone macroH2A1. 2 relocates to the inactive X chromosome after initiation and propagation of X-inactivation. *J Cell Biol*. 1999; 147:1399–1408. [PubMed: 10613899]
57. Gendrel A-V, Apedaile A, Coker H, Termanis A, Zvetkova I, Godwin J, Tang YA, Huntley D, Montana G, Taylor S, et al. Smchd1-Dependent and -Independent Pathways Determine Developmental Dynamics of CpG Island Methylation on the Inactive X Chromosome. *Dev Cell*. 2012; 23:265–279. [PubMed: 22841499]
58. Lee JT, Davidow LS, Warshawsky D. Tsix, a gene antisense to Xist at the X-inactivation centre. *Nat Genet*. 1999; 21:400–404. [PubMed: 10192391]
59. Gayen S, Maclary E, Buttigieg E, Hinten M, Kalantry S. A Primary Role for the Tsix lncRNA in Maintaining Random X-Chromosome Inactivation. *Cell Rep*. 2015; 11:1251–1265. [PubMed: 25981039]
60. Maclary E, Buttigieg E, Hinten M, Gayen S, Harris C, Sarkar MK, Purushothaman S, Kalantry S. Differentiation-dependent requirement of Tsix long non-coding RNA in imprinted X-chromosome inactivation. *Nature communications*. 2014; 5:4209.
- 61•. Schulz EG, Meisig J, Nakamura T, Okamoto I, Sieber A, Picard C, Borensztein M, Saitou M, Blüthgen N, Heard E. The Two Active X Chromosomes in Female ESCs Block Exit from the Pluripotent State by Modulating the ESC Signaling Network. *Cell Stem Cell*. 2014; 14:203–216. This work describes characterize the transcriptome of female and male mouse pluripotent stem cell in undifferentiated and differentiated conditions. [PubMed: 24506884]
- 62•. Zvetkova I, Apedaile A, Ramsahoye B, Mermoud JE, Crompton LA, John R, Feil R, Brockdorff N. Global hypomethylation of the genome in XX embryonic stem cells. *Nat Genet*. 2005; 37:1274–1279. Zvetkova et al. show that mouse XX ESCs are globally hypomethylated compared to XY and XO ESCs. [PubMed: 16244654]
63. Habibi E, Brinkman AB, Arand J, Kroeze LI, Kerstens HHD, Matarese F, Lepikhov K, Gut M, Brun-Heath I, Hubner NC, et al. Whole-Genome Bisulfite Sequencing of Two Distinct Interconvertible DNA Methylomes of Mouse Embryonic Stem Cells [Internet]. *Cell Stem Cell*. 2013; 13:360–369. [PubMed: 23850244]
64. Ooi SK, Wolf D, Hartung O, Agarwal S, Daley GQ, Goff SP, Bestor TH. Dynamic instability of genomic methylation patterns in pluripotent stem cells. *Epigenetics & Chromatin*. 2010; 3:17. [PubMed: 20868487]
65. Marks H, Kalkan T, Menafrà R, Denisov S, Jones K, Hofemeister H, Nichols J, Kranz A, Francis Stewart A, Smith A, et al. The transcriptional and epigenomic foundations of ground state pluripotency. *Cell*. 2012; 149:590–604. [PubMed: 22541430]
66. Ying Q-L, Wray J, Nichols J, Batlle-Morera L, Doble B, Woodgett J, Cohen P, Smith A. The ground state of embryonic stem cell self-renewal. *Nature*. 2008; 453:519–523. [PubMed: 18497825]
67. Leitch HG, McEwen KR, Turp A, Encheva V, Carroll T, Grabole N, Mansfield W, Nashun B, Knezovich JG, Smith A, et al. Naive pluripotency is associated with global DNA hypomethylation. *Nat Struct Mol Biol*. 2013; 20:311–316. [PubMed: 23416945]
68. Ficiz G, Hore TA, Santos F, Lee HJ, Dean W, Arand J, Krueger F, Oxley D, Paul Y-L, Walter J, et al. FGF Signaling Inhibition in ESCs Drives Rapid Genome-wide Demethylation to the Epigenetic Ground State of Pluripotency. *Cell Stem Cell*. 2013; 13:351–359. [PubMed: 23850245]

69. Hackett JA, Dietmann S, Murakami K, Down TA, Leitch HG, Surani MA. Synergistic Mechanisms of DNA Demethylation during Transition to Ground-State Pluripotency. *Stem Cell Reports*. 2013; 1:518–531. [PubMed: 24371807]
70. Lee D-S, Shin J-Y, Tonge PD, Puri MC, Lee S, Park H, Lee W-C, Hussein SMI, Bleazard T, Yun J-Y, et al. An epigenomic roadmap to induced pluripotency reveals DNA methylation as a reprogramming modulator. *Nat Commun*. 2014; 5:1–10.
71. Tomoda K, Takahashi K, Leung K, Okada A, Narita M, Yamada NA, Eilertson KE, Tsang P, Baba S, White MP, et al. Derivation conditions impact X-inactivation status in female human induced pluripotent stem cells. *Cell Stem Cell*. 2012; 11:91–99. [PubMed: 22770243]
72. Barakat TS, Ghazvini M, de Hoon B, Li T, Eussen B, Douben H, van der Linden R, van der Stap N, Boter M, Laven JS, et al. Stable X Chromosome Reactivation in Female Human Induced Pluripotent Stem Cells. *Stem Cell Reports*. 2015; 4:199–208. [PubMed: 25640760]
73. Tchieu J, Kuoy E, Chin MH, Trinh H, Patterson M, Sherman SP, Aimiwu O, Lindgren A, Hakimian S, Zack JA, et al. Female human iPSCs retain an inactive X chromosome. *Cell Stem Cell*. 2010; 7:329–342. This study was the first to report lack of X chromosome reactivation in human iPSCs, which suggested implications for disease modeling. [PubMed: 20727844]
74. Pomp O, Dreesen O, Leong DFM, Meller-Pomp O, Tan TT, Zhou F, Colman A. Unexpected X chromosome skewing during culture and reprogramming of human somatic cells can be alleviated by exogenous telomerase. *Cell Stem Cell*. 2011; 9:156–165. [PubMed: 21816366]
75. Kim K-Y, Hysolli E, Tanaka Y, Wang B, Jung Y-W, Pan X, Weissman SM, Park I-H. X Chromosome of Female Cells Shows Dynamic Changes in Status during Human Somatic Cell Reprogramming. *Stem Cell Reports*. 2014; 2:896–909. [PubMed: 24936474]
76. Mekhoubad S, Bock C, de Boer AS, Kiskinis E, Meissner A, Eggan K. Erosion of Dosage Compensation Impacts Human iPSC Disease Modeling. *Cell Stem Cell*. 2012; 10:595–609. This study resolves a discrepancy regarding XCR in human iPSCs. It confirms that human iPSCs first have an Xi, which then erodes leading to reactivation and DNA demethylation of the Xi. Differentiation or further reprogramming does not restore erosion. Erosion involves loss of promoter DNA methylation and transcriptional repression in hES lines, impacting disease modeling. [PubMed: 22560080]
77. Pasque V, Gillich A, Garrett N, Gurdon JB. Histone variant macroH2A confers resistance to nuclear reprogramming. *EMBO J*. 2011; 30:2373–2387. [PubMed: 21552206]
78. Nazor KL, Altun G, Lynch C, Tran H, Harness JV, Slavin I, Garitaonandia I, Muller F-J, Wang Y-C, Boscolo FS, et al. Recurrent variations in DNA methylation in human pluripotent stem cells and their differentiated derivatives. *Cell Stem Cell*. 2012; 10:620–634. [PubMed: 22560082]
79. Vallot C, Huret C, Lesecque Y, Resch A, Oudrhiri N, Bennaceur-Griscelli A, Duret L, Rougeulle C. XACT, a long noncoding transcript coating the active X chromosome in human pluripotent cells. *Nat Genet*. 2013; 45:239–241. Vallot et al. discovered the lncRNA XACT that is expressed on the active X chromosomes in human pluripotent stem cells, and which is not found in the mouse. This paper paved the way to further analyses of XACT expression during erosion of the Xi in human pluripotent stem cells. [PubMed: 23334669]
80. Vallot C, Ouimette J-F, Makhoul M, Féraud O, Pontis J, Côme J, Martinat C, Bennaceur-Griscelli A, Lalande M, Rougeulle C. Erosion of X Chromosome Inactivation in Human Pluripotent Cells Initiates with XACT Coating and Depends on a Specific Heterochromatin Landscape. *Cell Stem Cell*. 2015; 16:533–546. [PubMed: 25921272]
81. Anguera MC, Sadreyev R, Zhang Z, Szanto A, Payer B, Sheridan SD, Kwok S, Haggarty SJ, Sur M, Alvarez J, et al. Molecular signatures of human induced pluripotent stem cells highlight sex differences and cancer genes. *Cell Stem Cell*. 2012; 11:75–90. [PubMed: 22770242]
82. Yildirim E, Kirby JE, Brown DE, Mercier FE, Sadreyev RI, Scadden DT, Lee JT. Xist RNA is a potent suppressor of hematologic cancer in mice. *Cell*. 2013; 152:727–742. [PubMed: 23415223]
83. Nolen LD, Gao S, Han Z, Mann MRW, Gie Chung Y, Otte AP, Bartolomei MS, Latham KE. X chromosome reactivation and regulation in cloned embryos. *Dev Biol*. 2005; 279:525–540. [PubMed: 15733677]
84. Inoue K, Kohda T, Sugimoto M, Sado T, Ogonuki N, Matoba S, Shiura H, Ikeda R, Mochida K, Fujii T, et al. Impeding Xist expression from the active X chromosome improves mouse somatic cell nuclear transfer. *Science*. 2010; 330:496–499. [PubMed: 20847234]

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.

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.

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].

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
XY ESCs	Serum / LIF	High
XX ESCs	Serum / LIF	Low
XO ESCs	Serum / LIF	High
XX ESCs Differentiated	Embryonic Bodies	Low
XX Somatic Cells	Kidney	High
XY ESCs	2i	Low
XX ESCs	2i	Very Low
XX iPSCs	-	? / (Minor Satellites Low)
XY iPSCs	-	?
XO iPSCs	-	?