Topic: Reversing X Chromosome Inactivation as a New Therapeutic Treatment for X-linked Diseases

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Reversal of X chromosome inactivation: lessons from pluripotent reprogramming of mouse and human somatic cells

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ABSTRACT

X chromosome inactivation (XCI) is a strategy used by mammals to silence genes along one of the two female X chromosomes and equilibrate expression dosage between XY males and XX females. This epigenetically-inherited silencing is established during early embryonic development and maintained thereafter through cell divisions. Seeding of multiple repressive epigenetic marks along the inactive X chromosome (Xi) makes inactivation extremely robust and difficult to reverse upon single genetic perturbations. Reversal of XCI has, however, been observed when somatic cells are reprogrammed towards pluripotency, and *in vitro* reprogramming techniques have been used in recent years to dissect Xi gene reactivation mechanisms. These studies pave the way for developing novel therapeutic approaches for X-linked diseases. Here, the author reviews Xi reactivation during pluripotent reprogramming of mouse and human somatic cells, highlight recent advances and species-specific differences, and discuss the relevance for human diseases.

INTRODUCTION

Dosage compensation between XX females and XY males is achieved in placental mammals by the random inactivation of one of the two female X chromosomes[1,2]. This process - named X chromosome inactivation (XCI) - is a major example of epigenetic gene regulation that leads to global chromatin condensation and transcriptional silencing along the future inactive X chromosome (Xi)[2]. The choice of which X chromosome to inactivate is completely random and, once established in early embryonic precursors it is stably maintained through cell divisions. As a consequence, females are mosaics of cells expressing either the maternal or paternal allele that co-exist in a 50:50 ratio. Mosaicism makes females more refractory to diseases that are caused by X-linked mutations because cells expressing the wild-type allele can functionally compensate the deficit in heterozygous carriers. In addition, the X chromosome that carries mutations

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or structural abnormalities is often found as inactive in the majority of somatic cells due to selection[3,4]. Many X-linked diseases are indeed asymptomatic in heterozygous females and manifest only in their male progeny[5]. However, some X-linked mutations are lethal in males and lead to severe disease in heterozygous females[6]. For example, in the case of gene mutations with a dominant negative effect, expression of a wild-type allele from 50% of cells is not able to rescue the function in heterozygosity. This is exemplified by *UBQLN2* mutations that cause abnormal protein aggregation and consequent neurodegeneration in X-linked amyotrophic lateral sclerosis[7]. Importantly, there are other diseases in which the compensatory effect of XCI in heterozygous females is not effective due to tissue-specific sensitivity to dosage unbalance and/or to skewed XCI patterns that favor the expression of mutated genes. For example, expression of the wild-type allele only from half of the cells may lead to loss of function only in certain tissues but not in others, as for mutations in the X-linked *MECP2* gene that specifically cause a severe brain deficit even if the gene is constitutively expressed[8,9]. In addition, in human some genes escape inactivation and are variably expressed from the Xi in different tissues of the same individual and even in-between different females[10]. This variability in Xi expression has been suggested to influence the penetrance and expressivity of X-linked diseases as hypothesized for oro-facial-digital syndrome type 1[11]. Finally, it has been shown that XCI skewing can also favor cells in which the wild-type allele is on the inactive X chromosome thus leading to the outbreak of disease in heterozygous females[12-16].

An important implication of the epigenetic regulation of Xi gene expression is the preservation of the genetic material and, indeed, the presence of a silent set of alleles for over a thousand genes on the X chromosome. Reactivation of wild-type alleles in heterozygous females could potentially be harnessed and might represent a therapeutic approach for many X-linked diseases. This is probably best exemplified by Rett syndrome, for which a phenotypic reversal of advanced neurological symptoms has been shown in both young and adult mice upon expression of a wild-type *MeCP2*[17]. Importantly, rescue of disease symptoms has also been observed in human and mouse upon gene therapy of X-linked adrenoleukodystrophy[18] and Hunter syndrome[19,20], two recessive X-linked diseases that also affect heterozygous females[16,21]. This suggests that the usefulness of Xi reactivation in clinical approaches might be extended beyond dominant X-linked diseases. Understanding the molecular mechanisms of Xi inactivation and reactivation will be instrumental in the future to engineer selective Xi gene reactivation as a novel clinical approach for several X-linked diseases.

Our current understanding of XCI and its molecular mechanisms derives from studies in the mouse. During mouse embryonic development, Xi gene silencing is triggered by *Xist*, a long non-coding RNA that is up-regulated from the future Xi and spreads along the chromosome domain[22]. Although the repertoire of *Xist* interacting proteins has been recently identified, the precise molecular mechanisms by which *Xist* initiates silencing are still unknown[23-27]. It is currently believed that *Xist* might act as a scaffold to recruit further factors on the Xi, including Polycomb repressive complex 1 (PRC1), histone deacetylases, histone variants and the DNA methylation machinery[24,25,28-30]. Upon *Xist* coating the inactive X chromosome indeed becomes progressively devoid of RNA polymerase II and chromatin marks associated with transcriptionally active regions[31]; whereas it is enriched of repressive marks such as H3K27me3[32], H3K9me2/me3[33], H2AK119ub[34,35], macroH2A1[36] and promoter DNA methylation[37]. The multitude of epigenetic modifications that are deposited along the Xi are believed to maintain silencing in a redundant manner as removing single factors does not lead to global gene reactivation. Supporting this hypothesis, it has been shown that Xi gene silencing is maintained in the absence of macroH2A1 histone variant[38] and upon functional loss of Polycomb repressive complex PRC2 and some PRC1 components, which catalyze the deposition of the repressive chromatin marks H3K27me3 and H2AK119ub respectively[39,40]. A recent study of several *Xist* interacting proteins has also shown that knock-down of any single interactor could not reactivate an Xi-integrated GFP reporter, whereas combining targeting of each interactor with inhibition of DNA methylation and topoisomerases leads to reactivation of 75-100 Xi genes out of around 200 analyzed[25]. On the other hand, depletion of *Xist* in somatic cells leads to a stochastic reactivation of single Xi genes[41] that can be potentiated when combined with histone deacetylase (HDAC) inhibitors and DNA demethylation[42]. Importantly, *Xist* depletion in mouse fibroblasts has been shown to alter the Xi chromosome conformation making it more similar to the one of the active X chromosome (Xa)[25]. In addition, silencing of factors that affect *Xist* expression and/or localization has been shown to partially reactivate the Xi[43,44]. Altogether these findings support the hypothesis that multiple epigenetic layers are in place to prevent Xi reactivation and suggest a central role for *Xist* both in the initiation and maintenance of XCI.

Cell fate reprogramming studies offer the opportunity
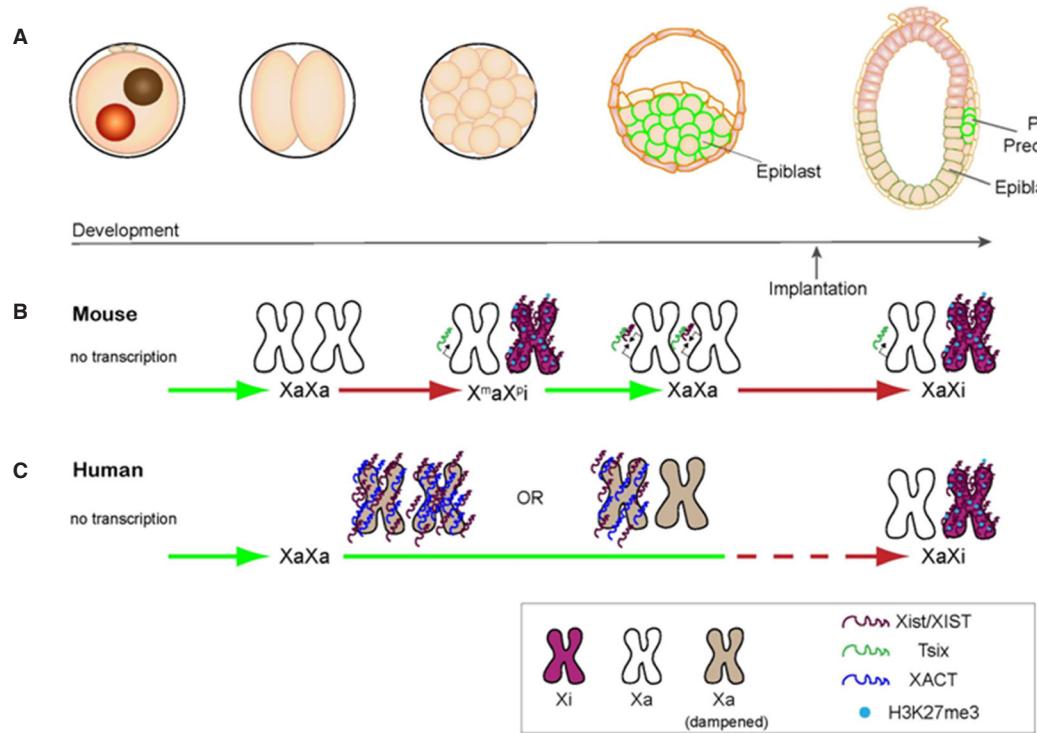
Figure 1: X chromosome state in mouse and human female embryos. (A) Schematic of early developmental stages; (B) in mouse embryos, both X chromosomes are transcribed upon the zygote genome activation (ZGA) at 2-cell stage. Imprinted inactivation of the paternal X chromosome then takes place at the 4-cell stage and is followed by reactivation in the epiblast that will give rise to the embryo proper. Upon implantation, the epiblast undergoes random XCI whereas imprinted XCI is maintained in extraembryonic lineages. As depicted, mouse Xi is characterized by the expression and coating of *Xist*, whereas the Xa expresses Tsix, a long non-coding RNA that antagonizes *Xist* and functions specifically in mouse but not in human; (C) in human embryos, the two X chromosomes are transcribed upon ZGA at 4-cell stage and remain transcriptionally active throughout pre-implantation development. *XIST* and another human-specific long non-coding RNA, XACT, coat both active X chromosomes in human embryos. Notably, *XIST* RNA does not tightly localize on the X chromosomes but shows a diffuse pattern and its coating is not accompanied by enrichment of H3K27me3. H3K27me3 enrichment is instead observed along the Xi upon XCI initiation. XCI: X chromosome inactivation; Xi: inactive X chromosome; Xa: active X chromosome; PGC: primordial germ cell

to model Xi reactivation and unravel its molecular mechanisms. Reversal of X inactivation has, in fact, been observed when mouse somatic cells are reprogrammed towards a pluripotent state. A tight link between pluripotency and reversal of XCI has been demonstrated[45-48], and reprogramming of somatic cells towards pluripotency has been widely used to investigate its dynamics and molecular mechanisms in mouse[49-52]. In human, the connection between pluripotency and Xi reactivation has instead been controversial as different Xi states have been reported in human embryonic stem cells (ESCs)[53-57] and in induced pluripotent stem cells (iPSCs)[55-59]. The lack of defined culture conditions that stabilize an embryonic-like pluripotent state and the resulting epigenetic instability of human pluripotent cells contributed to the observed variability. Recent studies in human embryos and ESCs suggest substantial differences in the mechanisms of XCI between human and mouse thus highlighting the need for model systems that allow to directly investigate the human Xi and its reactivation[57,60-62]. Here, I review studies of Xi reactivation during pluripotent reprogramming of both

mouse and human cells, emphasize specie-specific differences and recent advances in reprogramming-mediated human Xi reactivation.

REPROGRAMMING-MEDIATED Xi REACTIVATION IN MOUSE

In mouse embryos, the two X chromosomes undergo several rounds of inactivation and reactivation[63,64] [Figure 1A and B]. The first round of XCI takes place at the 4-cell stage and leads to the inactivation of the paternally-inherited X chromosome. This imprinted form of XCI is followed by reactivation in the epiblast cells of the blastocyst where random XCI is later established around implantation. Imprinted XCI is instead maintained in extraembryonic lineages. Interestingly, reversal of X inactivation has also been observed during mouse development when pluripotency genes are re-expressed in primordial germ cells (PGCs)[65-67]. In addition, several mouse pluripotency factors have been shown to inhibit XCI[45-48] thus suggesting an intimate connection between pluripotency and the presence of two active

X chromosomes.

Cell fusion-mediated Xi reactivation

The earliest evidence of an association between reversal of XCI and pluripotent reprogramming of somatic cells is dated back to 1983 when Takagi et al.[68] showed that fusions between female mouse thymocytes and pluripotent mouse embryonic carcinoma cells (ECCs) shifted the replication timing of the thymocyte Xi from late to early S phase, as observed for transcriptionally active X chromosomes. Early replication timing was associated with reactivation of the X-linked Pgk1 gene from the Xi and was observed upon fusions of several differentiated cells from thymus, spleen or bone marrow with ECCs, but not when two differentiated cell types were fused together. Importantly, hybrids obtained between mouse somatic cells and ECCs acquired the pluripotent differentiation potential of the parental carcinoma cells suggesting that reprogramming towards pluripotency might trigger Xi reactivation. Later studies confirmed that embryonic pluripotent cells of different origin (including ECC, ESC and embryonic germ cells, EGC) are able to reprogram the somatic cell genome and reverse XCI upon fusion. Specifically, it has been shown that the somatic Xi acquires epigenetic features of the active X chromosome (Xa), including early replication timing, loss of Xist coating and Xi-associated histone marks (e.g. H3K27me3 and H2Aub1), and re-expresses an Xi-linked GFP transgene[69-71]. Importantly, somatic-ESC hybrids are able to re-initiate random XCI upon differentiation suggesting a complete erasure of epigenetic memory along the Xi[70,72]. However, the fusion of ESC with somatic cells does not lead to erasure of genomic imprinting[69,73]. This extended reprogramming potential has instead been observed in fusions with EGCs that can induce both Xi reactivation and loss of DNA-methylation imprinting marks in the somatic nuclei[73,74].

Cell fusion studies have also demonstrated that the reprogramming capacity of embryonic pluripotent cells is ascribed to the transfer of pluripotency-associated transcriptional factors into the somatic nucleus[71,75]. Specifically, it has been shown that mouse ESCs (mESCs) overexpressing Nanog have an enhanced reprogramming capacity whereas ESCs lacking Oct4, but not Sox2, fail to induce pluripotent reprogramming in the somatic fusion partner. This is consistent with findings in mESCs where Oct4, Nanog, Sox2 and Rex1 repress Xist expression, while Klf4, c-Myc and Rex1 up-regulate Tsix[45-48], a long noncoding RNA that antagonizes Xist and protects the Xa from inactivation[76-78]. These results suggest that pluripotency factors within the mESCs might induce Xi reactivation

by directly repressing Xist and activating Tsix. Consistently with this hypothesis, re-expression of Oct4 from the somatic genome has been shown to precede Xi reactivation and repression of Xist expression upon cell fusion-mediated reprogramming[50]. The delayed repression of Xist has been associated with the kinetics of DNA methylation at its 5' regulatory sequences and is enhanced by the activation of Dnmt3a and Tsix via HDAC inhibitors. This suggests that chromatin remodeling is required together with pluripotency factors in order to reverse XCI, although delocalization of Xist rather that loss of its expression has been associated with Xi gene reactivation[79].

Somatic cell nuclear transfer-mediated Xi reactivation

The somatic cell genome can restart the entire developmental program upon nuclear transfer into eggs or oocytes. Conversely to other reprogramming techniques, somatic cell nuclear transfer (SCNT) reprograms differentiated cells to a totipotent state from which both embryonic and extra-embryonic lineages develop into cloned animals[80,81]. In this model system, reprogramming is triggered by maternally-inherited factors that are present in the cytoplasm of metaphase II oocytes. Interestingly, it has been shown that the zygote and blastomeres of early embryonic stages are not capable of reprogramming somatic nuclei unless they are in mitosis[82,83]. The enhanced reprogramming capacity of mitotic cells has been ascribed to the release of chromatin-bound factors during mitosis and depends upon cell cycle synchronization between the somatic donor and the embryonic recipient[84,85]. This evidence suggested a model in which the mitotic environment of the recipient cell induces premature chromatin condensation and consequent release of chromatin-bound factors in somatic nuclei, thus facilitating genomic access of transcriptional regulators that re-establish a totipotent transcriptional program[86].

The success of nuclear reprogramming has been associated with erasure of epigenetic memory from the somatic nucleus[87] and the reactivation of the somatic Xi has been used as a model to investigate epigenetic reprogramming. Initial experiments by Eggan et al.[49] showed that the reactivation of a GFP transgene on the somatic Xi occurs by the morula/blastocyst stage and is followed by random XCI in the embryonic lineages. In the extraembryonic cells, instead, the somatic Xi is re-inactivated suggesting an incomplete erasure of epigenetic memory in this lineage, in which imprinted inactivation of the paternal X chromosome occurs during normal development. Consistently with this hypothesis, random XCI was observed in the extraembryonic lineages when

mESCs were used as donors for nuclear transfer. This most likely results from the failure in resetting imprinting marks of donor nuclei[88], which are instead erased during normal development in primordial germ cells and re-established in the gametes in a parent-specific manner[89]. Further studies confirmed that cloned embryos fail to establish imprinted XCI in preimplantation stages and extraembryonic tissues, and revealed heterogeneity in random XCI within cloned embryos in which some cells undergo XCI while others do not inactivate any X chromosome[90]. Although progressive loss of *Xist* coating has been observed within 30 min after SCNT, the precocious appearance of H3K27me3 and H3K9me2 on the original Xi suggests an incomplete reprogramming of the somatic nucleus[91]. The extent of Xi gene reactivation remains however unknown. Altogether these studies suggest that nuclear transfer cannot fully erase Xi epigenetic marks during pre-implantation development and further reprogramming events are required for re-establishing the normal developmental program. Interestingly, ectopic *Xist* accumulation has been observed upon SCNT in both male and female embryos and loss or depletion of *Xist* have been associated with increased efficiency of reproductive cloning[92,93]. In order to get some mechanistic insights, it would be important to determine to which extent genes along the Xi are reactivated in single blastomeres and their correlation with the observed Xi epigenetic changes.

Xi reactivation has also been studied by injecting somatic nuclei into the germinal vesicle of *Xenopus* oocytes[51]. This study showed that the developmental state of the donor influences the reactivation of an X-linked transgene located on the Xi. Specifically, reactivation was observed when mouse post-implantation epiblast stem cells (EpiSCs) were injected into frog oocytes but not upon transfer of embryonic fibroblasts or extraembryonic cell nuclei. Comparative Xi chromatin analysis showed loss of Xi-associated *Xist* both in EpiSCs and reactivation-resistant cells, whereas H3K27me3 and DNA methylation were invariably maintained. Accumulation of the histone variant macroH2A was instead observed on the Xi of reactivation-resistant cells but not in EpiSCs. MacroH2A depletion upon transfer of fibroblast nuclei lead to partial Xi reactivation that could be enhanced when it was combined with HDAC inhibitors or activation of Oct4 and Sox2. This suggests that macroH2A contributes to the stability of Xi but other factors are required for full Xi reactivation. Notably, it has been shown that in human somatic cells macroH2A association with the Xi is cell-cycle dependent being most prominent in early S phase and

declining from late S through mitosis[94]. Nonetheless, macroH2A is retained on the Xi during mitosis whereas human XIST and other Xi-associated histone marks (e.g. H2A ubiquitination) are lost[95]. As cell cycle has been shown to influence the efficiency of SCNT-reprogramming (discussed above) and mouse *Xist* is instead associated with the Xi throughout mitosis, it will be interesting to determine whether the developmental state of the donor nucleus and/or its cell cycle phase also influence Xi reactivation and investigate mechanistic differences between mouse and human.

iPSC-mediated Xi reactivation
Global epigenetic resetting has been observed upon induction of pluripotency by transduction of four pluripotency factors (i.e. Oct4, Sox2, Klf4 and c-Myc) into mouse fibroblasts[96,97]. Similarly to mESCs, miPSCs have two active X chromosomes and undergo *de novo* random XCI upon differentiation[97]. Recent studies have indeed used iPSC reprogramming to dissect the molecular mechanisms of Xi reactivation in mouse[52,98].

A tight association between the reversal of XCI and the sequential activation of pluripotency factors has been detected by investigating the kinetics of Xi epigenetic changes during mouse iPSC reprogramming[52]. Specifically, it has been shown that loss of *Xist* from the Xi follows Nanog expression, consistently with Nanog role in repressing *Xist* expression. Xi gene reactivation was instead observed in a subset of Nanog positive cells that reactivate additional factors (i.e. DPPA4 and PECAM1) at later reprogramming stages. This suggests that the hierarchical activation of pluripotency factors is required for complete reversal of XCI[52,99]. Consistently with this hypothesis, depletion of Nanog impaired Xi reactivation, whereas its overexpression during late reprogramming stages promoted the formation of iPSC colonies expressing DPPA4 and bi-allelically transcribing *Tsix* from both X chromosomes. These data suggest that Nanog expression is required but not sufficient for efficient reversal of XCI. A further link between pluripotency and Xi reactivation has been provided by Prdm14, a germline factor that has been implicated in the epigenetic reprogramming of PGCs and whose expression correlates with Xi reactivation[100]. It has been shown that depletion of Prdm14 during iPSC reprogramming decreases Xi reactivation and hampers both the derivation and maintenance of iPSC colonies[98]. Prdm14 overexpression in mouse EpiSCs instead induced efficient conversion to ESCs and Xi reactivation[101]. Mechanistically, Prdm14 has been shown to repress *Xist* in a dual manner. First, it represses Rnf12, a E3-ubiquitin ligase that targets Rex1 for proteosome

degradation during differentiation[48]. Second, it directly binds *Xist* in a Tsix-dependent manner. Interestingly, depletion of *Tsix* during iPSC reprogramming does not compromise Xi reactivation neither iPSC colony formation[52,98], although it affects *Xist* repression and Xi reactivation in the mouse embryonic epiblast[102].

iPSC reprogramming studies also gave further mechanistic insights in the role of *Xist* during Xi reactivation[52]. Kinetic studies during iPSC reprogramming showed that loss of *Xist* RNA in Nanog positive cells precedes bi-allelic expression of X-linked genes, suggesting that *Xist* repression might be required for Xi gene reactivation. Supporting this hypothesis, constitutive *Xist* expression decreased the reactivation of Xi genes within Nanog positive cells without affecting reprogramming, whereas *Xist* depletion did not alter Xi reactivation events neither its kinetics. This suggests that *Xist* repression might be necessary but insufficient for Xi gene reactivation. Notably, bi-allelic expression of X-linked genes was enhanced upon combined deletion of *Xist* and inhibition of DNA methylation, thus supporting the hypothesis that Xi reactivation requires both *Xist* silencing and DNA demethylation.

REPROGRAMMING-MEDIATED Xi REACTIVATION IN HUMAN

Recent studies in human embryos have shown substantial differences between human and mouse XCI[60,61,103] [Figure 1A and C]. Differences concern both the status of the female X chromosomes in pre-implantation embryos and the factors involved in the regulation of XCI. It has been shown that the two female X chromosomes are transcriptionally active throughout human pre-implantation development and both in embryonic and extraembryonic lineages[60]. This contrasts with mouse embryos where several rounds of Xi reactivation/inactivation take place in the epiblast cells that give rise to the embryo proper [Figure 1B], whereas imprinted XCI of the paternally-inherited chromosome is maintained in extraembryonic cells[104]. Random XCI initiates in mouse epiblast cells around the time of implantation and is essential for proper development[63,64,105,106], while its precise timing is unknown in human[60]. Furthermore, a recent single cell RNA-seq study showed a progressive downregulation of both X chromosomes beyond the zygote genome activation stage through human preimplantation development, suggesting that a different dosage compensation mechanisms might be in place ahead of XCI[61]. Consistently with this hypothesis, it has been observed that human *XIST* RNA coats both X chromosome by the morula and

blastocyst stages[60,61,103]. However, whether bi-allelic accumulation of *XIST* leads to dampening of X-linked gene expression remains to be determined. Interestingly, a human-specific long non-coding RNA, named *XACT*, has been shown to localize to either one or both X chromosomes in human embryos and has been hypothesized to compete with *XIST* for binding along the chromosome[103,107]. Notably, *XIST* RNA signal has a dispersed nuclear localization in human embryos suggesting that it might be delocalized from Xi chromatin or bind only some chromosomal domains[60,103].

Xi reactivation in human pluripotent cells

Human pluripotent stem cells (i.e. hESCs and hiPSCs) are epigenetically unstable *ex vivo* and heterogeneous in their XCI state that varies between different cell lines and also among cells of the same culture[53-59,108,109]. Three different classes of hESCs have been originally described based on *XIST* nuclear pattern and the transcription of CotI repeats before and after differentiation[54]. Class I hESCs were characterized by the absence of *XIST* RNA signal and the presence of CotI transcripts from both X chromosomes. Upon differentiation of these hESCs, *XIST* was upregulated and coated one of the two X chromosomes whereas CotI transcription within the *XIST*-coated domain ceased. Collectively these results suggested that class I hESC retain two transcriptionally active X chromosomes and are able re-initiate XCI. *XIST*-coating and CotI exclusion, however, were shown to occur spontaneously when class I cells were maintained in culture, thus generating XaXi XIST+ hESCs (class II). Finally, class III hESCs were observed upon progressive loss of *XIST*-coating in class II cultures. Notably, loss of *XIST* nuclear domain was associated with promoter DNA demethylation and transcriptional reactivation of some Xi genes[53,56]. However, these class III hESCs could not re-establish *XIST* expression neither Xi gene silencing upon differentiation and were considered subject to an "erosion" of dosage compensation[53,54,59,110]. Interestingly, Xi reactivation occurring during erosion was shown to localize preferentially within chromosomal domains enriched with H3K27me3[107]. These H3K27me3 domains are lost upon erosion whereas H3K9me3-enriched domains, which spatially segregate along the human Xi, remain unaffected. Surprisingly, loss of *XIST* is not directly associated with Xi gene reactivation as it occurs afterwards. Reactivation and accumulation of *XACT* RNA along the Xi was instead observed ahead of *XIST* loss and Xi gene reactivation [Figure 2A] and was indeed hypothesized to trigger the reorganization of Xi heterochromatin domains and consequent gene reactivation. As *XACT* coats the active X
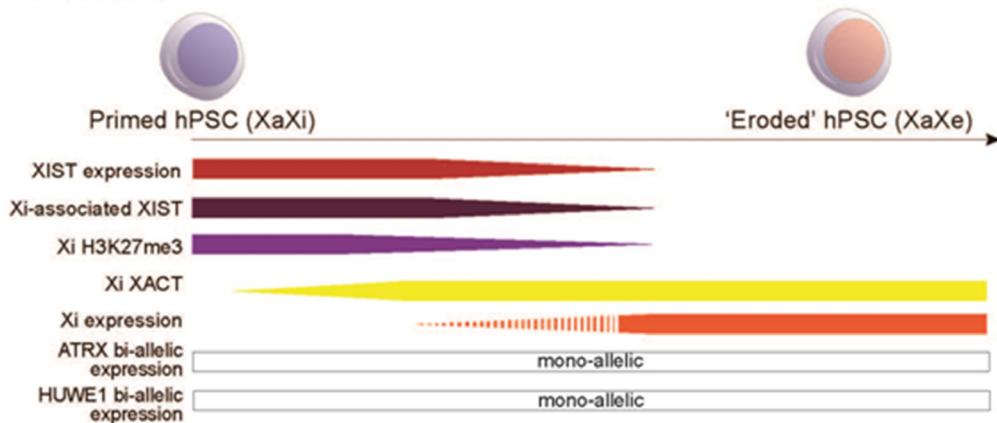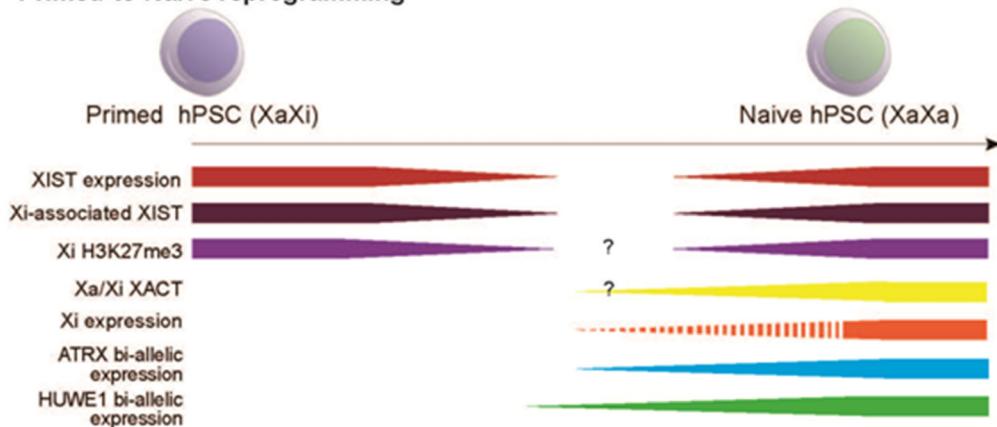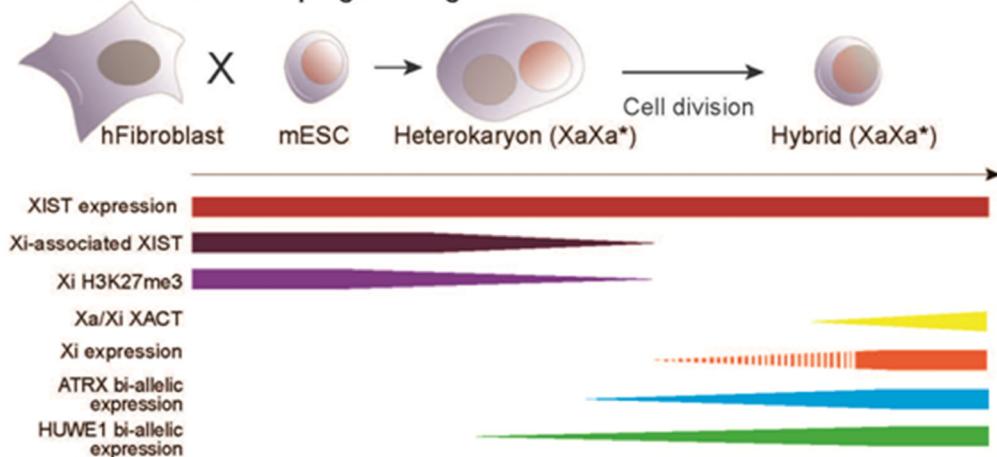
Figure 2: Human Xi reactivation in pluripotent cells. Schematic representation of epigenetic changes that are associated with Xi reactivation in different model systems. (A) Erosion of XCI in primed human pluripotent cells (i.e. ESCs and iPSCs) leads to partial Xi gene reactivation that leads to the "eroded" X chromosome (Xe). Sequential Xi chromatin changes occur ahead of gene reactivation and include XACT re-expression and Xi localization followed by loss of Xi-associated XIST and H3K27me3[107]. The extent of Xi reactivation and the reactivated genes vary in different cell lines. Xi genes that are refractory to erosion (i.e. *HUWE1* and *ATRX*) remain mono-allelically expressed; (B) reprogramming of primed human pluripotent cells to the naïve state is represented accordingly to 5iLAF[116]. XIST expression and its enrichment on the human Xi (i.e. Xi-associated XIST) are transiently lost during the transition from the primed (XaXi XIST-expressing) to naïve (XaXa XIST-expressing) state[62]. No data is available for H3K27me3 enrichment on the Xi neither for XACT relative to the timing of transient XIST loss (highlighted by question marks); (C) reprogramming of human somatic cells by fusion with mouse ESCs induces partial Xi gene reactivation (Xa*) ahead of cell division[126]. In this system, loss of H3K27me3 enrichment and XIST delocalization from the Xi are early chromatin changes that precede Xi gene reactivation. Importantly Xi genes that are refractory to erosion show bi-allelic expression in the subset of cells where *XIST* is delocalized. *XACT* re-expression is a later event and is not associated with Xi gene reactivation. XCI: X chromosome inactivation; Xi: inactive X chromosome; Xa: active X chromosome; Xp: paternal X chromosome; PSC: pluripotent stem cell; ESC: embryonic stem cell

chromosome in XaXi class II hESCs and in the human epiblast ahead of XCI, it has been suggested to antagonize *XIST* by competing for Xi binding or limiting chromosome accessibility[103,109]. Consistently with this hypothesis, overexpression of a *XACT* transgene in mESCs has been shown to prevent inactivation of the X chromosome from which it is expressed, whereas its downregulation restores random XCI[103].

The variable XCI state and its progressive epigenetic alterations in hESCs have been suggested to result from inappropriate culture conditions that are unable to stabilize the XCI state during the derivation from the *in vivo* epiblast and further *in vitro* passaging[111]. Derivation in low oxygen levels allowed to obtain hESCs with two active X chromosomes and was suggested to preserve this state[112]. However, a separate study showed that hypoxia rather stabilize hESCs that have already undergone XCI[113]. Other studies reported the derivation of hESCs that preserved the ground state of the *in vivo* epiblast but they did not fully characterize the status of the X chromosomes[114-116]. In light of recent findings showing that the two X chromosomes in human female embryos are characterized by dual *XIST* coating and dampening of X chromosome expression, a recent work highlights the importance of characterizing chromosome-wide X-linked gene expression and *XIST* nuclear localization before and after differentiation[57]. Multi-gene RNA-FISH and allele-specific X-linked gene expression revealed that hESC derived and propagated in standard FGF2-containing medium maintain their XCI state upon differentiation. Notably, it has been shown that XIST⁻ XaXa hESCs (previously defined as class I) cannot re-express *XIST* neither undergo XCI upon differentiation similarly to cells in the XaXe eroded state. These aberrant cells arise from blastocyst outgrowth as early as 48 h after plating onto feeders and can be stably maintained in this state upon establishment and propagation of hESCs. Although it cannot be formally excluded that these cells represent an intermediate state in human XCI, they are currently believed to result from epigenetic adaptation to *in vitro* culture.

Similar epigenetic instability has been shown to occur in human iPSCs and it is probably the cause of controversial results in different labs[58,59,117-121]. Some groups, in fact, reported that XCI is maintained upon human iPSC reprogramming[58,59,117,119] while others claimed Xi reactivation[55,118,120,121]. Most of these studies, however, analyzed indirect markers of Xi reactivation, such as *XIST* and H3K27me3 nuclear localization and expression of X-linked genes compared to autosomes, or directly assessed allele-specific expression of only few Xi genes. These analyses could easily confuse Xi reactivation with erosion, as it has been recently shown

for hiPSC that were initially supposed to reactivate the Xi upon culture on LIF producing feeders. The latter have instead been shown to undergo extensive erosion of XCI by multi-gene RNA-FISH[57].

Recently, two culture conditions[116,122] have been shown to reprogram hESCs and hiPSCs to a state similar to human blastocysts[123]. Detailed analysis of XCI state in these "naive" human pluripotent cells showed that they retain some features of the epiblast, including dual *XIST* and *XACT* coating, bi-allelic expression of X-linked genes and dampening of X-linked gene expression on both X chromosomes[62,103] [Figure 2B]. However, the same Xi was re-inactivated upon differentiation and only a minority of cells within the culture (< 10%) showed *XIST*-coating on both X chromosomes. Collectively, these results suggest that an improvement of culture conditions is still required for stabilizing human naïve pluripotency and careful X chromosome-wide analysis of Xi-specific expression need to be performed in the future to unequivocally define XCI state in pluripotent cells.

Xi reactivation by interspecies cell fusion-mediated reprogramming

Cell fusion between somatic and ESCs from different species has been used to investigate human pluripotent reprogramming[75,124]. This system allows the analysis of early reprogramming events because specie-specific features in nuclear organization (e.g. the presence of chromo centers in mouse nuclei) and DNA sequence differences can be used to track each fusion partner by imaging and molecular techniques. Importantly, cell nuclei remain separated within a shared cytoplasm and this transient heterokaryon state persists until the first mitosis, when nuclei fuse and generate hybrids[125]. We have, indeed, recently used cell fusion between human female fibroblasts and mouse ESCs to reprogram the somatic nucleus and investigate human Xi reactivation[126] [Figure 2C]. We showed that expression of pluripotency genes from the human nucleus occurs as early as two days after fusion at a time when the majority of cells are heterokaryons. This observation allowed us to discriminate pre- and post-mitotic reprogramming events in heterokaryons and hybrids, respectively. Single cell analyses demonstrated that *XIST* delocalization and loss of H3K27me3-enrichment from the human Xi occur in heterokaryons and hybrids 2-3 days after fusion, and precede bi-allelic expression of *ATRX* and *HUWE1*, two X-linked genes that are not subject to XCI erosion in human ESCs[57,107]. RNA-FISH analysis of nascent *ATRX* and *HUWE1* transcripts together with *XIST* or *XACT* RNAs showed that bi-allelic expression of X-linked genes only occurs in cells that have lost a localized *XIST* signal (i.e. about 30% at

3 days after fusion) but not in all of them (50% at day 3). *XACT* instead re-associates with either one or two X chromosomes later during reprogramming in a minority of both mono-allelic and bi-allelic cells (< 1% at day 6). These results suggest that human Xi reactivation is induced by cell fusion-mediated reprogramming ahead, or immediately after, cell division and that it requires *XIST* delocalization, but not *XACT* re-association along the Xi. Notably, delocalization of *XIST* upon cell fusion-mediated reprogramming results in a diffuse nuclear signal that resembles the one observed in human blastocysts and might represent remaining binding at foci from where *XIST* initially spreads[127,128]. In addition, a minority of heterokaryon and hybrid nuclei (7% at day 3 *vs*. 1% at day 0; unpublished data) have two diffused *XIST* clouds suggesting that cell fusion-mediated reprogramming might be able to recapitulate some features of the *in vivo* epiblast cells. Consistently with this hypothesis, genome-wide expression analysis upon cell fusion-mediated reprogramming showed the reactivation of pluripotency genes associated with both primed and naïve human pluripotent cells[129]. In the future, single cell studies might indeed be used to segregate different human pluripotent states and help us refine culture conditions for naïve pluripotency.

To investigate the extent of Xi gene reactivation along the entire X chromosome, we derived human fibroblast clones with reciprocal Xi chromosomes (e.g. Xa^1Xi^2 and Xi^1Xa^2) and performed allele-specific RNA-seq analysis of these clones before and after reprogramming[129]. This analysis showed that cell fusion-mediated reprogramming induces partial human Xi reactivation with 10% of the sampled genes being consistently reactivated at different times after fusion and in different clones. Notably, clones with opposite Xi haplotype reactivate the same set of genes suggesting that neither parent of origin nor mutations in regulatory sequences influence reactivation. We instead observed a preferential reactivation of Xi genes localized within XCI "escape" domains at the telomeric end of the short Xp arm suggesting a higher susceptibility of these loci to reactivation. Similar results were obtained in a large iPSC population study that identified Xi domains reactivating with higher frequency[130]. Conversely to iPSC erosion, however, Xi genes that were reactivated upon cell fusion-mediated reprogramming could not be predicted by H3K27me3 domains suggesting that the mechanism leading to reactivation might be different in these two systems[107]. Cell fusion-mediated Xi reactivation could instead be predicted by variable Xi expression among single cell-derived fibroblast clones ahead of reprogramming. This probably reflects an intrinsic predisposition of certain loci to transcriptional activation that can already occur in the

somatic cells with low frequency and is enhanced upon reprogramming. Interestingly, recent studies of three-dimensional X chromosome organization in mouse suggested that genes variably expressed from the Xi in a cell-specific context co-localize within topologically associated domains and showed reduced expression upon disruption of the domain architecture[131]. As loci that escape X inactivation vary between different cell types and also between females[10], future population analysis of somatic cells from different tissues will help us to understand the molecular basis for this higher reactivation susceptibility.

Another important finding of our Xi reactivation study is the possibility of using cell fusion-mediated reprogramming to identify distinct subsets of Xi genes based on their susceptibility to reactivation upon further epigenetic perturbations. Indeed, DNA demethylation of human fibroblasts allowed us to identify a second subset of genes that was reactivated only after reprogramming. This suggests that the extensive Xi chromatin remodeling that takes place during reprogramming might unmask limiting factors in the reactivation of Xi gene subsets.

FINAL REMARKS

Pluripotent reprogramming of somatic cells has been widely used to induce the reversal of X chromosome inactivation and investigate its molecular mechanisms. In the last decade, mouse somatic cell reprogramming allowed major steps forward in unraveling the molecular connections between pluripotency and X chromosome inactivation/reactivation[51,52,71,98]. Reprogramming, indeed, represents a promising system to investigate the functional role of newly discovered XCI factors[23-27].

In light of recent findings in human embryos, the interpretation of mouse XCI studies need to be carefully considered when translated to human. Recent analyses of human pre-implantation development show remarkable differences between the events and the molecular players that characterize X chromosome inactivation in human and mouse, suggesting unforeseen specie-specific mechanisms[60,61,103] [Table 1]. In addition, the epigenetic instability and heterogeneity of human pluripotent cells upon time in culture limit the possibility of modelling human XCI and its reversal *in vitro*. Recently, comparison of transcriptome profiles of single epiblast cells with human pluripotent cells allowed the identification of culture conditions that better preserve naïve pluripotency *ex vivo*[116,122,123]. XCI state, however, cannot be fully recapitulated in these cultures[62,103] thus highlighting the need for further improvements. We propose that cell fusion-mediated reprogramming might

Table 1: Differences between mouse and human X chromosome inactivation

	Mouse	Human
Random XCI initiation	Late blastocyst	Unknown
X chromosome dampening	Not detected	Dosage compensation mechanism (pre-implantation)
Imprinted XCI (embryonic)	Xp inactivation (4-cell stage) /reverted in epiblast (mid-blastocyst stage)	Not detected
Imprinted XCI (extra-embryonic)	Xp inactivation (mantained in trophoectoderm)	Not detected
XIST expression and localization	Mono-allelic Xi expression and coating upon random XCI	Bi-allelic expression and XaXa coating ahead of random XCI
Tsix/TSIX expression and function	Xa expression; Xist antagonist	No expression (pre-implantation)/ Xi expression (foetal cells); unknown function
XACT expression and function	No orthologue identified	XaXa expression and coating ahead of random XCI; XIST antagonist?

XCI: X chromosome inactivation; Xi: inactive X chromosome; Xp: paternal X chromosome; Xa: active X chromosome

be used to segregate different pluripotent states with distinct X chromosome epigenetic features in single cells, as it can induce the expression of genes associated with both the primed and naïve human pluripotent states[129]. This information might be useful for identifying pathways that stabilize naïve pluripotency and for refining culture conditions.

The controversies regarding XCI state in human pluripotent cells also highlight the importance of standardized protocols for determining XCI status. Indirect measurements of XCI, such as the presence of *XIST* nuclear foci and/or the expression ratio between X and autosomes, as well as analyses of few X-linked genes with only one methodology (e.g. RNA-FISH or allele-specific RT-PCR) might be misleading[57]. The advent of genome-wide techniques that can be used to assess allele-specific expression at the single cell level will be fundamental in defining different XCI states and investigating the susceptibility of distinct Xi loci to reactivation. In addition, it will be important to reach an agreement about whether Xi expression should be defined as percentage of expression relative to the Xa or based on more sophisticated statistical methods[129,132-134]. Notably, the combination of molecular and cell biology techniques applied to human pluripotent cells before and after differentiation is required to distinguish naïve from primed and eroded pluripotency. Finally, another important aspect to consider is the variability of XCI in different tissues and individuals[10]. This variable Xi expression will need to be taken into account, and studies of XCI in a large number of subjects and in different tissues will be required to achieve a better understanding of how cellular context influence locus susceptibility to reactivation.

In perspective, studies of human pluripotent reprogramming will allow us to dissect the precise molecular mechanisms of Xi reactivation and to unravel locus-specific susceptibilities to reactivation.

This information might help us to achieve locus-specific control of Xi reactivation and to engineer novel therapeutic strategies for X-linked diseases that will be based on the re-expression of the silent allele from the Xi. Furthermore, these studies will give us mechanistic insights into human diseases in which the reactivation of genes along the Xi has been observed, including cancer[135-137], age-related[138,139] and autoimmune diseases[140].

DECLARATIONS

Authors' contributions
I. Cantone contributed solely to the paper.

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