Developmental regulation of X-chromosome inactivation

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Abstract

With the emergence of sex-determination by sex chromosomes, which differ in composition and number between males and females, appeared the need to equalize X-chromosomal gene dosage between the sexes. Mammals have devised the strategy of X-chromosome inactivation (XCI), in which one of the two X-chromosomes is rendered transcriptionally silent in females. In the mouse, the best-studied model organism with respect to XCI, this inactivation process occurs in different forms, imprinted and random, interspersed by periods of X-chromosome reactivation (XCR), which is needed to switch between the different modes of XCI. In this review, I describe the recent advances with respect to the developmental control of XCI and XCR and in particular their link to differentiation and pluripotency. Furthermore, I review the mechanisms, which influence the timing and choice, with which one of the two X-chromosomes is chosen for inactivation during random XCI. This has an impact on how females are mosaics with regard to which X-chromosome is active in different cells, which has implications on the severity of diseases caused by X-linked mutations.

Key Words

X-Chromosome Inactivation, Epigenetics, Development, Reprogramming, Pluripotency, Noncoding RNA

Abbreviations

E	embryonic day of development post fertilization
EpiSCs	epiblast stem cells
ESCs	embryonic stem cells
iPSCs	induced pluripotent stem cells
MSCI	meiotic sex chromosome inactivation
PE	primitive endoderm
PGCs	primordial germ cells
PRC2	Polycomb Repressive Complex 2
TE	trophectoderm
<i>Xic</i>	<i>X-inactivation center</i>
XCI	X-chromosome inactivation
XCR	X-chromosome reactivation
Xp	paternally inherited X-chromosome
Xm	maternally inherited X-chromosome
Xi	inactive X-chromosome
Xist	X-inactive specific transcript
Xa	active X-chromosome
<i>Xce</i>	<i>X-chromosome controlling element</i>

1. Introduction

Epigenetic marks serve as memory for the ON- and OFF-state of genes and thereby maintain cellular identity across cell divisions [1,2]. The mammalian X-chromosome provides an unusual example for epigenetic regulation, as it can switch from an active to an inactive state affecting all the genes on a chromosome-wide level, with the exception of few so-called escapee genes [3]. The process of X-chromosome inactivation (XCI) thereby induces the OFF-state, which affects one of the two X-chromosomes in females, in order to ensure the same X-linked gene dosage levels as in males, which have only one X-chromosome [3-5]. XCI is a tightly controlled, multilayered epigenetic event essential for mouse development [6], and defects in XCI maintenance are associated with cancer in mice and humans [7-9]. XCI is not a permanent state, as it is reversed in the mouse embryo by X-chromosome reactivation (XCR), which occurs in the pluripotent epiblast and in the germ cell lineage [10,11]. *In vitro*, XCR is associated with mouse pluripotent stem cells like embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). When being differentiated, they undergo XCI, reflecting the events during differentiation in female postimplantation mouse development. XCI and XCR are thereby linked to differentiation and pluripotency, respectively, and the molecular intertwining between X-chromosome dosage compensation and the cellular differentiation state is recently becoming increasingly uncovered. At the core of XCI/XCR regulation lies the *X-inactivation center* (*Xic*), which is a locus on the X-chromosome containing a number of mostly non-coding RNA genes, the expression of which is controlled by pluripotency factors [10]. The most

prominent long non-coding RNA at the *Xic* is Xist (X-inactive specific transcript), which is expressed only from the future inactive X-chromosome (Xi), which Xist coats and silences *in cis* [12-15]. Xist initiates the XCI process by recruiting epigenetic regulators and by changing the 3D structure of the X-chromosome [16-19]. This leads to establishment of the unique epigenetic makeup of the Xi, which includes chromosome-wide enrichment for repressive marks like histone H3 lysine 27 tri-methylation (H3K27me3), histone H2A lysine 119 mono-ubiquitination (H2AK119ub1) histone H4 lysine 20 mono-methylation (H4K20me1), DNA methylation of X-linked gene promoters and incorporation of the histone variant macroH2A [5,20]. Combined, this ensures faithful maintenance of XCI until it is reversed by XCR again.

In this review, I will summarize, what is known about the crosstalk between pluripotency/differentiation and the XCR/XCI state during mouse development and in cell culture systems. Interesting in this aspect is the hematopoietic lineage, which displays some properties in regard to XCI, which are normally associated with pluripotent cells only [21-23]. Furthermore, I will give an overview of the causes and consequences of the mosaicism of females in respect to their X-chromosome activity. This is caused due to skewing of random XCI, where either the paternal or maternal X is inactivated. The deviation from randomness has helped to define regions on the X-chromosome, which regulate the likelihood of an X to be chosen to be either active or inactive.

2. Imprinted XCI

XCI occurs in two distinct forms during mouse development – imprinted and random. Imprinted XCI (Fig. 1A), in which always the paternally inherited X (Xp) is inactivated, takes place in the early embryo during preimplantation development, when *Xist* becomes expressed on the Xp from the 2-cell stage onwards [24,25] and is maintained in the extraembryonic tissues of the placenta [26]. After imprinted XCI has been erased by X-chromosome reactivation (XCR) in the epiblast of the late blastocyst (Fig. 2A) [25,27], random XCI occurs in the differentiating postimplantation epiblast (Fig. 2B) [28,29], in which either the maternal or paternal X is randomly chosen for inactivation. Imprinted XCI is essential for development, as female embryos defective in *Xist* [6] or the polycomb protein Eed [30], fail in imprinted XCI and die during postimplantation development due to placental defects. Nevertheless, imprinted XCI seems to be less strictly controlled in the placenta than random XCI in the embryo. X-linked genes can get spontaneously reactivated in trophoblast giant cells *in vivo* [31,32] and also in trophoblast stem cells *in vitro* [33], in which imprinted XCI can even be transiently completely reversed [34]. The reason for this infidelity in silencing could be the unusual chromatin state of the Xp in extraembryonic tissues, which consists both of repressive as well as active chromatin marks [32] and shows less involvement of DNA-methylation, when compared to random XCI [35].

The imprint for XCI in the preimplantation stages is set up in the germ line, but it has been a matter of debate, if exclusively in the maternal [36], in the paternal [24], or in both parental germ lines [37]. The maternal imprint (Fig. 1B) is required to ensure that *Xist* is kept silent on the maternally inherited X-chromosome (Xm), so that the Xm remains active. Evidence for this comes from parthenogenetic mouse embryos, in which both X-chromosomes are maternally

inherited and are kept active during early preimplantation development until the morula stage, when the imprint seems to be overcome or erased and *Xist* starts to become expressed [38]. Nuclear transfer experiments have shown that the repressive maternal imprint on *Xist* is established late during oocyte development [39,40], as embryos established with non-growing oocyte nuclei expressed *Xist*, while embryos with nuclei from fully-grown oocytes did not. Mechanistically, trimethylation of histone H3 lysine 9 (H3K9me3) at the *Xist* promoter on the Xm in preimplantation embryos is critical to keep *Xist* silent and thereby the Xm active [41]. The H3K9me3 mark is required to counteract the function of the *Xist* activator RNF12/RLIM on the Xm [41], which is deposited maternally in the oocyte and which is required for expression of *Xist* on the Xp during imprinted XCI [42]. However, it seems that H3K9me3 is not the primary imprinting mark, as there is no difference in H3K9me3 at the *Xist* promoter before and after oocyte growth, when the imprint is established [43]. On the other hand, the *Xist* locus appears more condensed after imprint establishment during oocyte growth, which could influence *Xist* repression on the Xm, although the opposite has been postulated for *Xist* expression from the Xp (see below [44]). It still remains open, what the exact nature of the maternal repressive imprint on *Xist* is and how it is established.

Another critical regulator with a function in imprinted XCI is *Tsix*, the noncoding antisense regulator of *Xist*. *Tsix* is expressed from the Xm during imprinted XCI and mouse embryos with a *Tsix* mutation on the Xm express *Xist* from both the paternal and maternal X-chromosomes in extraembryonic tissues and die during early postimplantation development [45,46]. During preimplantation stages, *Tsix* is not required for correct Xp-specific imprinted *Xist* expression [47,48],

therefore its function for imprinted XCI seems to be mainly restricted to the extraembryonic tissues. DNA-methylation, which is essential for autosomal imprints, is not believed to play a major role in the maternal repressive imprint on *Xist*, as maternal deletion of the *de novo* DNA methyltransferase genes *Dnmt3a* and *Dnmt3b* does not interfere with imprinted XCI [49]. Nevertheless, depletion of the DNA-methyltransferase DNMT1o, which is deposited in the oocyte and needed for maintenance of autosomal imprints during preimplantation development, is also important for imprinted XCI in extraembryonic tissues [50]. *Tsix* and its enhancer *Xite* [51], which are normally expressed only from the Xm in placentae, are expressed both from the Xp and the Xm in the absence of DNMT1o, resulting in repression of *Xist* and biallelic expression of X-linked genes [50]. The major function of DNMT1o thereby seems to be to maintain a DNA-methylation imprint on *Xite* during preimplantation development, which is established during spermatogenesis (Fig. 1C) and therefore specific to the Xp [52]. The mechanism of the establishment of the paternal imprint on *Xite* and *Tsix* still needs to be investigated.

Further indications for paternal imprinting of the Xp (Fig. 1C) come from a recent study using transgenic experiments [44]. Transgenes, containing the *Xic* region including *Xist*, *Jpx*, *Tsix* and *Xite*, have been inserted on autosomes and showed correct imprinted *Xist* expression – silent *Xist* when inherited through the maternal and active *Xist* when through the paternal germline. A similar study using single-copy transgenes has previously concluded that this is evidence that the imposed imprint is exclusively a maternal, repressive one and that *Xist* is expressed by default when inherited paternally without the need of a paternal imprint [36]. In the more recent study [44], however, in which multicopy

transgenes were used, a different conclusion was drawn, which implied both a maternal and a paternal imprint (Fig. 1). When the transgene array was inherited from a father, which was homozygous for the transgene, transgenic *Xist* was not efficiently upregulated during preimplantation development in the offspring. However, when the transgene was inherited from a hemizygous father (transgene only present on one member of an autosome pair), transgenic *Xist* was expressed. This correlated with a more condensed transgenic *Xic* locus during spermatogenesis in the hemizygous and a more decondensed, open chromatin state in the homozygous state. How would this explain establishment of a paternal imprint for imprinted XCI? It could mean that the hemizygous transgene array cannot pair during meiosis, as it is absent on the second chromosome, which reflects the situation of the endogenous *Xic* locus, in which the X- and Y-chromosomes pair incompletely and form the heterochromatic XY-body during the pachytene stage of male meiosis. This incomplete pairing leads to transcriptional repression of the sex chromosomes through the process of meiotic sex chromosome inactivation (MSCI) [53,54]. How this could promote *Xist* expression later on during imprinted XCI is still unclear, as the molecular nature of this permissive *Xist* imprint still needs to be established. Potentially it could involve the repressive DNA-methylation imprint on the *Xist*-repressors *Xite* and *Tsix* mentioned above [50,52]. Alternatively, it could also have to do with the fact that *Xist* is one of few genes expressed in a heterochromatic environment and therefore the condensed state during meiosis could facilitate later *Xist* expression during embryogenesis [55]. The previous study using single-copy transgenes did not report differences in *Xist* expression between inheritance of the transgene from homozygous or hemizygous fathers [36].

Possibly the length of unpaired chromatin in the single-copy transgene is insufficient to attract differential epigenetic marking between hemizygous and homozygous fathers, which only a multi-copy transgene array or the XY body can. Also no impaired viability or spermatogenesis phenotype was reported in the case of single-copy transgenes in contrast to the multi-copy arrays, and therefore the single-copy transgene might not fully recapitulate the silencing function of *Xist* and not completely reflecting the endogenous situation. In any case, it will be necessary to further elucidate how the permissive paternal imprint on *Xist* is established and which molecular players are involved.

Another aspect of imprinted XCI with a potential root in the male germline is the silencing of repeats on the Xp (Fig. 1C). Genes on the Xp are initially active after fertilization and become progressively inactivated during preimplantation development in an *Xist*-dependent fashion [25,37,56]. One study also reported some X-linked genes to be silenced independently of *Xist* by an unknown mechanism [57], although stable gene silencing requires *Xist* RNA [37]. Repeat sequences on the Xp, on the other hand, are already silent by the 2-cell stage both in wildtype and *Xist*-mutant embryos, showing that repeat-silencing is *Xist*-independent [37]. The repeats are silenced during MSCI in male meiosis and likely inherited in the silent state to preimplantation embryos. Thereby they might contribute to the formation of a silencing compartment for efficient *Xist*-dependent gene silencing during imprinted XCI [37,58]. In conclusion, evidence for both maternal and paternal imprints on the X-chromosome exist. A maternal repressive imprint on *Xist* and a paternal permissive imprint on *Xist* – possibly through silencing of its negative regulators *Xite* and *Tsix* and finally a paternal imprint on repeat elements, which facilitates efficient gene silencing by *Xist*-RNA.

It remains open what the molecular nature of these imprints are, how they are established, and how and when they are finally erased, to allow random XCI later on.

3. Random XCI – a hallmark of cell differentiation

One of the major properties of random XCI is its link to the differentiated cell state, while in contrast, XCR is a characteristic mark of naive pluripotent stem cells. Historically, the fact that XCI occurs during cell differentiation *in vivo* has long been known and described in postimplantation mouse embryos based on X-linked enzymatic activities, histological stains and late replication timing of the inactive X-chromosome [28,59,60]. These early studies defined the time window for random XCI between E(embryonic day)4.5 and E6.5 in the mouse epiblast during early differentiation (Fig. 2), before the full specification of individual cell types. This is in agreement with cell culture studies, in which XCI has been observed during the differentiation of female mouse pluripotent stem cells [61,62] and since then, mouse ESCs have become the model system of choice to dissect the mechanisms of random XCI *in vitro*.

In contrast to the view that random XCI is completed in all embryonic cells by E6.5, has been a report using an X-linked *lacZ* reporter transgene [63]. The authors observed delayed downregulation of LacZ-staining activity, being completed in some tissues only as late as E11.5, prompting the conclusion that XCI occurs at different timepoints in different tissues and cell types. A likely explanation of this discrepancy is the longevity of the LacZ protein, which has

been shown to persist for some time in embryos even after the downregulation of its mRNA, [64,65], making it unsuitable to study the kinetics of gene silencing by XCI. Indeed, the same authors came to the conclusion in a later study [66] that endogenous X-linked genes are not silenced in a cell-lineage-dependent fashion, providing further evidence that transgene-reporter-based evaluation of XCI has to be interpreted with caution. More recently, studies employing X-linked fluorescent reporter genes in mice also came to the conclusion that XCI is completed in the postimplantation epiblast (Fig. 2B) at the latest between E6.5 and E7.5 [31,67]. This provides further evidence that XCI is an early differentiation event during the exit from naïve pluripotency.

The tight link between XCI and differentiation is additionally strengthened by observations of a feedback of X-chromosome dosage on the differentiation state itself. A first indicator for XCI affecting differentiation came from the difference in developmental speed between Xm0, XY, XX and XXY mouse embryos [68,69]. Xm0 and XY embryos, which do not undergo XCI, generally develop faster than XX or XXY embryos, which do undergo XCI. This indicates that XCI slightly retards developmental progression, which is in accordance with the fact that also X0 and XY ESCs differentiate quicker than XX ESCs [70]. The slower differentiation kinetics of cells with two X-chromosomes has been explained by the generally lower DNA methylation levels (=hypomethylation) in female ESCs when compared to their male counterparts, [70-73]. Genome-wide DNA hypomethylation is not the only epigenetic characteristic of female ESCs, as they also show X-chromosome-specific hyperacetylation of core histones, hypermethylation of histone H4K4 and hypomethylation of histone H3K9 [74]. The double dosage of X-linked genes in undifferentiated XX ESCs [75] inhibits the

MAPK/Erk signaling pathway through an yet unknown mechanism, which in turn reduces expression of the *de novo* DNA methyltransferases DNMT3A and DNMT3B when compared to male ESCs [70,71]. However, this seems not to be necessarily the case in all ESC lines [73] and hypomethylation of female ESCs might be also caused by additional mechanisms, like reduced DNMT recruitment or higher DNA demethylation activity than in male ESCs. Due to the reduced DNA-methylation levels in female ESCs, pluripotency gene methylation and downregulation is delayed, which in turn slows down their differentiation [70]. This crosstalk between X-linked gene dosage and differentiation likely ensures that dosage compensation by XCI is achieved before cells are becoming differentiated, as a double X-dosage is detrimental to embryonic development [6,76].

While X-dosage affects differentiation, the pluripotent state inhibits XCI [10,11,77,78] and this inhibition is released during differentiation. This feedback of the cell differentiation state on XCI regulation is directly controlled by pluripotency-associated transcription factors, which bind along the *Xic* in multiple locations. The most prominent and well-described binding sites are within *intron 1* of the *Xist* gene [79] and nearby the *Tsix* promoter region [80,81]. *Xist intron 1*, which is bound in pluripotent stem cells by multiple factors such as NANOG, OCT4, SOX2 and PRDM14, has been postulated to act as a repressive element on *Xist* expression and thereby XCI [47,82-84]. However, deletion of *Xist intron 1* does not lead to de-repression of *Xist*, showing that it is not the only element responsible for keeping *Xist* silent in pluripotent cells [85,86]. Therefore, while binding of pluripotency factors to *Xist intron 1* is not essential to repress *Xist* during XCR, it might play a role in safeguarding the process in

collaboration with other pluripotency factor binding regions. *Xist* intron 1 also might play more important functions in some developmental contexts than in others, for example during XCR in germ cells versus in the epiblast, something which will need to be tested in the future. The situation appears to be complex, with a multitude of elements ensuring the fine-tuning and fidelity of XCI-regulation. The pluripotency factor binding hubs surrounding *Tsix* on the other hand, which are bound by OCT4, REX1, KLF4, C-MYC and SOX2, seem to have an activating effect on *Tsix*-expression and thereby indirectly a repressive one on *Xist* [47,80,81,83]. *Xist* is also directly repressed by REX1, which binds to the *Xist* promoter and which in turn is degraded during differentiation by ubiquitination by the ubiquitin ligase RNF12/RLIM [87]. *Rnf12*, which is thereby a promoter of X-inactivation [86,88], interestingly lies itself on the X-chromosome about 500kb upstream of *Xist*. Also the *Rnf12* promoter is bound by the pluripotency factors NANOG, OCT4, SOX2, KLF4 and PRDM14 [47,89]. PRDM14 recruits the Polycomb Repressive Complex 2 (PRC2) to repress *Rnf12* in pluripotent stem cells and during XCR [47]. Another activator of *Xist* is the non-coding RNA gene *Jpx*, which lies just 5' of *Xist* and which becomes expressed during differentiation [90]. *Jpx* RNA evicts the repressor CTCF from the *Xist* promoter, thereby allowing *Xist*-upregulation during XCI [91]. How *Jpx* is regulated in a differentiation-dependent manner remains to be investigated. In summary, there are a manifold of inputs from the pluripotency program to ensure faithful suppression of XCI in pluripotent stem cells, which are released and counteracted by *Xist*-activators during differentiation, thereby tightly coupling XCI with the differentiation process.

At the beginning of ESC-differentiation, *Xist*-upregulation occurs during a critical time window, in which it can cause chromosome-wide silencing and establishment of an epigenetic memory on the Xi [92,93]. While at first the silencing is reversible and strictly *Xist*-dependent, it becomes irreversibly locked-in afterwards, most likely due to the attraction of multiple epigenetic silencing marks [94] like DNA-methylation and incorporation of the histone variant macroH2A [95]. After cells are fully differentiated, *Xist* expression is usually not sufficient to re-initiate silencing again [92]. An exception to the norm seems to be the hematopoietic system, where inducible *Xist* expression has been shown to lead to *de novo* silencing [22]. The AT-rich binding protein SATB1 has been put forward as a mediator for the ability of *Xist* to initiate silencing. SATB1 is expressed in hematopoietic cells, and together with its homologue SATB2, also in ESCs during the critical time-window of XCI [23]. SATB1 and SATB2 depletion has been described to lead to defects in *Xist* RNA localization and gene silencing, while overexpression of SATB1 and SATB2 in mouse embryonic fibroblasts enabled ectopic *de novo* gene silencing by *Xist* [23]. However a later study did not detect XCI abnormalities in *Satb1/Satb2* double-mutant mice [96], which does not necessarily exclude a role for SATB1 and SATB2 in XCI, but points toward yet unidentified compensatory mechanisms in their absence [97]. Nevertheless, the hematopoietic system possesses an unusual epigenetic plasticity regarding XCI for a differentiated cell lineage. This might also be responsible for the observation, that conditional *Xist*-deletion in hematopoietic cells can lead to blood cancer in mice – likely due to reactivation of X-linked genes [7]. This is in line with the recent observation of incomplete X-chromosome silencing in hematopoietic cells in both mice and humans [21].

Identifying the commonalities between hematopoietic cells and pluripotent stem cells will lead to further insights into the mechanisms of the X-inactivation process.

4. Resetting the XCI-stage by X-chromosome reactivation (XCR)

In order to allow for the switch from imprinted XCI of the Xp to random XCI, in which either the Xp or Xm are inactivated, it is necessary that XCI is reversed by XCR, which takes place in the epiblast of the late mouse blastocyst (Fig. 2A). Furthermore, XCR serves the purpose to erase random XCI in the germ cell lineage, to avoid inheritance of an inactivated X-chromosome to the next generation. This would cause the lack of X-linked gene products in male embryos, which only inherit the maternal X and also in female embryos, which inactivate the Xp during preimplantation stages. Therefore XCR is equally critical as XCI and also needs to be tightly coupled to key decision points during mouse development.

The mouse blastocyst consists of three cell types – epiblast, primitive endoderm (PE) and trophoctoderm (TE) (Fig. 2A). While PE and TE give rise to extraembryonic cell lineages like the placenta, the epiblast is the precursor of all embryonic cell lineages as well as embryonic stem cells (ESCs). Imprinted XCI is maintained in the PE and TE but erased by XCR specifically in the epiblast between E3.5 and E4.5 [25,27]. XCR is characterized by the downregulation of Xist, X-chromosome-wide removal of XCI silencing marks, like H3K27me3 and reactivation of X-linked genes. Based on Immunostaining and RNA-FISH

experiments, it has been claimed that X-linked gene reactivation occurs prior to *Xist*-downregulation and loss of H3K27me3 from the Xp [98]. However, it could be that epigenetic remodeling takes place locally at X-linked gene promoters before it can be detected by conventional microscopy like in this study and investigations using higher resolution methods are needed to confirm this. XCR in the blastocyst is confined to the epiblast cells expressing the pluripotency factor NANOG [27], which becomes biallelically expressed at the time of XCR [99]. Indeed, NANOG represses *Xist* by binding to *Xist intron 1* [79] and to the promoter of the *Xist*-activator *Rnf12* in ESCs [89]. Furthermore, *Nanog*-mutant mouse blastocysts do not undergo XCR, although this might be also partially due to indirect effects, because *Nanog* $-/-$ embryos lack proper epiblast cells, in which normally XCR would take place [100]. Nevertheless, it is likely that NANOG plays a critical role during XCR in the blastocyst. Another factor important for XCR in the blastocyst is the germ cell determinant and pluripotency factor PRDM14 [47]. PRDM14 is expressed in all instances when XCR takes place including the epiblast, in germ cells and in pluripotent stem cells [101,102]. *Prdm14*-mutant blastocysts fail to undergo XCR efficiently as indicated by remaining of the H3K27me3 mark on the Xp [47]. Like NANOG, PRDM14 binds to *Xist intron 1* and the *Rnf12* promoter in ESCs [47,84], where it represses *Rnf12* by recruiting the PRC2 complex, which lies down the H3K27me3 mark. As RNF12 is essential for imprinted XCI [41,42] and gets downregulated in the epiblast [103], this could provide a mechanism, how *Xist* is repressed during XCR. A facilitating role in the XCR process in the blastocyst plays the *Xist* repressor *Tsix*, which becomes biallelically expressed in the epiblast during XCR [45,104]. Forced *Tsix* expression is sufficient to repress *Xist* precociously at earlier stages of

development [105] and *Tsix*-mutant blastocysts show a delay in H3K27me3 erasure [47]. Therefore, albeit *Tsix* is not essential for XCR in the blastocyst [48], it appears to ensure the correct timing of the process, potentially by facilitating the binding of pluripotency factors like PRDM14 to *Xist intron 1* [47].

The second time during mouse development, when XCR takes place, is within the germ cell lineage to erase random XCI, which occurs earlier in the postimplantation epiblast, from which primordial germ cells (PGCs) are derived. XCR happens during the time when PGCs migrate through the hindgut and colonize the embryonic gonads [106-108]. In contrast to the blastocyst, where XCR takes place within a day, XCR in germ cells is an asynchronous process, which spans several days and cell divisions. Therefore the rapid XCR of imprinted XCI in the blastocyst might be mechanistically distinct from the slow XCR of random XCI in PGCs. A major reason for this difference could be the need to remove DNA-methylation from X-linked promoters after random XCI, which is less prevalent after imprinted XCI [109]. The process of XCR in PGCs follows a stepwise fashion, in which first *Xist* RNA becomes downregulated concomitant with loss of the X-chromosome-wide H3K27me3 mark [107,110]. Only then, X-linked genes become reactivated, with some genes being reactivated earlier than others. This kinetics mirrors the passive loss of genome-wide DNA-methylation in migrating PGCs [111]. The completion of XCR in the gonads, which roughly coincides with the erasure of autosomal imprints, seems to be dependent on a gonadal signal [107]. XCR has been also observed in human fetal PGCs, where H3K27me3 erasure, DNA-demethylation and X-linked gene reactivation appeared to occur already during the migration period before PGCs enter the gonads [112,113]. Therefore XCR in human PGCs might be controlled differently

than in the mouse. Besides the XCR kinetics in germ cells, not much is known mechanistically and identification of the critical players will be needed to fully understand the process. Newly developed *in vitro* systems of germ cell derivation both from mouse [114,115] and human [116-118] pluripotent stem cells will be instrumental in that.

Besides occurring naturally during development in the blastocyst and in PGCs, XCR is also a hallmark of naïve pluripotent stem cells and can be modeled using several *in vitro* reprogramming systems. For example, XCR takes place during de-differentiation of female somatic cells by cell fusion with pluripotent stem cells, which has shown that not only female, but also male ESCs contain all the necessary factors to induce XCR of the Xi in the female somatic fusion partner [119]. Another system to study XCR *in vitro* is during the conversion of Epiblast Stem Cells (EpiSCs) into ESCs through culture conditions or overexpression of ESC-factors like NANOG, KLF4, or KLF2 and PRDM14 [100,120-122]. While mouse ESCs reflect the naïve pluripotent state of the epiblast in the blastocyst at implantation, epiblast stem cells (EpiSCs) are the *ex vivo* equivalent of the early postimplantation epiblast and exhibit so-called primed pluripotency [123]. One of the main properties of primed pluripotent EpiSCs, besides not being able to efficiently contribute to chimeras when injected into blastocysts, is the display of random XCI, which is reversible in culture when EpiSCs are forced back to ESC-fate. EpiSC to ESC reversion and the associated XCR was shown to be particularly rapid when both the pluripotency factors KLF2 and PRDM14 were overexpressed together [120]. The effect on XCR is thereby both indirect by speeding up the EpiSC to ESC cell fate change, but also direct as PRDM14 binds at key XCI regulatory regions at *Xist* intron 1 and the *Rnf12* promoter, whereby it

suppresses *Xist* expression [47,84]. EpiSC to ESC conversion is a useful model system to study XCR due to its high efficiency and speed. However, not every feature of XCI is present in EpiSCs, like for example the histone variant macroH2A, which is incorporated towards the end of the XCI process, but absent on the Xi in EpiSCs [95]. Therefore other model systems than EpiSCs need to be used to study all aspects of the XCR process.

Particularly useful to address XCR mechanistically has been the iPSC reprogramming system [124], as it allows for a better accessibility and scalability when compared to studying XCR in embryos. During iPSC generation, XCR seems to be a gradual, late event [125,126]. This might be due to the fact that like in PGCs, random XCI has to be reversed in iPSCs. Therefore iPSC reprogramming might better reflect XCR in PGCs [107,110] than the reversal of imprinted XCI in the blastocyst [25,27,98], where XCR is much more accelerated. XCR in iPSCs involves multiple steps, of which some occur in reverse order to the XCI process, while others follow a different kinetics [126]. One of the earliest events during iPSC reprogramming is an initial enrichment of the PRC2 complex and its associated H3K27me3 mark on the Xi. This likely reflects an intermediate reprogramming stage and mirrors the global increase in nuclear PRC2 and H3K27me3 as also seen in XCR-competent cells *in vivo*, like in the epiblast and in PGCs [127,128]. Later however, after *Xist* gets downregulated, the H3K27me3 mark is erased from the Xi during XCR [126]. These steps occur approximately at the time, when endogenous pluripotency genes like *Nanog* become reactivated [47,125,126]. *Xist* is repressed both directly and indirectly by binding of pluripotency factors like NANOG or PRDM14 to *Xist* intron 1 and the promoter of the *Xist*-activator *Rnf12* [47,79,89]. Indeed depletion of PRDM14 or NANOG

decreases the efficiency of XCR during iPSC reprogramming [47,126]. At the same time, *Tsix* also becomes expressed first from the Xa and then from the Xi in response to pluripotency factor binding to its regulatory regions [80,81,126]. Interestingly, *Tsix* expression also facilitates binding of pluripotency factors like PRDM14 to *Xist* intron 1 in cis [47]. It is not yet known if this happens through direct recruitment of PRDM14 by *Tsix* RNA or by making the locus more accessible through transcription. On the other hand, neither *Tsix* nor *Xist* intron 1 are essential for *Xist* downregulation during XCR in iPSCs [47,85,126]. Nevertheless they might play a facilitating role [83] in concert with other pluripotency factor-bound regulatory regions along the *Xic*, which might be able to compensate for each other, if individual elements are missing. Another important event for XCR in iPSCs besides *Xist*-downregulation is DNA-demethylation of X-linked gene promoters. Only when both *Xist* is downregulated and DNA-methylation is removed, genes become reactivated during XCR [126], reflecting multiple layers of silencing which safeguard XCI maintenance in differentiated cells [16,94,129,130]. Depletion of the DNA-demethylases TET1 and TET2 does not interfere with XCR in iPSCs [126]. The mechanism of DNA-demethylation during XCR is still unknown, and it could be either based on different demethylating enzymes or on a passive dilution process. Despite the gains in our knowledge on *Xist* regulation and the kinetics of XCR in iPSCs, many questions related to the associated epigenetic changes and the responsible players remain to be investigated.

In order to get a full picture about XCR, we also need to keep in mind that at least in the case of imprinted XCI, marks on both X-chromosomes need to be erased. Importantly, silencing marks on the Xi (Xp in case of imprinted XCI) have to be

removed to reactivate X-linked genes. However also the imprints on *Xist* - the repressive imprint on the Xm [39] and the facilitating one on the Xp [44] need to be erased, in order to allow for random choice in the next round of XCI. Not all XCR events necessarily need to be coupled or take place at the same time. While the X-chromosome-wide epigenetic changes on the Xp take place during blastocyst maturation [25,27], the removal of the Xm imprint on *Xist* might occur already earlier. Parthenogenetic mouse embryos with two Xm's initially repress *Xist* during preimplantation development in a H3K9me-dependent manner [41]. However, this mark is removed from the *Xist*-promoter around the morula stage, leading to partial *Xist* de-repression in parthenogenetic embryos [41,131]. There is some evidence however that even ESCs retain to some degree memory of imprinted XCI. This can be unveiled when differentiating ESCs into extraembryonic cell lineages [132,133], although another study observed exclusively random XCI [134]. Further work will be needed to determine precisely when, to which degree and how the maternal and paternal imprints on *Xist* are erased during development.

5. Females are mosaics

An important outcome of random XCI is the fact that female eutherian (placental) mammals are mosaics, when it comes to X-chromosome activity. That means that in a proportion of the female's somatic cells, the paternal X-chromosome is active, while in the remaining cells the maternal X is active. This has an effect on the expression of different allelic variants present on the two X's,

leading for example to mosaic patterns in coat color in female mice, while male or XO female mice with one X express only one allele and therefore show uniform color. This phenotype led Mary Lyon to postulate her X-inactivation hypothesis, which marked one of the beginnings of the X-inactivation field [29]. A recent study using transgenic mice, in which gene expression from one X-chromosome is indicated by a green fluorescent reporter and from the other X-chromosome by a red fluorescent reporter gene, provided an impressive picture of this mosaicism down to cellular resolution [67]. It showed that depending on the individual mouse and the tissue type, the cells expressing one or the other X-reporter can be anything from completely intermingled to separated into left and right halves of the body. Except in skeletal muscle, where cell fusion occurs, no cells were found to express both X-reporter genes, indicating allelic maintenance of XCI in all adult somatic tissues including adult stem cells. The high variability in mosaicism will provide interesting insights into cellular mixing and selection events during tissue morphogenesis.

Apart from coat color this mosaicism also has important consequences for diseases in humans caused by X-linked mutations, which in general have a more dramatic impact on males than on females [135]. Females, which are heterozygous for an X-linked mutation, consist of cells in which the mutant allele is active and the wild-type allele is inactive and of cells, in which the opposite is the case. Therefore the cells with the active wildtype allele and the inactive mutant allele can at least partially compensate and alleviate the disease phenotype in females, which is not possible in males or XO females, which only carry the mutant allele. Classic examples for such diseases are hemophilia and color blindness, which are much more prevalent in males, as in heterozygous

females the cells expressing the wildtype allele can provide the necessary functionality. Other diseases, like the neurodevelopmental disorder Rett Syndrome, which is caused by mutations of the X-linked *MECP2* gene, show a severe phenotype in males (lethality before birth) and a postnatal neurodevelopmental phenotype in heterozygous females. The severity of the disease varies with the proportion of cells expressing the wildtype or the mutant copy of the gene, depending on the skewing of XCI in heterozygous females [136]. Re-expression of *MECP2* in *Mecp2*-mutant mice with symptoms of Rett Syndrome can reverse the neurological defects [137]. Therefore, approaches to reactivate the wildtype copy of the *MECP2* gene in heterozygous human Rett Syndrome patients might provide a potential avenue to treat this disease [130].

As the ratio of cells harboring the wildtype versus the mutant form of an X-linked gene on the active X-chromosome can affect the severity for a disease phenotype, it is important to understand what parameters determine the XCI skewing ratio. In females with two identical X-chromosomes, like in inbred female mice, random XCI occurs with the same likelihood for each X-chromosome leading on average to a 50:50 ratio of cells with inactivating either the maternal or the paternal X. This is also the case in healthy women, where most show a 50:50 ratio overall, with a normal distribution of patterns, where about 15-20% of adult women would display a strongly skewed (80:20 or more) XCI ratio [138]. In general, skewed XCI can occur for different reasons. Primary skewing can occur either per chance (low frequency), or more consistently, when the genes controlling the XCI process itself are allelically different between the two X-chromosomes, making one chromosome favored to be inactivated [139]. In this case most cells of the female will show skewing. Secondary skewing on the other

hand can take place, when XCI initially occurs in the embryo with a 50:50 ratio, but then afterwards a heterozygous X-linked allele gives the cells a selective proliferation or survival advantage when located on the active X. This type of skewing can affect either the whole female, but can also occur in a tissue-specific manner, depending on when and where the cells undergo selective pressure. When no secondary skewing due to cell selection is prevalent, blood or buccal swab samples can be used to relatively accurately predict the skewing ratio in many tissues of the individual [140,141]. Thereby they can provide diagnostic information for the prevalence of active X-linked disease alleles also for inaccessible tissues like the brain. Nevertheless, due to the unpredictable variation between individuals and organs in the degree of mosaicism, as shown in the mouse model [67], the deduction of XCI skewing in a specific tissue from these indirect tests is still only indicative and needs confirmation on a case by case basis.

Like for many other aspects of XCI, most information about the mechanisms of XCI skewing has been obtained in the mouse model. In inbred mouse strains the XCI ratio is close to 50:50 on average, whereas heterozygous females derived from crosses between different mouse strains consistently show XCI skewing. Depending on the strain of origin of an X-chromosome, it is either more or less likely to be inactivated compared to an X of a different strain [139]. This phenomenon was first linked to a region on the mouse X-chromosome named the *Xce* (*X-chromosome control element*) by Cattanaach and colleagues [142,143]. The *Xce* comes in different strengths, meaning that in heterozygous females the X-chromosome with the weaker *Xce* allele will be preferentially inactivated. So far the following *Xce* alleles have been identified in order of their strength

(starting with the weakest / most likely to be inactivated allele): $Xce^a \leq Xce^f \leq Xce^e < Xce^b < Xce^c < Xce^d$ [144]. In addition to the difference in skewing depending on *Xce* allele strength, a parent-of origin effect has been observed, with a tendency to slightly favor the paternal X-chromosome for inactivation, which might be caused by an incomplete erasure of epigenetic memory from imprinted XCI [132,133,144-147]. A number of genetic mapping studies have defined the mouse *Xce* to different regions sized between several megabases [145,148,149] and down to 176 kilobases [144], either overlapping and/or proximal/distal to the *Xic* region. The mutations and associated genes on the X-chromosome responsible for the *Xce* effect have not yet been identified, but it has been suggested that multiple regions within the *Xce* region might contribute [145]. In fact, many of the genes at the *Xic*, which have been shown or postulated to influence *Xist* expression *in cis*, like *Ftx* [150], *Jpx* [90], *Tsix* [151], *DXPas34* [152], *Xite* [51], *Tsx* [153], or *Linx* [154], are potential candidates for causing skewing, if differentially expressed between X-chromosomes [145]. Differential binding affinities of trans-acting factors between different *Xce* alleles have been postulated as the potential mechanism, by which choice could be skewed [155]. *Xce* allele differences could stem from SNPs (single nucleotide polymorphisms) in critical trans-factor binding sites. For example the human *XIST* promoter has a SNP at a binding site for the zinc finger protein CTCF, which influences the choice during X-chromosome inactivation [156,157]. Indeed, CTCF has also been found in mice to be a dosage-sensitive blocking factor, which blocks *Xist* from being upregulated from the active X-chromosome during X-inactivation [91]. CTCF is likely not the only trans-factor, of which binding can affect X-inactivation choice. A mutagenesis screen for autosomal mutations affecting XCI-skewing has

identified a number of candidate regions, however the associated genes/factors have not been identified yet [155,158]. Besides individual SNPs, also copy number variations of trans-acting factor binding sites through duplications and inversions could be responsible for the differences in *Xce* allele strength [144]. In summary, it appears that the *Xce* consists of multiple genes, which can affect X-inactivation choice, and this is likely controlled by a number of different trans-acting factors. Therefore, reducing the *Xce* effect to a single locus/gene might be not feasible and a systems approach with multiple players will need to be used to gain full mechanistic insight into the parameters affecting skewing during X-inactivation.

6. Concluding remarks and future outlook

A lot of knowledge has been gained over recent years on the XCI and XCR processes and their link to differentiation and pluripotency. However, many question still remain to be solved. For example it is not clear, why pluripotent cells do not require dosage compensation, while it is critical for differentiated cells. What are the genes on the X-chromosome specifically dosage-sensitive for differentiated cells and how does misregulation of these genes lead to cancer? The answer to this question could lead to novel cancer treatments - in particular for breast cancer, where the absence of an inactive X-chromosome and the presence of two active X-chromosomes has been observed, caused by either Xi-reactivation or by a combination of Xi-loss and Xa-duplication [8,9,159]. Furthermore, it will be critical to understand in more detail what sets the

hematopoietic lineage apart from other differentiated cell lineages, when it comes to its unique ability to initiate Xist-dependent gene silencing outside the critical silencing window of cellular differentiation [21-23]. Also in this aspect further findings might have an impact on future avenues of cancer treatments, as deletion of *Xist* in hematopoietic cells and the ensuing XCI-erosion is sufficient to cause blood-cancer in mice [7].

Related to the topic of imprinted XCI we now know that there are imprints established in both the maternal and the paternal germline. Despite the gain in knowledge we have about epigenetic marks detected in the preimplantation embryo, we still don't know, which type of marks are established in the germline and transmitted through the gametes. Interestingly, both during establishment of the paternal permissive, as well as the maternal repressive imprint on *Xist*, the compaction state of the *Xic* might play a role [43,44]. How these states correlate with potential key epigenetic imprints remains to be determined and finding the imprints will also allow establishing, how long into development these imprints persist and at which stage they are erased.

Another key question will be the evolutionary conservation of aspects of imprinted XCI between mouse and human. There is some evidence that human preimplantation embryos show a similar XIST expression pattern during like in mice [160]. However, there is also evidence to the contrary, where biallelic *XIST* expression has been detected in female human embryos without epigenetic signs of X-chromosome silencing [161]. In fact, a recent single-cell expression study of human preimplantation embryos indicated biallelic dampening of X-linked gene expression in females compared to males, instead of imprinted monoallelic silencing like in the early female mouse embryo [162]. If this is achieved by the

biallelic *XIST* expression detected in this study, or by an alternative mechanism, remains to be investigated. In addition, also in the human placenta either imprinted [163,164] or random XCI [165-167] has been detected, therefore displaying a deviation from the imprinted form in the mouse. This has been partially attributed to the different gene structure of *TSIX* in humans compared to mice [168,169], where *Tsix* is the key *Xist* regulator during imprinted XCI in the placenta [45,46]. A further difference between human and mouse relates to the status of XCI in pluripotent stem cells, in which human cells do not always display XCR as mouse cells but rather shows different states ranging from anywhere between complete XCR and XCI [170]. However, human ESCs and iPSCs derived under novel naïve culture conditions, follow the XCR pattern of mouse pluripotent stem cells [171,172]. In conclusion, it will be necessary to reassess many aspects of XCI and XCR carefully directly in human, as not all information we gained from the mouse model can be readily applied. For that purpose, naïve human pluripotent stem cells will be an invaluable tool for studying human XCI and XCR *in vitro*.

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Figure Legends

Fig. 1. Imprinted X-chromosome inactivation (XCI) mechanisms in mice

(A) Overview of imprinted XCI and its establishment in the germline. In the female germline, a repressive imprint on *Xist* (red stripes) is established during oocyte growth on the maternal X-chromosome (X_m, (B)). Furthermore, a permissive imprint (blue stripe), which ensures *Xist* expression in embryos, is established on the paternal X-chromosome (X_p) during meiosis in spermatogenesis. This occurs in the XY-body during meiotic sex chromosome inactivation (MSCI, (C)). In addition, repeat-sequences on the X_p are silenced chromosome-wide and inherited silent to the next generation (pink shading). From the 2-cell stage onwards, *Xist* is expressed from the X_p, leading to progressive silencing of X-linked genes during preimplantation development. Imprinted XCI is maintained in the blastocyst stage in the extraembryonic primitive endoderm (PE) and trophectoderm (TE), but erased in the epiblast by XCR. p, paternal pronucleus; m, maternal pronucleus.

(B) Maternal imprint establishment during oogenesis. Already at the non-growing oocyte stage, a H3K9me3 mark is established at the *Xist* promoter.

During oocyte growth the *Xic* region condenses, acquiring the maternal repressive imprint (red) on *Xist*. This imprint, together with H3K9me3 ensure *Xist* repression during early preimplantation development. In the placenta *Xist* is repressed by *Tsix*.

(C) Paternal Imprint Establishment during Spermatogenesis. While autosomes (A) pair during the pachytene stage, the X- and Y-chromosome pair incompletely and get silenced by MSCI in the heterochromatic XY-body (yellow). The *Xic* locus becomes condensed, establishing the permissive imprint on *Xist* (blue). Also during spermatogenesis, *Xite* and *Tsix* become methylated (DNA-me), keeping them off and *Xist* on during preimplantation development and in the placenta.

Fig. 2. XCR vs. XCI during embryonic development

(A) XCR takes place in the epiblast (Epi, cyan within dotted circle) lineage of the late (E4.5) blastocyst. Both X-chromosomes are active (Xa) and show expression of negative *Xist*-regulator genes (green), while *Xist*-activators and *Xist* itself are off (grey). Repressive chromatin marks and CpG-methylation (CpG-me) are absent while activating histone marks are enriched on X-linked gene promoters keeping them expressed.

(B) After implantation, random XCI takes place in the Epiblast, while imprinted XCI is maintained in extraembryonic ectoderm (ExE) and visceral endoderm (VE). Differentiation of the epiblast triggers shutting down of *Xist* repressor genes (grey), while *Xist*-activators and *Xist* become expressed (red). *Xist* RNA recruits silencing factors to the Xi, which results in chromosome-wide chromatin

changes. Repressive histone marks, CpG-methylation and incorporation of the histone variant macroH2A thereby ensure faithful silencing of X-linked genes.

(A) Imprinted XCI

(B) Maternal Imprint Establishment

(C) Paternal Imprint Establishment

(A) X-Chromosome Reactivation (XCR)

Xic region on Xa

Chromatin on Xa

E4.5 blastocyst

(B) X-Chromosome Inactivation (XCI)

Xic region on Xi

Chromatin on Xi

E5.5 postimplantation embryo