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X-inactivation and X-reactivation: epigenetic hallmarks of mammalian reproduction and pluripotent stem cells

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Abstract

X-chromosome inactivation is an epigenetic hallmark of mammalian development. Chromosome-wide regulation of the X-chromosome is essential in embryonic and germ cell development. In the male germline, the X-chromosome goes through meiotic sex chromosome inactivation, and the chromosome-wide silencing is maintained from meiosis into spermatids before the transmission to female embryos. In early female mouse embryos, X-inactivation is imprinted to occur on the paternal X-chromosome, representing the epigenetic programs acquired in both parental germlines. Recent advances revealed that the inactive X-chromosome in both females and males can be dissected into two elements: repeat elements versus unique coding genes. The inactive paternal X in female preimplantation embryos is reactivated in the inner cell mass of blastocysts in order to subsequently allow the random form of X-inactivation in the female embryo, by which both Xs have an equal chance of being inactivated. X-chromosome reactivation is regulated by pluripotency factors and also occurs in early female germ cells and in pluripotent stem cells, where X-reactivation is a stringent marker of naive ground state pluripotency. Here we summarize recent progress in the study of X-inactivation and X-reactivation during mammalian reproduction and development as well as in pluripotent stem cells.

Introduction

Acquisition of an XY sex chromosome system necessitates the need to resolve X-linked gene dosage imbalances between XX females and XY males (Graves 2006; Payer and Lee 2008). Ancient mammals may have solved this dosage dilemma by selectively inactivating the paternally derived (father's) X-chromosome in all female cells in a process called imprinted X-chromosome inactivation (X-inactivation). Non-placental extant mammals such

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as marsupials only possess this ancestral form of dosage compensation (Graves 1996; Sharman 1971) (Fig. 1). On the other hand, placental mammals (eutherians) additionally developed random X-inactivation: a process in which both X-chromosomes have an equal chance of being inactivated (Lyon 1961). Imprinted and random X-inactivation in placental mammals is controlled by a newly acquired regulatory genetic element, the X-inactivation center (*Xic*), which most prominently includes the non-coding *Xist* gene (Borsani et al. 1991; Brockdorff et al. 1991; Brown et al. 1991). In mice, imprinted X-inactivation first takes place in the early embryo and is maintained in the placenta, where the paternal X (X^P) is preferentially inactivated (Huynh and Lee 2003; Mak et al. 2004; Okamoto et al. 2004). The X^P is then reactivated specifically in the epiblast of the inner cell mass of the blastocyst that corresponds to the pluripotent status of embryonic stem cells (ES cells) (Mak et al. 2004; Okamoto et al. 2004), followed by random X-inactivation in the embryonic lineage. Acquisition of the *Xist* gene (Duret et al. 2006) and random X-inactivation may represent one of the critical events that contributed to the evolutionary advantage of placental mammals, as both parental X chromosomal alleles could be utilized.

To date, many questions remain unanswered in the regulation of X-inactivation in development. For example, it is unclear how the imprint information is programmed in the parental germlines, how chromosome-wide silencing is established, and how the X-chromosome is reactivated during epigenetic reprogramming in the peri-implantation embryo and in germ cells to achieve the two-active X state, which is also characteristic of pluripotent stem cells. In this article, we summarize recent advances in the study of X-inactivation and X-reactivation during germ cell and embryonic development as well as in pluripotent stem cells.

X-chromosome inactivation in germ cells and pre-implantation embryos

It remains elusive how the paternally derived X-chromosome is selectively inactivated in imprinted X-inactivation. Despite recent advances, controversies in the field remain, in particular regarding the regulation of imprinted X-inactivation (Huynh and Lee 2005; Okamoto and Heard 2009; Payer and Lee 2008). Our recent study suggests that the opposing models represent different aspects of imprinted X-inactivation and are not mutually exclusive (Namekawa et al. 2010). We try to unravel the reasons for current disparities in the field and aim to clarify the models regarding the regulation of X-inactivation.

Initiation of imprinted X-inactivation in vivo

The study of random X-inactivation has progressed greatly in the past 15 years because of the accessibility of an ex vivo system in which random X-inactivation can be recapitulated during the differentiation of ES cells (detailed in other reviews (Barakat et al. 2010; Chow and Heard 2010; Lee 2009; Payer and Lee 2008). At the same time, progress in studying imprinted X-inactivation in the mouse embryo has been slow, because the limited supply of early embryos and the limited cellular material makes it challenging to conduct the analyses, which are standard for the study of random X-inactivation in ES cells. Therefore, the status of X-inactivation during embryogenesis remained elusive until recently. Classically, imprinted X-inactivation was thought to occur in the extraembryonic lineage around the time of implantation (reviewed in (Huynh and Lee 2005), although the first sign of X-inactivation, such as the expression of Xist RNA from the paternal X, had been observed in preimplantation embryos (Kay et al. 1994). Groundbreaking discoveries were made in 2003 and 2004 from three laboratories, demonstrating that imprinted inactivation of the paternally derived X already takes place in preimplantation mouse embryos (Huynh and Lee 2003; Mak et al. 2004; Okamoto et al. 2004) and that this imprinted X-inactivation is reversed in the inner cell mass of blastocysts at the timepoint when pluripotent ES cells can be derived (Mak et al. 2004; Okamoto et al. 2004). Although the data in these three reports are

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generally consistent, subtle differences spawned two fundamentally different models regarding the origin of imprinted X-inactivation. Based on the absence of nascent transcription near the paternal *Xic* in female 2-cell embryos, the Lee laboratory proposed that imprinted X-inactivation originates from meiotic sex chromosome inactivation (MSCI) in male spermatogenesis and that the pre-inactivated X-chromosome is inherited from father to daughter (pre-inactivation hypothesis) (Huynh and Lee 2003). On the other hand, the Heard laboratory showed that transcriptional silencing on the X^p at the 2-cell stage could not be detected (Okamoto et al. 2004). Gradual accumulation of histone modifications related to gene silencing were seen on the paternal X only after the 4-cell stage of preimplantation development, leading to the model that imprinted X-inactivation is established de novo after fertilization, independent of MSCI (de novo model).

Recent studies tested the two models and revealed that genic silencing of imprinted X-inactivation takes place de novo rather than being continuously silent since its inheritance from the paternal germline. Using gene-specific RNA fluorescence in situ hybridization (FISH) it was shown that three X-linked genes on the paternal X are initially active at the 2-cell stage (Okamoto et al. 2005). Additionally, three recent independent studies using gene-specific RNA FISH confirmed that dozens of X-linked genes are initially active at the 2-cell stage and are then gradually inactivated during preimplantation development (Kalantry et al. 2009; Namekawa et al. 2010; Patrat et al. 2009). However, our recent study revealed the paternal X-chromosome is treated differently in the genic regions and the non-genic repeat regions, such as long interspersed elements (LINEs) and short interspersed repetitive elements (SINEs), and that the repeat silencing precedes genic silencing in imprinted X-inactivation (Namekawa et al. 2010). This study suggests that the X-linked repeat elements may be preinactivated and inherited from the paternal germline, although the genic silencing is established de novo in imprinted X-inactivation.

Epigenetic programming establishes the imprinting information in the germline, which is then inherited by the embryo. Although gene silencing during imprinted X-inactivation was shown to take place de novo, the underlying mechanisms of the two models are rooted in different parental origins. The preinactivation hypothesis predicts the events in the paternal germline are instrumental for paternal imprinted X-inactivation, while the de novo model favors a maternally derived imprint. Based on recent advances, we propose that epigenetic events of both parental origins contribute to establishing the imprinted X-inactivation in the embryo, reconciling different aspects of the two models.

Events on the paternal X in the male germline

The preinactivation hypothesis predicts that imprinted X-inactivation originates from the paternal germline and specifically from MSCI (Huynh and Lee 2003, 2005). Historically, the prevailing view has been that MSCI is transient and limited to meiotic prophase, and that the purpose of MSCI was to prevent the induction of a meiotic checkpoint for the sake of meiotic progression (McKee and Handel 1993). However, subsequent studies have revealed the transcriptional features of the X-chromosome during spermatogenesis, showing that the effects of MSCI persist throughout spermiogenesis after meiosis. Repressive histone modifications were shown to remain on sex chromosomes into the second meiotic division (Khalil et al. 2004). Cytological evidence revealed that the sex chromosomes occupy a silent compartment in round spermatids (Greaves et al. 2006; Namekawa et al. 2006; Turner et al. 2006), named post-meiotic sex chromatin (PMSC) (Namekawa et al. 2006). Chromosome-wide silencing of the sex chromosomes was also confirmed by microarray analysis in round spermatids (Namekawa et al. 2006). These findings challenged the prevailing view and unexpectedly illuminated potential new roles for MSCI during epigenetic regulation of the early embryo, in which the silent memory is maintained throughout the meiotic cell divisions, into spermiogenesis, and carried into daughter embryos.

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Several recent studies illuminate a potential mechanism of epigenetic silencing in MSCI. MSCI is known to be Xist-independent (McCarrey et al. 2002; Turner et al. 2002), though the underlying mechanism and *raison d'etre* of the epigenetic silencing in MSCI remain enigmatic (Inagaki et al. 2010; Namekawa and Lee 2009; Turner 2007; Yan and McCarrey 2009). Meiotic silencing is a general silencing mechanism, which represses unsynapsed chromatin in male germ cells and has been termed meiotic silencing of unsynapsed chromatin (MSUC) (Baarends et al. 2005; Schimenti 2005; Turner et al. 2005). MSCI is considered to be a manifestation of MSUC on the unsynapsed sex chromosomes in normal meiosis. The site of MSCI is decorated with various DNA damage response (DDR) factors, which were originally shown to accumulate at the site of DNA damage in somatic cells. First, ATR kinase and TOPBP1 (an ATR activator) accumulate at the site of MSCI (Moens et al. 1999; Perera et al. 2004; Reini et al. 2004). Then ATR phosphorylates histone variant H2AX (phosphorylated histone H2AX is called γH2AX) (Bellani et al. 2005; Turner et al. 2004). Although a previous study showed that MSCI does not occur in the H2AX knockout mouse (Fernandez-Capetillo et al. 2003), the function of γH2AX and the associated DDR pathway has not been explored by genetic experiments. Recent work in the Namekawa laboratory tested the role of the DDR pathway, focusing on mediator of DNA damage checkpoint 1 (MDC1), a binding partner of γH2AX (Ichijima et al. 2011). This study provides the first genetic evidence that γH2AX and the associated DDR pathway are the essential determinants for MSCI and also the potential mechanisms to recognize the chromosome-wide domain in MSCI. The study shows that MSCI consists of two genetically separable steps: the MDC1-independent recognition of the unsynapsed axis by DDR factors and the MDC1-dependent chromosome-wide spreading of DDR factors to the entire chromatin of the sex chromosomes (Fig. 2). Furthermore, it was demonstrated that the DDR pathway has a shared role in MSCI and the somatic response to replicative stress in S phase which is related to silencing. These results establish that the DDR pathway centered on MDC1 recognizes the chromosome-wide domain and induces epigenetic silencing of sex chromosomes in germ cells. The study concludes that the DDR pathway is a master regulator of sex chromosome inactivation in males.

Other independent studies also revealed potential mechanisms underlying MSCI. During meiosis, sex chromosomes undergo replacement of histone H3 by the histone variant H3.3 (van der Heijden et al. 2007), as well as incorporation of the histone variant H2A.Z (Greaves et al. 2006). Furthermore, X-linked microRNAs are not silenced during MSCI, suggesting a role for X-linked microRNAs in the initiation of MSCI (Song et al. 2009). These observations suggest that the DDR pathway acts in concert with histone replacement and the microRNA pathway to confer epigenetic silencing of the sex chromosomes in male germ cells.

As proposed in the preinactivation hypothesis, it is possible that MSCI is responsible for the inheritance of epigenetic information through sperm. In human sperm, various histone modifications are retained at gene promoters in accordance with their roles in early embryogenesis (Arpanahi et al. 2009; Brykczynska et al. 2010; Hammoud et al. 2009). Thus, chromatin modifications in sperm are potentially instrumental in governing gene expression during the critical phase of early development. Future challenges will be to determine if epigenetic modifications on the X-chromosome in sperm functionally contribute to the establishment of imprinted X-inactivation.

Maternal regulation of imprinted X-inactivation

The de novo model proposes that the X-chromosome imprint is transmitted from the maternal germline, which confers resistance to the maternal X-chromosome and prevents it from being inactivated, but that the paternal X lacks such a protective imprint. Autosomal transgenes from the *Xic* region can recapitulate specific expression of Xist RNA from the

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paternal allele when transmitted from the paternal germline, even though the autosomal transgene does not go through MSCI (Okamoto et al. 2005). Instead, when the transgene is transmitted from the maternal germline, Xist RNA is never expressed from the maternal allele (Okamoto et al. 2005). These observations are consistent with the previously postulated idea that the maternal X-chromosome carries an imprint that is acquired during oocyte maturation to resist being inactivated in embryos (Kay et al. 1994; Tada et al. 2000) and that the maternal *Xic* carries an imprint to repress Xist expression from the maternal allele in embryos (Goto and Takagi 2000; Lee 2000). Also, XX androgenetic embryos, in which both X-chromosomes are derived from a paternally derived genome, show a random pattern of X-inactivation and survive through implantation, suggesting that the paternal X-chromosome does not carry an imprint to control *Xist* expression (Okamoto et al. 2000). Therefore, it was predicted that the maternal *Xic* carries the imprint and that Xist RNA expression from the paternal allele is the critical determinant of gene silencing on the paternal X-chromosome during imprinted X-inactivation. *Tsix*, the anti-sense regulator gene of *Xist*, was proposed as a candidate region for carrying the maternal imprint on the *Xic* (Lee 2000). However, the nature of the maternal imprint on the *Xic* remains elusive. Although autosomal imprinting is regulated by de novo DNA methylation in the germline, it was recently shown that de novo DNA methylation is not essential for the establishment of the maternal imprint in imprinted X-inactivation (Chiba et al. 2008; Kaneda et al. 2004).

A recent study identified a novel maternal regulator of imprinted X-inactivation. Shin et. al. showed an essential role of the E3 ubiquitin ligase RNF12 in imprinted XCI using a mouse model with the conditional deletion of *Rnf12* that is encoded in the proximal region of *Xic.* When the mutant *Rnf12* allele is transmitted from the maternal germline, only female embryos are lethal, presumably due to defects in imprinted X-inactivation (Shin et al. 2010). RNF12 was originally proposed to be a dosage-dependent activator of *Xist* for random X-inactivation in ES cells (Jonkers et al. 2009). Importantly, in female embryos with a maternally inherited mutated allele of *Rnf12*, Xist cloud formation and gene silencing on the paternal X-chromosome is compromised. Based on these observations, it was proposed that the deposit of RNF12 in the maternal germline is the critical determinant of imprinted X-inactivation. Although this model appears to explain the nature of the previously postulated maternal imprint, there seems to be a functional difference between the maternal imprint acquired during oocyte maturation that confers resistance to inactivation on the maternal X-chromosome during preimplantation development (Tada et al. 2000) and the role of RNF12 in the regulation of imprinted X-inactivation. During oocyte maturation, RNF12 indeed accumulates in oocytes. However, the presence of maternally deposited RNF12 protein cannot rescue the viability of female embryos with the maternally inherited mutant allele (Shin et al. 2010), suggesting that the maternal RNF12 deposit is not sufficient for the initiation of imprinted X-inactivation. Furthermore, homozygous deletion of RNF12 in oocyte maturation (i.e. absence of maternally deposited RNF12) leads to viable male embryos, suggesting that maternally deposited RNF12 is not required for keeping the maternal X-chromosome active during male pre-implantation development. Given the quiescence in transcription after oocyte maturation until zygotic genome activation, it is possible that zygotic expression of RNF12 from the maternal allele is the critical determinant of Xist expression from the paternal allele *in trans*, which leads to imprinted X-inactivation in preimplantation embryos. This notion is consistent with the potential action of RNF12 in random X-inactivation in ES cells in that RNF12 acts *in trans* and activates *Xist* expression (Barakat et al. 2011). Thus, there can be two layers of the maternal imprint: one is the unknown imprint on the *Xic* acquired during oocyte maturation that protects the maternal X-chromosome from being inactivated (Lee 2000; Okamoto et al. 2005; Tada et al. 2000), and the other is the maternally inherited allele of RNF12 that activates Xist expression from the paternal allele (Fig. 1). Taken together, these observations further

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support the idea that Xist expression from the paternal X-chromosome is regulated by the rigorous maternal imprint.

Though these observations establish the role of the maternal imprint, it remains unclear whether the maternal imprint is the sole determinant of imprinted X-inactivation and whether the paternal imprint contributes to imprinted X-inactivation. However, paternally inherited autosomal *Xic* transgenes, which do not go through MSCI, can express their transgenic *Xist* copies during preimplantation development (Okamoto et al. 2005). These mice with autosomal *Xic* transgenes are viable and do not have any overt phenotype (Heard et al. 1996). This suggests that Xist RNA expressed from paternally derived *Xic* transgenes is not sufficient to fully silence the autosomes on which the transgenes are integrated. This observation raised the possibility of additional epigenetic mechanisms (possibly from the paternal germline) that would ensure chromosome-wide silencing of the X-chromosome during imprinted X-inactivation apart from the rigorous maternal imprint controlling *Xist* expression.

Role of Xist: a biparental model

The de novo model postulates that Xist induces gene silencing in imprinted X-inactivation during preimplantation development. However, the absence of *Xist* in marsupials suggests that the ancestral form of imprinted X-inactivation might be Xist-independent (Duret et al. 2006). MSCI and PMSC also exist in marsupials, raising the possibility of a germline-driven mechanism of imprinted X-inactivation in marsupials without the need for Xist (Hornecker et al. 2007; Namekawa et al. 2007). More than a decade ago, it was shown that a deletion of *Xist* on the paternal X causes embryonic lethality several days after implantation (Marahrens et al. 1997). These observation raised questions about the role of Xist at the onset of imprinted X-inactivation.

Recently, two independent studies addressing this question came to different conclusions: one study argues that genic silencing during imprinted X-inactivation occurs independently of Xist (Kalantry et al. 2009), whereas our study showed that genic silencing in imprinted X-inactivation requires the *Xist* gene (Namekawa et al. 2010). This disparity can be explained by technical differences during RNA FISH, RT-PCR, and *X-GFP* transgene analysis (see details in Namekawa and Lee 2011; Namekawa et al. 2010). For example, our results suggest that the low X-linked GFP expression in the extraembryonic ectoderm of early postimplantation embryos is seen both in females with a paternally inherited *X-GFP* transgene as well as in males and females with maternally inherited *X-GFP* and therefore is not caused by imprinted X-inactivation. Thus, low X-GFP expression cannot be used as faithful indicator for Xist-independent X-inactivation as done in the study by Kalantry et al. Furthermore, other independent studies also present genetic evidence that the expression of Xist RNA is the prerequisite for genic silencing in imprinted X-inactivation (Hoki et al. 2009; Senner et al. 2011; Shin et al. 2010). However, it is possible that some genes may behave like repeat elements and are inactivated in an Xist-independent manner. It is proposed that this mechanism of Xist-independent gene silencing in mice may share a similar mechanism with imprinted X-inactivation in marsupials in the absence of *Xist* (Kalantry et al. 2009).

Our recent study revealed that the chromosome-wide silencing during imprinted X-inactivation is established in two-steps: first by repeat silencing that occurs specifically on repeat elements, which is then followed by genic silencing (Namekawa et al. 2010). Repeat silencing occurs independently of Xist at the 2-cell stage on the paternal X, which localizes to the nucleolus. This can be observed both in female wildtype embryos and embryos with *Xist* deletion on the paternal X-chromosome. Thereafter follows the Xist-dependent phase of genic silencing. Given the Xist-independent mechanism of silencing in the male germline

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(McCarrey et al. 2002; Turner et al. 2002), the Xist-independent repeat silencing might represent the paternal imprint and could be maintained through nucleolar association. Consistent with the de novo model, Xist-dependent genic silencing is regulated by the maternal imprint that prohibits *Xist* expression from the maternal allele. In summary, we propose that imprinted X-inactivation is regulated by bi-parental imprints in placental mammals and that the preinactivation and de novo models represent different but not mutually exclusive aspects of imprinted X-inactivation (Fig. 1).

The two-step process is somewhat similar to the previous observation of initiation of random X-inactivation (Chaumeil et al. 2006; Clemson et al. 2006). In random X-inactivation, the *Xic* is responsible for choosing the X-chromosome to be inactivated, and exclusion of RNA polymerase II precedes individual genic silencing. Interestingly, the underlying mechanisms of repeat silencing are different between imprinted and random X-inactivation, although the genic silencing depends on Xist in both types of X-inactivation. Repeat silencing during random X-inactivation requires the repeat A region of the *Xist* gene (Chaumeil et al. 2006). The repeat A region, which is required for the function of Xist in the induction of gene silencing (Wutz et al. 2002), expresses the short 1.6 kb non-coding RNA RepA and functions to recruit Polycomb Repressive Complex 2 (PRC2) complex to the inactive X-chromosome during random X-inactivation (Maenner et al. 2010; Zhao et al. 2008). If repeat elements were already marked in the paternal germline, it would facilitate the spreading of Xist along the inactive X-chromosome during imprinted X-inactivation. Curiously, the repeat A region was also shown to be a critical region for the initiation of genic silencing in imprinted X-inactivation (Hoki et al. 2009). In the repeat A mutant, expression of Xist RNA from the paternal X-chromosome is diminished in pre-implantation embryos, indicating the regulatory role of the repeat A region in *Xist* expression in imprinted X-inactivation. Although genetic studies are revealing the action of Xist in imprinted X-inactivation, the mechanisms underlying Xist-independent repeat silencing have not been specified yet.

Evolution of X-inactivation: repeat versus genic silencing

In the male germline, PMSC represents the repeat silencing of the sex chromosomes during spermiogenesis in mammals (Namekawa et al. 2006, 2007). A genomic analysis revealed that the X-chromosome is enriched with multi-copy genes (Mueller et al. 2008). The expression of these multicopy X-linked genes is proposed to correlate with previous microarray data that showed that a small portion of X-linked genes are expressed specifically in round spermatids (Mueller et al. 2008; Namekawa et al. 2006). Also, single-copy X-linked genes showed a varying extent of reactivation during mouse spermiogenesis (Mueller et al. 2008). Interestingly, in marsupials, X-linked genes are largely reactivated in round spermatids (Mahadevaiah et al. 2009), despite the fact that repeat silencing persists with PMSC (Namekawa et al. 2007). These results suggest that even in the germline, the X-chromosome is treated differently regarding repeat and genic regions and that repeat silencing is evolutionally conserved among mammals. The two-step model of imprinted X-inactivation in placental mammals implies that an ancestral mechanism underlies repeat silencing from the paternal germline, while a more recently acquired mechanism underlies the maternal imprint in *Xic* (Fig. 1). The finding that genes are also silenced de novo during embryogenesis in marsupials, despite the lack of an *Xist* gene, raises questions about the possible mechanism (Mahadevaiah et al. 2009).

Studies in the past 2 years have revealed epigenetic modifications on the inactive X-chromosome in marsupials. One study identified trimethylation at H3K27 (H3K27me3) as a marker of the inactive X-chromosome in marsupial interphase nuclei (Mahadevaiah et al. 2009). H3K27me3 is also a characteristic modification of the inactive X-chromosome in placental mammals (Erhardt et al. 2003; Plath et al. 2003; Silva et al. 2003). H3K27me3 is induced by PRC2, which is recruited by the action of RepA RNA generated within the *Xist*

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gene (Maenner et al. 2010; Zhao et al. 2008). Based on the accumulation of H3K27me3 on the marsupial inactive X-chromosome, it was proposed that imprinted X-inactivation may have a common mechanism with marsupials and placental mammals (Mahadevaiah et al. 2009). However, subsequent studies revealed that trimethylation at H3K9 (H3K9me3) and HP1 are stable markers of marsupial inactive X-chromosomes and that H3K27me3 accumulation is transient and labile (Chaumeil et al. 2011; Rens et al. 2010; Zakharova et al. 2011). H3K9me3 and HP1 are well-characterized markers of pericentromeric heterochromatin and are also conserved markers of mammalian PMSC in spermiogenesis (Namekawa et al. 2007), supporting a notion that germline silencing is the ancestral driving force of imprinted X-inactivation in the absence of Xist RNA in marsupials. It will be particularly interesting to find out if gene silencing in marsupials is also controlled by a non-coding RNA or if the silencing is based on an alternative process, such as the chromatin-based mechanism observed in the male germline.

These studies suggest that repeat elements on the X-chromosome may have a specific role in sustaining epigenetic memories on the X-chromosome. In this respect, Mary Lyon's hypothesis was a noteworthy prediction regarding the role of repeat elements. Mary Lyon proposed the LINE hypothesis in which the X-chromosome is enriched with specific repeat elements, such as LINEs, to facilitate X-inactivation (Chow et al. 2010; Lyon 1998). Both imprinted and random X-inactivation in females and repeat silencing in PMSC in the male germline are in accordance with that hypothesis. The different types of regulation for repeat versus genic regions emerge as common characteristics of chromosome-wide silencing across mammalian species (Fig. 1).

X-chromosome reactivation: an epigenetic hallmark of pluripotency

While the inactive X-chromosome is stably maintained in adult female somatic tissues it undergoes dynamic changes during mouse embryogenesis (Fig. 3). The transition from the imprinted to the random form of X-inactivation in the embryo and from random X-inactivation to an active state in the germline requires the reactivation of the inactive X-chromosome by epigenetic reprogramming.

X-chromosome reactivation in vivo

The first instance of X-reactivation occurs at the blastocyst stage between embryonic day (E)3.5 and E4.5. This coincides with the time when blastocysts implant into the uterus and shortly after the first distinct cell lineages become apparent (Mak et al. 2004; Okamoto et al. 2004). Imprinted X-inactivation, which has been established during preimplantation development in all cells of the female mouse embryo, is maintained in the trophectoderm and primitive endoderm, which will later give rise to extra-embryonic tissues like the placenta. However, in the Nanog-positive epiblast lineage of the inner cell mass, which will form the future embryo, the imprinted inactivation of the paternal X-chromosome is reversed by reactivation. The characteristic signs of the X-reactivation process are the downregulation of *Xist* expression and the disappearance of the accumulation of Polycomb proteins Ezh2 and Eed and their associated histone H3 lysine 27 di/tri-methylation mark (H3K27me2/3) from the paternal X-chromosome (Mak et al. 2004; Okamoto et al. 2004). Surprisingly, a new study suggests that reactivation of some X-linked genes and of repeat sequences might even occur before the chromosome-wide removal of Xist RNA and H3K27me3 from the inactive X-chromosome (Williams et al. 2011). It is yet unknown though, if this is the case for all X-linked genes and if Xist RNA and H3K27me3 are indeed still localized to particular gene loci during their reactivation. Further studies will be needed to explain which potential mechanisms could lead to the proposed Xist-independent gene-reactivation.

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Soon after a short period of the reactivated state, random X-inactivation commences in the epiblast of early postimplantation mouse embryos around E5.5 to E6.5 (Rastan 1982; Takagi et al. 1982). This also applies to early primordial germ cells (PGCs), which initially show signs of random X-inactivation (Chuva de Sousa Lopes et al. 2008; Sugimoto and Abe 2007). During their migration and gonadal colonization between E7.0 and E10.5, PGCs downregulate *Xist* and loose the characteristic H3K27 trimethylation spot on the inactive X (Chuva de Sousa Lopes et al. 2008; de Napoles et al. 2007; Sugimoto and Abe 2007), which coincides with general genome-wide epigenetic reprogramming in PGCs (Hajkova et al. 2008; Hayashi and Surani 2009; Seki et al. 2005, 2007). Progressively X-linked genes become reactivated, which is a gradual process not completed until much later during oogenesis (Sugimoto and Abe 2007). Therefore, it appears that X-reactivation in PGCs is a slower and more passive process in contrast to the rapid reactivation in the blastocyst, which occurs within a day. This might have to do with the fact that random X-inactivation is maintained by multiple epigenetic marks including DNA methylation, while maintenance of imprinted X-inactivation is DNA methylation-independent and believed to be less stable (Payer and Lee 2008; Sado et al. 2000). In addition, the set of expressed pluripotency genes varies between PGCs and blastocyst epiblast cells, which also might contribute to the differences in X-reactivation kinetics. The importance of appropriate programming and re-programming of the X-chromosome in the germline is further underscored by nuclear transfer experiments. Cloned mouse embryos frequently display aberrant X-inactivation patterns with bi-allelic Xist expression in females and Xist being detected on the single X in males (Bao et al. 2005; Nolen et al. 2005). This contributes greatly to the low cloning efficiency and survival rate of those embryos and can be rescued by deleting *Xist* on the active X-chromosome (Inoue et al. 2010).

X-chromosome reactivation in vitro

In addition to blastocysts and PGCs, X-chromosome reactivation is also an epigenetic characteristic of pluripotent stem cells in vitro (Fig. 3), making them attractive model systems for dissecting the mechanisms of X-reactivation. Pluripotent cell lines have the capacity to self-renew in culture and differentiate into all cell types including somatic cells and germ cells.

Classic examples are mouse embryonic stem (ES) cells, which can be derived from ground state epiblast of E4.5 blastocysts (Nichols and Smith 2009) and mirror its epigenetic state by displaying two active X-chromosomes. The same is the case is for embryonic germ (EG) cells, which are derived from PGCs. Interestingly, EG cells derived from late PGCs also erase their autosomal imprints, while ES cells retain them (Shovlin et al. 2008), demonstrating differences in reprogramming capacity of different pluripotent stem cell types. When fused with female differentiated cells, factors present in ES and EG cells reprogram the somatic genome to a pluripotent state causing reactivation of the somatic inactive X-chromosome (Tada et al. 1997, 2001). It is worth mentioning that this activity is present both in female and male pluripotent stem cells, demonstrating that the necessary factors for X-reactivation are not female-specific but a general feature of the pluripotent cell state.

Epiblast stem cells (EpiSCs) are derived from the epiblast (E5.5-E6.5) of postimplantation embryos and share some common properties with mouse ES cells (Brons et al. 2007; Tesar et al. 2007), like the expression of the pluripotency markers Oct4, Sox2 and Nanog and the ability to differentiate into all three germ layers in vitro and in vivo in teratomas. However, in contrast to mouse ES cells, EpiSCs do not efficiently contribute to chimeras after blastocyst injection and exhibit X-inactivation, reflecting the epigenetic makeup of their tissue of origin in vivo (Guo et al. 2009). These different pluripotent states are now commonly referred to as primed pluripotency in EpiSCs versus the naive pluripotent ground

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state in mouse ES cells (Nichols and Smith 2009). Interestingly, the single overexpression of individual pluripotency factors, which are expressed in ES cells but are absent or only weakly present in EpiSCs like *Klf2/4*, *Nr5a1/2*, *c-Myc* or *Nanog* or the activation of the Jak/Stat3 pathway can convert EpiSCs into an ES cell-like state (reviewed in Gillich and Hayashi 2011). Even without overexpression of external factors, EpiSC to ES-like cell conversion can occur spontaneously during prolonged culture of EpiSCs under ES-cell conditions albeit at very low frequency (Bao et al. 2009). After this conversion from primed to naive pluripotency, EpiSC-derived ES-like cells are competent to form germline chimeras and also display reactivation of the previously inactive X-chromosome as indicated by disappearance of H3K27me3 accumulation from the previously inactive X-chromosome (Guo et al. 2009). Therefore, X-reactivation mirrors precisely the naive pluripotent state, making X-reactivation a true hallmark of ground state pluripotency.

ES cells are not the only cell lines, which can be derived from mouse blastocysts. Also the two extraembryonic cell lineages of the blastocyst, trophectoderm (TE) and primitive endoderm (PE), can give rise to trophoblast stem (TS) cells and extraembryonic endoderm (XEN) cells, respectively (Fig. 3). Like their in vivo counterparts, female TS (Mak et al. 2002) and XEN (Kunath et al. 2005) cells display imprinted inactivation of the paternal X-chromosome. Recently it has been demonstrated that TS cells can be converted to ES-like cells by overexpressing *Oct4, Sox2, Klf4* and *c-Myc* (Kuckenberg et al. 2011) or by *Oct4* alone (Wu et al. 2011). After this conversion the X-chromosome becomes reactivated, demonstrating the successful erasure of imprinted X-inactivation in vitro (Wu et al. 2011). To this point successful reprogramming of XEN cells has not yet been reported. On the contrary, ES cells can also be converted in vitro to TS cells by overexpression of *Cdx2* (Niwa et al. 2005) and to XEN cells by overexpression of *Gata4* or *Gata6* (Shimosato et al. 2007). Analysis of the X-inactivation status of ES cell-derived TS and XEN cells showed random rather than imprinted X-inactivation, which was also observed in trophectoderm cells of blastocysts cloned from ES cells (Murakami et al. 2010). This suggests that the X-chromosome imprints have been erased in ES cells leading to random X-inactivation in extraembryonic lineages, which normally would display imprinted X-inactivation.

Differentiated somatic cells can be reprogrammed into induced pluripotent stem (iPS) cells by overexpressing the four transcription factors Oct4, Sox2, c-Myc and Klf4 (Takahashi and Yamanaka 2006), reviewed by (Plath and Lowry 2011; Stadtfeld and Hochedlinger 2010). Mouse iPS cells are pluripotent, contribute to all germ layers when injected into blastocysts and share many similarities with ES cells regarding gene expression profiles and chromatin modifications. During reprogramming, female iPS cells reactivate the inactive X-chromosome (Maherali et al. 2007) as a relatively late reprogramming event around the time when they start expressing telomerase and endogenous pluripotency genes like *Oct4*, *Sox2* and *Nanog* (Stadtfeld et al. 2008). When re-differentiated in culture, iPS cells undergo again random X-inactivation, indicating that the epigenetic memory of the previously inactive X-chromosome got erased during iPS cell generation (Maherali et al. 2007). The relatively slow X-reactivation kinetics during iPS cell reprogramming might have several reasons. First, X-reactivation might rely on the expression of pluripotency factors like Nanog (see below; Fig. 4) and indeed, adding Nanog to the four factors appears to accelerate iPS-reprogramming (Hanna et al. 2009). Furthermore, reversal of random X-inactivation requires multiple steps including *Xist*-downregulation, H3K27me3 removal and DNA demethylation of X-linked genes. Thereby iPS-reprogramming of somatic cells might be similar to X-reactivation in PGCs, which requires several days to be completed (Chuva de Sousa Lopes et al. 2008; Sugimoto and Abe 2007). It would be interesting to see, if the kinetics of imprinted X-reactivation during the conversion of TS to ES/iPS cells (Wu et al. 2011) occurs faster, thereby resembling more the erasure of imprinted X-reactivation in the blastocyst.

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The function of pluripotency factors in X-reactivation

A common property of all known cell types displaying X-reactivation both in vivo and in vitro is the expression of a number of pluripotency factors including Oct4, Sox2 and Nanog. Both functional and biochemical evidence recently suggested a direct involvement of these factors in the X-reactivation process. A key step in X-reactivation is the downregulation of Xist expression from the inactive X-chromosome. Binding of Oct4, Sox2 and Nanog has been demonstrated to *Xist* intron 1 in ES cells (Donohoe et al. 2009; Navarro et al. 2008). Furthermore, Oct4 (Donohoe et al. 2009), Klf4, c-Myc and Rex-1 (Navarro et al. 2010) bind *DXPas34* and Oct4, Sox2 and Klf4 (Donohoe et al. 2009; Navarro et al. 2010) bind *Xite*, both of which are enhancers of *Tsix*, the non-coding antisense regulator gene of *Xist* during X-inactivation (Fig. 4). Depletion of Oct4 from ES cells by RNAi knockdown or inducible downregulation results in upregulation of Xist from both X-chromosomes in female cells (Donohoe et al. 2009) and depending on experimental conditions even from male ES cells (Navarro et al. 2008). Conversely *Tsix* is downregulated after Oct4 (Donohoe et al. 2009), Rex1 or c-Myc (Navarro et al. 2010) knockdown.

Nanog in particular seems to be important for X-reactivation in blastocysts, as Nanog-mutant female embryos fail to erase the characteristic H3K27 trimethylation from the inactive X in the inner cell mass (Silva et al. 2009). However, these Nanog-mutant embryos are missing viable epiblast cells, which make it hard to assess, if the effects observed on X-reactivation are direct or indirect. Nanog-mutant ES cells show some *Xist* upregulation albeit at lower levels than after Oct4 depletion (Navarro et al. 2008). A key role for Nanog during reprogramming and X-reactivation is supported by the observations that Nanog overexpression greatly increases the reprogramming efficiency after ES-somatic cell fusion (Silva et al. 2006) and during the in vitro conversion of EpiSCs to ES cells (Silva et al. 2009). Nanog dosage seems to be important in this case, as EpiSC also express endogenous Nanog, albeit at lower levels than ES cells. Furthermore, generation of fully reprogrammed iPS cells requires Nanog (Silva et al. 2009) and X-reactivation in iPS cells coincides approximately with the onset of endogenous Nanog expression (Stadtfeld et al. 2008).

The *Xist* gene is repressed by pluripotency factors both directly (via binding to *Xist* intron 1) and indirectly (via upregulation of *Tsix*), coupling X-reactivation and X-inactivation tightly to the pluripotent and differentiated state, respectively. Neither deletion of *Xist* intron 1 (Barakat et al. 2011) nor of *Tsix* (Lee and Lu 1999) lead to full *Xist* upregulation, showing that the two repressive mechanisms might be able to compensate for each other, or that additional regulatory elements influence *Xist* expression. Analysis of *Xist* intron 1 and *Tsix* double mutant ES cells will be needed to answer this question. Besides the repression of *Xist* by pluripotency factors, also the lack of *Xist* activators in pluripotent cells might be an important contributor. Indeed, two activators of *Xist* expression, both of them located on the X-chromosome, have been recently identified. One of them is the long non-coding RNA gene *Jpx*, which is located 10 kb 5′ of *Xist* (Chureau et al. 2002; Johnston et al. 2002; Nesterova et al. 2003). Jpx RNA is nearly absent in undifferentiated ES cells but gets increasingly expressed during differentiation (Tian et al. 2010). Jpx is required for *Xist* upregulation during X-inactivation and can act *in trans*, which has not been described before for a non-coding RNA from the X-inactivation center. The *Jpx* deletion can be rescued by truncating *Tsix* on the same chromosome, indicating that the two non-coding RNAs have opposing roles in *Xist* regulation. *Ftx*, a non-coding RNA gene located upstream of *Jpx* has been also recently described as an activator of *Xist* expression in male ES cells (Chureau et al. 2011). If *Ftx* plays a role in *Xist* upregulation during X-inactivation in female cells still needs to be tested.

Another important activator of *Xist* is the ubiquitin ligase RNF12, which has been proposed to act in a dosage-dependent manner and is able to transactivate *Xist* independently of *Tsix*

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and *Xist* intron 1 (Barakat et al. 2011; Jonkers et al. 2009; Shin et al. 2010). Also RNF12 is expressed at low levels in undifferentiated ES cells and gets upregulated during differentiation. Interestingly, expression of *Rnf12* is mutually exclusive with Nanog expression, which is caused by direct repression through binding of Nanog and other pluripotency factors like Oct4 and Sox2 to the *Rnf12* promoter (Barakat et al. 2011; Kim et al. 2008; Navarro et al. 2011). RNF12 expression peaks during X-inactivation and then gets repressed in differentiated cells by X-inactivation, suggesting an involvement in the initiation but not in the maintenance of *Xist* expression (Jonkers et al. 2009). While one study found an absolute requirement for RNF12 in random X-inactivation (Barakat et al. 2011), another study observed random X-inactivation even in *Rnf12* mutant cells (Shin et al. 2010). This suggests either differences between the mutant alleles used in the two studies, or that under certain experimental conditions other *Xist* activators might compensate for a lack in functional RNF12. Mechanisms underlying how Jpx or RNF12 activate *Xist* transcription remain elusive. As RNF12 is an E3 ubiquitin ligase, specific degradation of *Xist* repressors might be a hypothetical mechanism. Suggestively, both Oct4 and Nanog have been shown to be targets of ubiquitination-mediated decay (Moretto-Zita et al. 2010; Xu et al. 2004). Future experiments will be needed to identify RNF12 targets to further understand its mode of *Xist* regulation.

Multiple open questions remain to be addressed before we fully understand the mechanisms of X-chromosome reactivation in mice. For example, which factors confer developmental specificity to the timing of X-reactivation? In the early blastocyst at E3.5, Oct4, Sox2, Nanog, Rex1, Klf4 and c-Myc are all already expressed before X-reactivation takes place, but are not sufficient to induce *Xist* repression and H3K27 trimethylation loss at that stage (Mak et al. 2004; Okamoto et al. 2004; Silva et al. 2009). Additional factors might be necessary to trigger X-reactivation specifically in epiblast cells between E3.5 and E4.5. In addition it is unclear by which mechanism the H3K27 trimethylation mark is removed from the inactive X-chromosome and how X-linked genes become reactivated again. Especially it is unclear, if X-reactivation in the different in vivo and in vitro systems presented here (Fig. 3) is achieved by similar or distinct mechanisms. What is the root cause for the difference in reactivation kinetics between the blastocyst and germ cells and is it more difficult for the pluripotency machinery to erase random compared to imprinted X-inactivation? It also remains untested, if repeat sequences become reactivated alongside X-linked genes, or if there are also mechanistic differences between repeat- and gene-reactivation as in the X-inactivation process. Although substantial progress has recently been made regarding X-chromosome reactivation, we are still in the infancy of understanding this critical reprogramming event on the path to achieve naive pluripotency.

X-Chromosome reactivation in human embryos and pluripotent stem cells

The X-inactivation status of female human preimplantation embryos is currently under debate. A previous study (van den Berg et al. 2009) reported XIST RNA coating of one X-chromosome beginning at the morula stage which was accompanied by H3K27me3 accumulation and X-linked gene silencing. Therefore, this paper suggested that the mechanisms of early X-inactivation and reactivation might be conserved between mice and humans. However, the study did not address, if early human X-inactivation is imprinted rather than random and if X-reactivation occurs in the epiblast of the blastocyst as it does in the mouse. In a more recent study (Okamoto et al. 2011), very different observations have been made. According to Okamoto et al., *XIST* gets upregulated on both X-chromosomes in female and the single X in male human embryos, which surprisingly does neither result in H3K27me3 accumulation on the X-chromosomes nor in X-linked gene silencing. In this study, *XIST* is also reported to be biallelically expressed in the inner cell mass of blastocysts, which is different from mice. In conclusion, the authors claim that no imprinted

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X-inactivation during human preimplantation development exists and that X-inactivation and X-reactivation mechanisms vary substantially between different eutherian mammalian species. The divergent results between the studies of van den Berg and Okamoto could be explained by varying culture conditions, the quality of donated human embryos and differences in RNA FISH methodology. Further independent studies will be required to finally answer the question, how different or similar X-inactivation and reactivation mechanisms are between mouse and man.

Indirect evidence in favor of X-reactivation in the female human blastocyst comes from studies of human ES cells (reviewed by Kim et al. 2011). Most female human ES cell lines, which have been derived under ambient (20%) oxygen conditions, already show signs of X-inactivation in the undifferentiated state while only few lines show two active X-chromosomes (Hall et al. 2008; Hoffman et al. 2005; Shen et al. 2008; Silva et al. 2008). However, when female human ES cells are derived under low oxygen concentration (5%) resembling the physiological condition of the embryo, they frequently have two active X-chromosomes equivalent to mouse ES cells (Lengner et al. 2010). Derivation culture conditions can therefore determine if human ES cells display a naive pluripotent phenotype reflecting probably the epigenetic status of the epiblast of the blastocyst or rather show a primed pluripotent phenotype similar to mouse EpiSCs. Like mouse EpiSCs, also human ES cells with primed pluripotency characteristics can be converted into a more naive pluripotent mouse ES cell-like state (Hanna et al. 2010). This can be accomplished by culture with GSK3β and ERK1/2 inhibitors and LIF in addition to overexpression of Oct4 and Klf4/2. The same can be done for human iPS cells (Buecker et al. 2010; Hanna et al. 2010), which frequently fail in X-reactivation during reprogramming and show an inactive X-chromosome also in the undifferentiated state (Tchieu et al. 2010).

The gold standard to test for pluripotency of stem cells in mice is the generation of fully stem cell-derived mice by injection into tetraploid blastocysts. Out of ethical considerations this is not an option for human stem cells and the best test for pluripotency in human cells to date is the generation of teratomas by injection into immunocompromised mice. While this test can accurately check for the ability of stem cells to differentiate into all three germ layers ecto- meso- and endoderm, it is not stringent enough to test if the stem cells can differentiate into all cell types including the germ line. For example, primed pluripotent stem cells like mouse EpiSCs are not able to efficiently contribute to mouse chimeras, but can form all germ layers in the teratoma assay (Brons et al. 2007; Tesar et al. 2007). Therefore, other more stringent markers for human stem cells are needed to assess their quality as naive pluripotent stem cells, with a mouse ES-cell like pristine epigenetic makeup. One marker identified to distinguish between mouse iPS cells with high or low developmental potential is the normal expression of the imprinted *Dlk1–Dio3* cluster (Stadtfeld et al. 2010). If transcripts from this locus are aberrantly silenced, iPS cells fail to form fully iPS cell-derived mice. As outlined above, X-reactivation in female stem cells is a promising indicator to distinguish between the primed and naive pluripotent state. Only female undifferentiated stem cells with two active X-chromosomes can be considered as having truly reached ground state pluripotency. Needless to say, X-reactivation can be only used in female stem cells as a direct readout of epigenetic quality. However, the X-reactivation program is not exclusive to female cells, as male pluripotent stem cells also contain the necessary reprogramming factors as shown after cell fusions between male mouse ES cells with female somatic cells (Tada et al. 2001). Therefore, once we better understand the roots and regulators of X-reactivation in female cells and their link to pluripotency, we might also be able to extrapolate these findings to assess the epigenetic quality of male stem cells. Consequently, understanding the mechanistic differences and commonalities between X-reactivation in mice and humans is not only a scientifically interesting question by itself, but

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it also has important implications on stem cell research and its medical application in general.

Concluding remarks

In the past 3 years, the field of X-inactivation has entertained substantial progress regarding the initiation of imprinted X-inactivation. This progress has revealed that X-inactivation is regulated by a multilayer process to establish chromosome-wide silencing. In all types of X-inactivation (imprinted and random X-inactivation in females and sex chromosome inactivation in male germ cells), repeat elements and unique coding elements are treated differently. Although the mechanism governing the multi-layer process remains elusive, X-inactivation is regulated locally at the *Xic* but also extensively in a chromosome-wide manner. These different regulations would explain the nature of the imprints that are programmed in parental germlines; the local imprint at the *Xic* from the maternal germline versus the chromosome-wide imprint on repeat elements from the paternal germline. On the other hand, X-reactivation also might be regulated by a multilayer process. Although local action of pluripotency factors at the *Xic* has been reported, it is still unclear how the entire X-chromosome is reprogrammed. Many questions remain about the molecular mechanisms that govern the parental imprints, initiation of X-inactivation, and X-reactivation.

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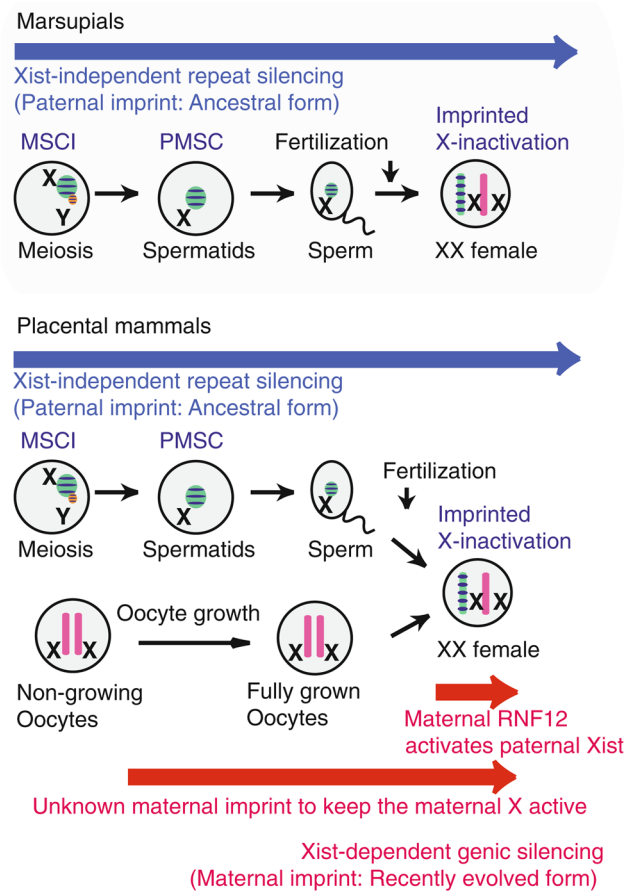

Fig. 1.

Models about the origin of imprinted X-inactivation. In marsupials, MSCI and PMSC may be the driving force of imprinted X-inactivation (*upper panel*). In placental mammals such as mice, imprinted X-inactivation is regulated by the bi-parental imprints (*lower panels*). *Barred* chromosomes represent silent imprints on the paternal X-chromosome

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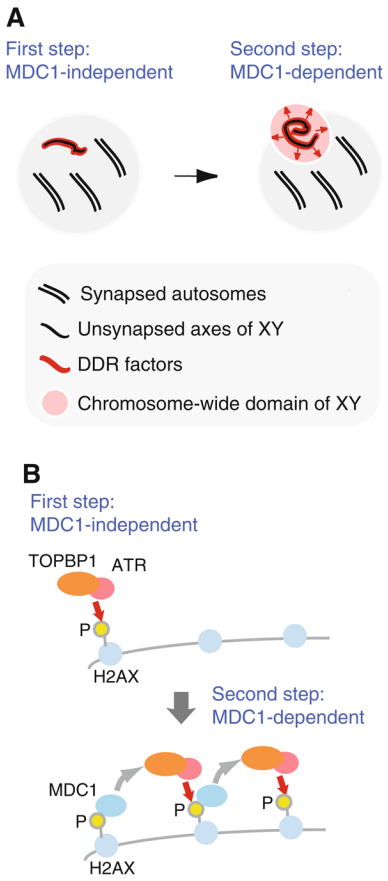

Fig. 2.

Models of the initiation of meiotic sex chromosome inactivation (MSCI). **a** Pictorial representation of the role of MDC1 in establishing chromosome-wide inactivation in MSCI (Ichijima et al. 2011). The first step is MDC1-independent recognition of the unsynapsed axis. The second step is MDC1-dependent spreading of DDR factors to the chromosome-wide domain. **b** Action of MDC1 in signal amplification of DDR factors. MDC1 binds γH2AX and recruits the ATR and TOPBP1 complex to spread γH2AX to the chromosome-wide domain of the sex chromosomes (Ichijima et al. 2011)

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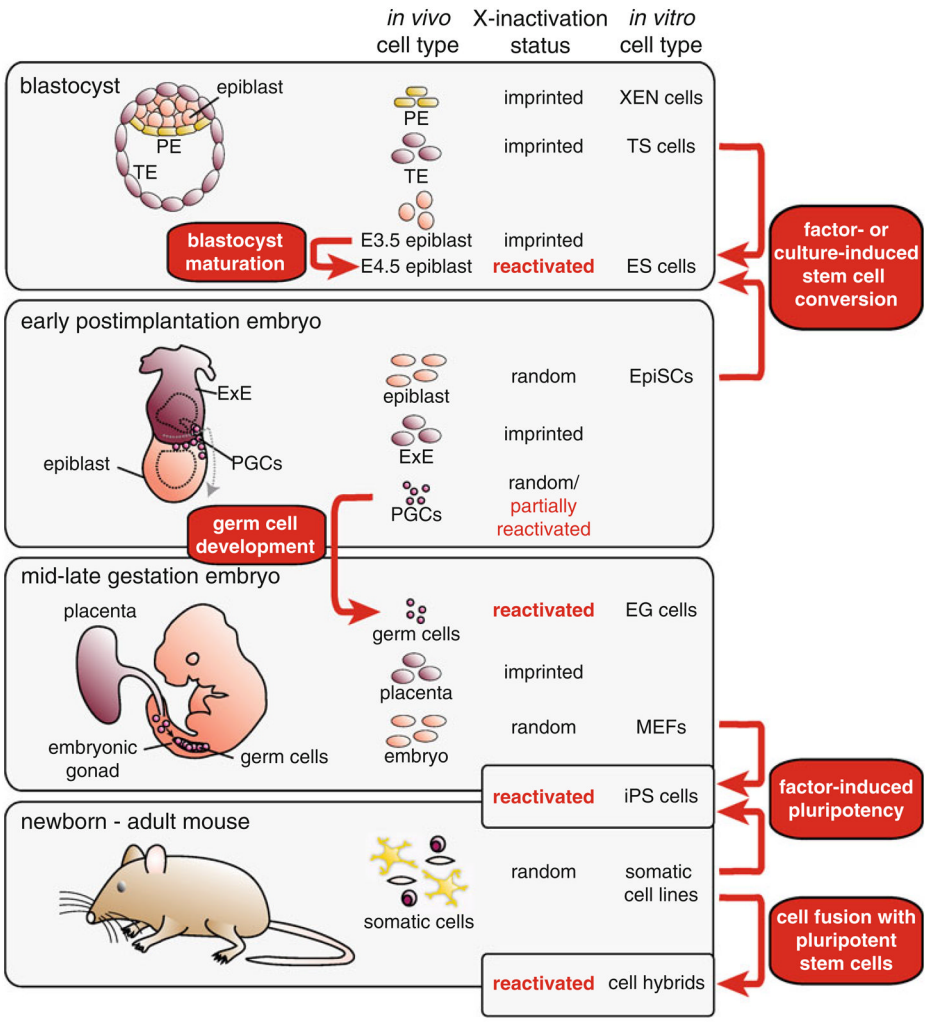

Fig. 3.

A developmental timeline of X-chromosome reactivation in mice. In vivo, X-reactivation (*red arrows*/ *boxes*) occurs in the epiblast of late blastocysts and during germ cell development. In vitro, X-reactivation is associated with reprogramming toward the naive pluripotent stem cell state like the conversion of trophoblast stem (TS) cells and epiblast stem cells (EpiSCs) to embryonic stem (ES) cells by overexpression of external factors or specific culture conditions (see text). Furthermore, X-reactivation happens during the reprogramming of adult somatic cells or mouse embryonic fibroblasts (MEFs) into induced pluripotent stem (iPS) cells by defined factors. The inactive X-chromosome in somatic cells is also reprogrammed when these cells are fused with pluripotent stem cells. *EG cells* embryonic germ cells, *ExE* extraembryonic ectoderm, *PE* primitive endoderm, *PGCs* primordial germ cells, *TE* trophectoderm, *XEN cells* extra-embryonic endoderm cell lines

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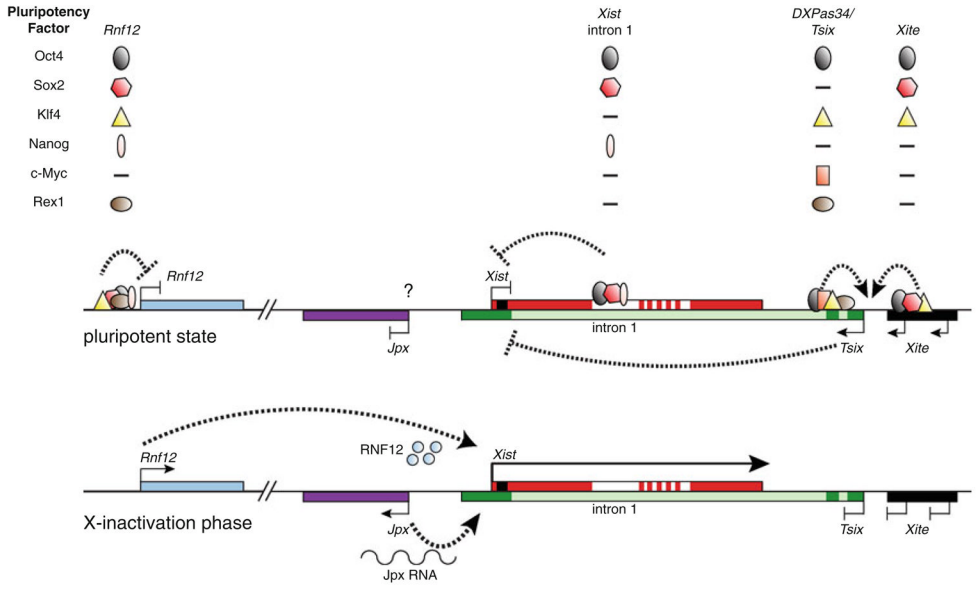

Fig. 4.

Model for the molecular link between pluripotency factors and *Xist* repression. In the pluripotent state (*top*), Oct4, Sox2 and Nanog bind to *Xist* intron 1 and thereby repress *Xist* transcription directly. Furthermore, Oct4, Sox2, Klf4, c-Myc and Rex1 also bind regions within the *DXPas34* and/or *Xite* enhancers resulting in *Tsix*-activation. *Tsix* in turn is an inhibitor of *Xist* expression. The two activators of *Xist* expression *Rnf12* and *Jpx* are expressed at low levels in pluripotent cells. In case of *Rnf12*, this is achieved by repressive action of pluripotency factors at regulatory regions (Barakat et al. 2011; Kim et al. 2008; Navarro et al. 2011). It is also unknown, what regulates *Jpx* (*question mark*). Thereby pluripotency factors appear to be involved in both direct and indirect mechanisms to repress *Xist* in pluripotent cells. During the X-inactivation phase (*bottom*), pluripotency factors dissociate from their binding sites, which has two effects. First, repression of *Xist* by pluripotency factors at intron 1 and *Tsix* is released and second, the *Xist* activators RNF12 and Jpx get upregulated, thereby elevating *Xist* expression