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REVIEW

X chromosome inactivation in human development

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ABSTRACT

X chromosome inactivation (XCI) is a key developmental process taking place in female mammals to compensate for the imbalance in the dosage of X-chromosomal genes between sexes. It is a formidable example of concerted gene regulation and a paradigm for epigenetic processes. Although XCI has been substantially deciphered in the mouse model, how this process is initiated in humans has long remained unexplored. However, recent advances in the experimental capacity to access human embryonic-derived material and in the laws governing ethical considerations of human embryonic research have allowed us to enlighten this black box. Here, we will summarize the current knowledge of human XCI, mainly based on the analyses of embryos derived from *in vitro* fertilization and of pluripotent stem cells, and highlight any unanswered questions.

KEY WORDS: X chromosome inactivation, Human, Germline, Embryo, XIST, XACT, Pluripotent stem cells

Introduction

The formation of heteromorphic (see Glossary, Box 1) sex chromosomes during the evolution of mammals has created a dosage imbalance for sex-linked genes between sexes. Although this imbalance is well tolerated for the male-specific Y chromosome, probably because of its highly specialized and low gene content, this is not the case for the X chromosome, which covers ∼1.5 Mb of DNA and harbors up to a thousand genes, many of which serve fundamental cellular functions. In metatherians and eutherians, the increased dose of X-linked genes in females is compensated for by the functional exclusion of one of the two X chromosomes, a process referred to as X chromosome inactivation (XCI; see Glossary, Box 1). During XCI, several hundreds of physically linked loci are concomitantly and stably silenced (Robert Finestra and Gribnau, 2017). Of note, some genes are refractory to XCI and escape this process, but the proportion of such genes varies from one species to another (Carrel and Brown, 2017).

Mice have long been the leading model for X-inactivation studies in placental mammals, and have thus served to elucidate the developmental regulation of this process and to identify most of the molecular mechanisms and factors involved. XCI is tightly regulated, established early during embryonic development and then stably maintained for the entire *in utero* and adult life. In the germline, however, reactivation of the X chromosome occurs concomitantly to global epigenetic reprogramming during primordial germ cell (PGC; see Glossary, Box 1) specification (Payer et al., 2011). More subtle exploration revealed two waves of XCI in mouse (Fig. 1). First, soon after fertilization and zygotic genome activation, XCI is established in an imprinted manner, with systematic inactivation of the paternal X chromosome (Fig. 1; Mak et al., 2004; Okamoto et al., 2004). This form of X-inactivation is maintained in the extra-embryonic lineages, but reversed in the inner cell mass of early blastocysts, in which the two X chromosomes are transiently active (Mak et al., 2004; Okamoto et al., 2004; Takagi, 2003). Subsequently, random XCI (rXCI; see Glossary, Box 1) is initiated in cells of the embryo proper, and gives rise to individuals in whom the maternal and paternal X chromosomes are active in roughly half of the cells each (Dupont and Gribnau, 2013).

At the molecular level, XCI is triggered by the long noncoding RNA (lncRNA; see Glossary, Box 1) X-inactive specific transcript (*XIST*; see Glossary, Box 1), which is expressed from and remains associated with one of the two X chromosomes (Borsani et al., 1991; Brockdorff et al., 1991; Brown et al., 1991). *XIST* accumulation forms a cloud-like structure in the nucleus, visible using RNA fluorescence in situ hybridization (RNA-FISH), and acts as a platform for the recruitment of various complexes (Moindrot and Brockdorff, 2016). Through mechanisms that remain unclear, this modifies the organization of the decorated chromosome at multiple levels and prevents gene expression. One of the hallmarks of the inactive X chromosome (Xi; see Glossary, Box 1) is its heterochromatic and condensed nature (Barr and Bertram, 1949). Heterochromatization of the Xi involves a sequential, yet rapid, switch in post-translational modifications of histones, with pan-acetylation and tri-methylation of lysine 4 of histone H3 (H3K4me3) being removed and replaced by other marks such as tri-methylation of H3 lysine 9 and 27 (H3K9me3 and H3K27me3, respectively), and ubiquitination of H2A at lysine 119 (H2AK119Ub) (Zylicz et al., 2019). The Xi is also enriched for specific histone variants, such as macroH2A, and displays CpG island hypermethylation (Nora and Heard, 2010). All of these events are triggered, directly or indirectly, by the accumulation of *XIST* RNA, the expression of which depends on *trans*-acting factors and on elements located in the vicinity of the *XIST* gene, in a region called the X-inactivation center (*XIC*, see below). Several of these elements are noncoding themselves, and act through a variety of mechanisms that are not yet fully deciphered (Furlan and Rougeulle, 2016).

Despite the compulsory nature of XCI, the hierarchy of events leading to XCI and the XCI regulatory network, although largely conserved, display species specificities (Okamoto et al., 2011; Petropoulos et al., 2016; Vallot et al., 2017). In particular, there is mounting evidence that X chromosome activity, whether during early pre-implantation development or in the germline, embraces specific dynamics in humans (Fig. 1), raising questions as to the universality of the underlying regulatory network in eutherian mammals. In this Review, we discuss recent advances and highlight unanswered questions in the area of XCI initiation in human development.

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Box 1. Glossary

Embryo genome activation (EGA). A complex process occurring in the embryo after fertilization. It allows the embryo to take control over its development through a faithful reprogramming and restructuring of the two parental genomes before transcription occurs.

ESHRE Istanbul consensus. Defines the morphological criteria to classify a pre-implantation embryo at different stages of its development (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011).

Heteromorphic. A chromosome pair, such as the sex chromosomes X and Y, which have some homology but differ in size, shape and genetic content.

Hysterectomy. Surgical ablation of the uterus.

Long noncoding RNA (lncRNA). An RNA molecule longer than 200 nucleotides that does not encode a protein.

Pluripotency. The property of some cells to form all somatic lineages and germ cells; pluripotency exists in two states, naïve and primed, representing the distinct cellular features of the pre- and post-implantation epiblast, respectively.

Primordial germ cells (PGC). Precursors or primary undifferentiated stem cells that will give rise to the gametes.

Random XCI (rXCI; see XCI). Refers to the equal probability of each X chromosome (maternal and paternal) to be inactivated. Random XCI results in a mosaic individual, in which the maternal X chromosome is active in roughly half of the cells and the paternal one in the other half.

RNA-seq. Next-generation sequencing of all cellular RNAs, which can be performed on a cell population (bulk RNA-seq) or on individual cells (scRNA-seq).

Topologically associated domains (TAD). Chromosome domains of preferential interaction in the 3D space.

Xa. Active X chromosome.

***XACT* (X-active coating transcript).** An lncRNA that coats active X chromosomes in early development. *XACT* is found only in hominoids. The function of *XACT* is unknown.

XCI. X chromosome inactivation, the process whereby one of the two X chromosomes is transcriptionally shut down in female mammals.

Xe. An originally inactive X chromosome that has been partially reactivated owing to erosion of XCI, a process that takes place spontaneously when pluripotent stem cells are grown in culture.

Xi. Inactive X chromosome, with most genes being silenced.

***XIST* (X-inactive specific transcript).** An lncRNA produced by the X chromosome that is retained in the nucleus and triggers XCI. *XIST* is found in all placental mammals.

Dynamics of X chromosome activity during early human development

To gain insight into the initial phases of human X chromosome inactivation during early development, scientists rely on embryos derived from *in vitro* fertilization (IVF), which raises ethical as well as technical issues (see Box 2). Below, we summarize our current knowledge in this area of research.

Sex-dependent differences in early embryonic development

As the Y chromosome is enriched in transcription-regulating genes, which could accelerate growth development (Bellott et al., 2014), it has been suggested that male and female embryos may differ in several parameters during the human pre-implantation period, as in other mammalian species (Kochhar et al., 2001). As these differences appear before sexual differentiation, they cannot be linked to sex-related hormonal differences; instead, they could be attributed to a bias in the expression level of sex chromosome-encoded genes, which may indirectly affect the expression of autosomal genes. In particular, several studies have reported that sex could affect human embryo kinetics. The number of cells in day 2 IVF-conceived male embryos is greater than that in female embryos (Ray et al., 1995). This observation was later corroborated by the finding that male embryos tend to reach the final blastocyst stages faster than females (Alfarawati et al., 2011; Hentemann et al., 2009; Luna et al., 2007; Ménézo et al., 1999). Because the faster-developing and most expanded blastocysts are often selected for embryo transfer, this could explain why the sex ratio at birth after IVF has been reported to be skewed towards the male sex (Chang et al., 2009; Luna et al., 2007; Ménézo et al., 1999). Increased mitotic rates in male embryos could be attributed to early expression of sex determining region Y gene (*SRY*) from the Y chromosome, which is known to have mitogenic properties and is expressed as early as the eight-cell stage in humans (Fiddler et al., 1995). These differences in proliferation kinetics, which only become apparent around the time of embryo genome activation (EGA; see Glossary, Box 1), may also be explained by the dose-dependent transcription levels of mRNAs, notably from the X chromosome. Indeed, the differential expression of genes between sexes during pre-implantation development has recently been confirmed (Zhou et al., 2019). The majority of early differential gene expression (detected between day 3 and day 5) is X-linked, whereas at day 6 and day 7 it is mainly autosomal and may result from indirect effects of early X-linked gene expression imbalance. Different proliferation rates of male and female embryos may alternatively result from different metabolic activity between the two sexes. Some X-linked genes are related to amino acid turnover and transport, including solute carrier family 38 member 5 (*SLC38A5*), which transports asparagine, one of the amino acids differentially metabolized in female and male embryos (Picton et al., 2010); other X-linked genes, such as glucose-6-phosphate dehydrogenase (*G6PD*), are involved in the metabolism of glucose.

However, other studies have challenged the sex dependence of early human development as they did not confirm that male human pre-implantation embryos cleave at a faster rate (Csokmay et al., 2009; Richter et al., 2006), have a higher developmental potential (Ben-Yosef et al., 2012) or exhibit different kinetic parameters (Bronet et al., 2015; Serdarogullari et al., 2014) or inner cell mass and trophectoderm (TE) morphology (Alfarawati et al., 2011) compared with female embryos. Therefore, although the existence of sex-based difference in early embryonic development is an area of active debate, and thus requires further investigation, there is some indication of the differential expression of X-linked genes between early male and female human embryos, which raises the issue of dosage compensation.

X chromosome dosage compensation

Our knowledge of X chromosome dynamics in early development has mostly come from studies in mice, in which XCI is imprinted during the pre-implantation window and in extra-embryonic tissues that give rise to the placenta (Takagi, 2003). However, it has been known for decades that sex chromosome aneuploidies in humans have different consequences than those in mice. For example, mouse embryos with a supplementary copy of an X chromosome (XXX or XXY) die only when this supplementary chromosome is inherited from the mother (Xm) (Goto and Takagi, 1998). This has been interpreted as a resistance of the Xm to early XCI, which is acquired during the growth phase of the oocyte and involves a maternal-specific H3K27me3 imprint at *Xist* (Inoue et al., 2017). In contrast, humans with supernumerary X chromosomes survive and develop normally, even if the supplementary chromosome is maternally inherited (Skakkebaek et al., 2014). In both XXX females or XXY men (Klinefelter syndrome), only one X chromosome is active owing to XCI of all extra chromosomes (Tuttelmann and Gromoll, 2010), independent of the parental origin, thus arguing against imprinted XCI in humans. Taking

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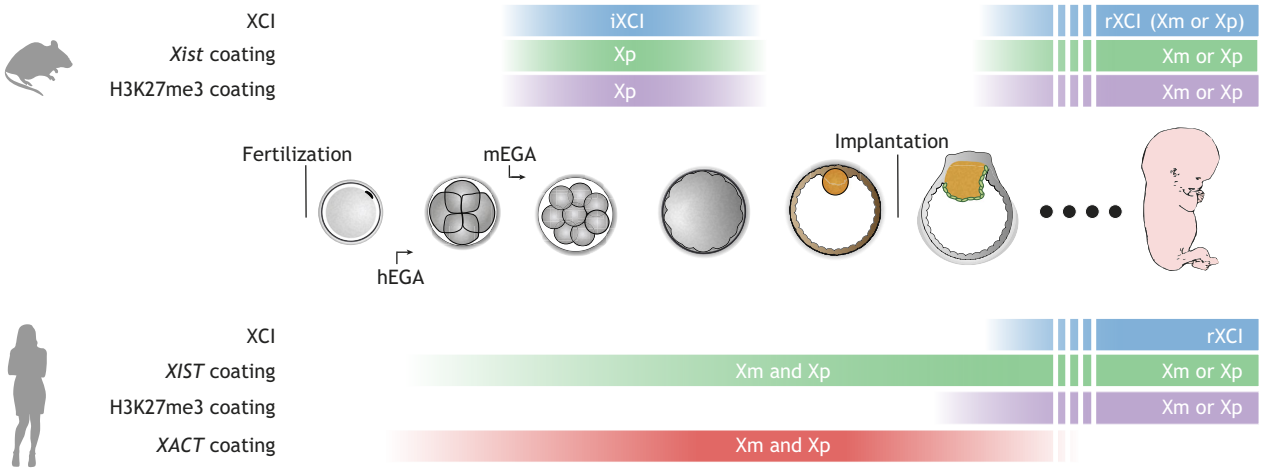

Fig. 1. Comparison of XCI timing and hallmarks between mouse and human early development. During female mouse pre-implantation development (top), X chromosome inactivation (XCI) is tightly coupled to *Xist* expression and occurs in two independent waves (blue and green, respectively). Imprinted XCI (iXCI), with systematic inactivation of the paternal X (Xp) is established rapidly in the mouse, following embryonic genome activation (EGA), and is maintained up to the formation of the early blastocysts, where iXCI is reversed in the cells of the inner cell mass (but maintained in the extra-embryonic lineage, not depicted). This is followed by random XCI (rXCI), in which either the maternal X (Xm) or the Xp is inactivated. Throughout mouse development, H3K27me3 patterns (purple) follow *Xist* coating on the X-chromosome (green). Human female pre-implantation development (bottom) unfolds without iXCI, despite expression of *XIST* upon EGA from both Xm and Xp. The X-linked *XACT* lncRNA is also expressed throughout pre-implantation development (red). H3K27me3 accumulation on the X chromosome does not follow *XIST* expression but correlates with the repression of *XACT*. rXCI initiates at the peri-implantation stage, with kinetics that remain to be determined (see text).

advantage of the large number of human X-linked single nucleotide polymorphisms (SNPs), analysis of X chromosome expression has shown that the term placenta is composed of relatively large clonal populations with either the paternal or the maternal X chromosome inactivated, suggesting random XCI in this organ (Moreira de Mello et al., 2010). This may explain why earlier studies performed on single placenta isolates, which may be composed of a clonal population of cells, concluded that XCI is completely skewed (Goto et al., 1997; Harrison, 1989; Ropers et al., 1978). However, recent data based on RNA sequencing and methylation analysis supports a slight bias towards inactivation of the paternal X chromosome in the human placenta (Hamada et al., 2016).

The analysis of pre-implantation embryos confirmed the lack of imprinted XCI and provided striking findings regarding dosage compensation in early human development. The initial observation that *XIST* is expressed from the maternal X chromosome in pre-implantation embryos and blastocysts further confirmed lack of paternal-specific XCI in humans (Daniels et al., 1997; Ray et al., 1997). This finding was later supported by analyses at the single cell level (Briggs et al., 2015; Okamoto et al., 2011; Petropoulos et al., 2016; Vallot et al., 2017). *XIST* expression starts from the four-cell stage (Fig. 1), coincident with the onset of EGA, and gradually increases in an asynchronous manner in individual blastomeres (Briggs et al., 2015). What is particularly striking is the observation that *XIST* is expressed in male embryos and from both X chromosomes in females (Okamoto et al., 2011; Petropoulos et al., 2016; Vallot et al., 2017). *In situ* studies revealed bi-allelic *XIST* coating in the majority (~85%) of cells of day 6 and day 7 female blastocysts (Fig. 2; Okamoto et al., 2011; Petropoulos et al., 2016; Vallot et al., 2017). In male embryos, ~60% of cells exhibit accumulation of *XIST* RNA on the single X chromosome in day 5 blastocysts and less than 10% at day 7 (Okamoto et al., 2011; Petropoulos et al., 2016). Notably, *XIST* expression is significantly higher in female than in male embryos (Moreira de Mello et al., 2017; Petropoulos et al., 2016).

Given the major contribution of *Xist* to XCI demonstrated in the mouse, this unusual pattern of *XIST* expression immediately raised questions regarding the activity of the X chromosomes during the pre-implantation period in humans. Analysis of selected X-chromosomal genes revealed bi-allelic expression in the morula and blastocyst (days 5 and 6) and a lack of H3K27me3 enrichment,

Box 2. Issues related to the use of human embryos

In contrast to mice, and with the exception of studies involving women undergoing elective hysterectomy (see Glossary, Box 1; Hertig et al., 1956), all observations on human embryo development come from embryos derived from *in vitro* fertilization (IVF). The evolution of bioethical laws authorizing, in many countries, research on human embryos to be performed under specific legal conditions has recently permitted increased research efforts into early human development. It is, however, important to remember that most IVF embryos have been conceived in a context of parental infertility, using potentially abnormal or immature gametes, and with assistance from several *in vitro* steps, including fertilization and the culturing of gametes and embryos. Human embryos are also characterized by a high level of aneuploidy, most of them originating from meiotic errors in oocytes (5-20% for oocytes), which is higher than in other species (0.5-1% for murine germ cells), and increases with maternal age (Nagaoka et al., 2012). All these parameters are likely to impact embryo quality per se. Another important issue is the relative inefficiency of human embryos to reach the blastocyst stage. Only up to 40-60% of cleaved embryos develop further into blastocysts *in vitro* (Glujovsky et al., 2016), a number similar to the 50% *in vivo* estimates (Hertig et al., 1956), which reflects the low fecundity rates of humans. Therefore, only few human embryos exhibit typical kinetics of development. Finally, in most basic research studies, embryos are not classified according to international guidelines used routinely in IVF practice, such as the ESHRE Istanbul consensus (see Glossary, Box 1; Alpha Scientists in Reproductive and ESHRE Special Interest Group of Embryology, 2011). They are rather described as 'typical' day-5 or day-6 embryos rather than with strict kinetic-morphological criteria, which occasionally leads to difficulties in defining their exact developmental stage. This imprecise morphological description may explain some discrepancies between studies.

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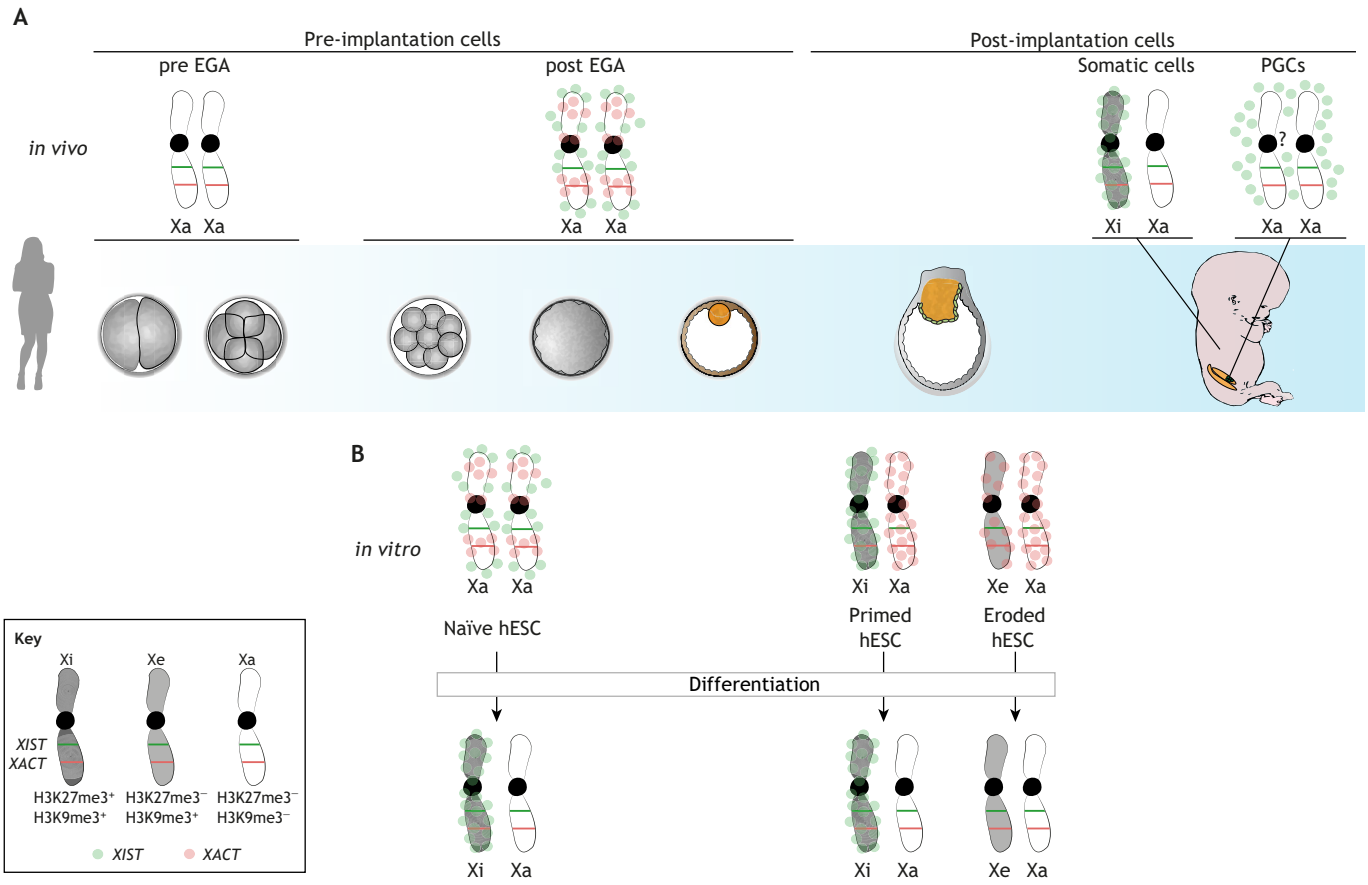

Fig. 2. The various flavors of X-chromosome activity during human development and in cellular models. (A) In pre-implantation embryos, *XIST* and *XACT* lncRNAs are expressed following embryonic genome activation (EGA) and co-accumulate on active X chromosomes (Xa). At these stages, *XIST* coating appears to be more diffuse than in differentiated cells. Following implantation, *XACT* expression is lost, *XIST* becomes mono-allelic in female cells and triggers XCI on one randomly chosen X (Xi); this status is thought to be stably maintained and propagated in somatic cells. Reversal of XCI is observed in primordial germ cells (PGCs), which maintain a certain level of *XIST* that is possibly not tethered to the X chromosomes. (B) *In vitro* pluripotent cellular models include human embryonic stem cells (hESCs) that can be maintained in naïve and primed states of pluripotency, which bear resemblances to pre- and post-implantation stages of *in vivo* development, respectively. In the naïve state, *XIST* and *XACT* may co-accumulate on two active X chromosomes, with *XIST* being more dispersed than in differentiated cells, similar to pre-implantation embryos. Of note, in naïve hESCs, *XIST* is often expressed from only one X chromosome. Primed hESCs are in a post-XCI state in which one chromosome is inactivated (Xi; coated by *XIST* and enriched in H3K9me3/H3K27me3 marks) and the other one is active (Xa; coated by *XACT*). These primed hESCs spontaneously undergo erosion of XCI, in which expression and coating of *XIST* and enrichment of H3K27me3 marks, but not H3K9me3 marks, are lost from the eroded X (Xe). Erosion of XCI initiates with coating of the Xe by *XACT* and leads to re-expression of a subset of genes from Xe. Differentiation of naïve cells triggers XCI on one chromosome, whereas differentiation of primed hESCs does not appear to alter their XCI status; although shuffling of epigenomic marks may occur (see text), eroded cells maintain their partially inactivated Xe (Mekhoubad et al., 2012; Vallot et al., 2015).

despite the accumulation of *XIST* (Okamoto et al., 2011). Bi-allelic expression of X-linked genes has since been confirmed at the chromosome-wide level and across pre-implantation development as early as EGA at day 3 (Zhou et al., 2019) and with the degree of bi-allelic X chromosome expression remaining stable from day 4 to day 7 (Petropoulos et al., 2016). When normalized to the total amount of X chromosome transcripts per embryonic cell, females, but not males, show a decrease in X chromosomal gene expression between the morula (day 4) and late blastocyst stages (day 7), which was interpreted as X chromosome dampening (Petropoulos et al., 2016). The model of X chromosome dampening was, however, questioned when different criteria for computational analysis of the data were applied (Moreira de Mello et al., 2017).

All of these observations indicate that human pre-implantation development proceeds in the absence of complete XCI and corresponds to a pre-inactive status (pre-XCI, Box 3 and Fig. 2), thus providing the first and only context known so far in which *XIST* accumulation is not correlated to XCI. This highlights the need to monitor multiple parameters and combine various approaches to assess the XCI status (Box 3) and raises questions as to the mechanisms and timing of XCI in humans.

Timing of XCI: when does it take place?

Owing to limitations in accessing relevant biological material, only few studies have so far addressed the timing of XCI. In an *in vitro* model for human implantation, H3K27me3 foci, characteristic of the Xi in female somatic cells, were observed in ∼25% of TE cells and in 7.5% primitive endoderm (PE) cells at day 8, but not in epiblast (EPI) cells (Teklenburg et al., 2012). It has also been proposed, based on the presence of H3K27me3, that one X chromosome is inactivated in 4-week somatic embryonic cells (Tang et al., 2015). XCI thus initiates between early implantation and the end of the first month of pregnancy, possibly in a progressive manner and with different kinetics depending on the cell lineage and its timing of specification. Because extended *in vitro* culture in humans has long been inefficient or not allowed for ethical reasons, this period of human development and the associated gene regulatory processes remained unexplored.

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Box 3. How to probe XCI status in human cells

Partial assessment of XCI status based on limited criteria may, and has in the past, lead to inaccurate conclusions. Cells can either be considered pre-XCI [containing two active X chromosomes (XaXa)] or post-XCI (once XCI has been initiated). Classical post-XCI cells carry one active X and one inactive X (XaXi). In hESCs however, XCI is unstable and thus post-XCI cells may harbor one active X and one partially reactivated 'eroded' X (XaXe). The following experimental strategies are used to determine the XCI status *in vivo* and *in vitro*.

X chromosome expression

The most robust criterion to infer XCI status is to monitor the transcriptional state of X chromosomal genes. Mono-allelic expression of X-linked genes is indicative of a post-XCI state, whereas bi-allelic expression suggests a pre-XCI state. However, 12-25% of human X-linked genes escape XCI (Carrel and Willard, 2005), resulting in their constitutive or stage/tissues-specific expression, even from the Xi. In addition, allelic expression (mono- versus bi-) has to be assessed at the single cell level because XCI is, in theory, random. X chromosome expression can be assessed using RNA-FISH, which provides visual information for discrete X-linked genes, independently of the presence of allelic SNPs. Using chromosome paint probes, RNA-FISH can also be used to monitor chromosome-wide expression, although the identity of the detected targets remains unknown (Vallot and Rougeulle, 2016). Alternatively, scRNA-seq simultaneously addresses the expression of all X-linked genes and, depending on the presence of informative polymorphisms, provides allele-specific information regarding the XCI status.

***XIST* expression**

XIST expression can be monitored using RNA-seq or RNA-FISH, the latter providing additional information regarding the distribution of the RNA at the single cell level (compaction of dispersed cloud versus pinpoint localization). However, in humans, expression and accumulation of *XIST* is not, per se, indicative of XCI.

Chromatin profiling

H3K27me3 marks the Xi in humans as it does in the mouse. However, as for *XIST*, presence or absence of this mark does not allow firm conclusions of pre- or post-XCI state. H3K9me3 appears to better discriminate post- from pre-XCI cells (Vallot et al., 2017), but this requires further investigation, notably in embryos. Histone marks can be monitored using immunofluorescence (IF) or chromatin immunoprecipitation followed by sequencing (ChIP-seq). DNA methylation of X-linked CpG islands is also a marker of human X-inactivation (Weber et al., 2007).

For routine analysis, RNA-FISH for selected X-linked genes, combined with *XIST* RNA-FISH and/or H3K27me3 IF is sufficient to distinguish between the various X chromosome states described so far.

However, the recent design of novel *in vitro* systems allowing human embryos to progress and organize beyond the blastocyst stage (Deglincerti et al., 2016; Shahbazi et al., 2016) will be helpful in lifting the veil on XCI initiation. Allele-specific expression of X-linked genes from day 6 to day 12 *in vitro* cultured embryos suggests that rXCI initiates during the implantation window, but is not fully completed by day 12 (Zhou et al., 2019). More studies will be needed to ascertain these observations.

X chromosome reactivation in the germline

The second round of whole-genome reprogramming occurs in the germline. The X chromosome is particularly concerned as the Xi is reactivated in the female germline. Although germline X chromosome reactivation (XCR) has been substantially studied in mice (Chuva de Sousa Lopes et al., 2008; de Napoles et al., 2007; Sugimoto and Abe, 2007), its timing in humans is still unclear.

PGCs are the precursors of mature gametes – the sperm and oocytes in mammals. Using global analysis of X chromosome expression and allelic investigation of selected genes, of which some were known to escape XCI, it was initially concluded that XCR has already taken place in the earliest female human PGCs (hPGCs) analyzed, at 4 or 5.5 weeks after fertilization (Guo et al., 2015; Li et al., 2017). Intriguingly however, the total expression level of X-linked genes in female PGCs is less than twofold that of males between 4 and 11 weeks of development (Guo et al., 2015). This could indicate either a global increase of the single X chromosome expression in male PGCs, a decrease in activity of the two X chromosomes in females ('dampening'), or a combination of both. The conclusion that XCR has fully occurred in 4-week PGCs has later been challenged and a more heterogeneous pattern, with ∼30% of hPGCs at 4-9 weeks still exhibiting incomplete XCR, was reported (Vértesy et al., 2018). This is consistent with some of these early hPGCs displaying a faint but characteristic perinuclear spot of H3K27me3, indicative of XCI (Vértesy et al., 2018).

Together, these results show that XCR in hPGCs is a heterogeneous and complex process, starting from 4 weeks of development onward and occurring in an asynchronous manner. This correlates with the global wave of DNA demethylation, which is already underway in 4-week PGCs (Guo et al., 2015; Tang et al., 2015). The onset of XCR appears to be related to the transcriptional signature of the cell rather than the fetal age (Vértesy et al., 2018). Importantly, *XIST* is expressed in the male and female human germline independently of the XCR status and at all stages analyzed (Gkountela et al., 2015; Vértesy et al., 2018); *XIST* is also expressed in oocytes, albeit to a lower extent (Daniels et al., 1997; Ernst et al., 2018). It remains to be determined whether *XIST* accumulates around the X chromosomes in hPGCs but, in any case, such widespread *XIST* expression is not associated with H3K27me3 enrichment on the X chromosomes (Tang et al., 2015). Altogether, these observations suggest that, similar to pre-implantation development, the activity status of the X chromosome in the female human germline does not depend on the presence of *XIST*, but rather on its ability to trigger chromosome silencing, although the underlying mechanisms still remain elusive. The development of protocols to generate PGC-like cells from pluripotent stem cells (PSCs) (Irie et al., 2015; von Meyenn et al., 2016) opens new perspectives for studying XCR in the germline.

PSCs as a cellular model for investigating XCI

Even though recent advances have been made regarding XCI in human development using pre-implantation embryos, the paucity of such material and, more importantly, the associated ethical issues prevent their extended use. PSCs, which possess the formidable capacity of surviving quasi indefinitely in culture, provide a promising *ex vivo* counterpart to these early developmental stages, and thus stand as the ultimate cellular model for human XCI studies. Indeed, mouse PSCs have been instrumental for deciphering features, mechanisms and regulators of XCI in rodents. Two main types of PSCs exist: embryonic stem cells (ESCs), which are directly derived from embryos, and induced pluripotent stem cells (iPSCs), which are obtained through *in vitro* reprogramming of differentiated cells. Because the question of whether reprogramming of differentiated cells to iPSCs is accompanied by XCR in human as it has been shown in the mouse (Maherali et al., 2007) is still debated (Talon et al., 2019), we have chosen to only discuss ESCs.

Human ESCs in a pre-XCI status

Analogous to mouse ESCs, initial reports on female undifferentiated human ESCs (hESCs) showed that they carry two active X chromosomes (Xa; see Glossary, Box 1) that do not express

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XIST ($Xa^{XIST-}Xa^{XIST-}$) (Dhara and Benvenisty, 2004). The activity of both X chromosomes is stabilized when cells are derived in hypoxia (Lengner et al., 2010), although the impact of oxygen concentrations is under debate (Patel et al., 2017). However, it rapidly transpired that the X chromosome status of hESCs is far more complex than initially thought, with various patterns found between, and even co-existing within, hESC lines and cell populations (Hoffman et al., 2005; Shen et al., 2008; Silva et al., 2008; Vallot et al., 2015). In addition, the ability of $Xa^{XIST-}Xa^{XIST-}$ hESCs to undergo XCI upon differentiation is now questioned; instead, the X chromosome status of most undifferentiated hESCs is maintained during differentiation (Patel et al., 2017). The reason for this discrepancy is unknown, but may be linked both to the criteria used to define the XCI status (Box 3) as well as to the heterogeneous nature of the starting population, with differentiation selecting one population (*XIST*+) over another (*XIST*–). Nevertheless, studies in pre-implantation embryos have now established that the pre-XCI state in humans is $Xa^{XIST+}Xa^{XIST+}$ (Okamoto et al., 2011; Petropoulos et al., 2016). This has launched the race to define hESC culture conditions that sustain a pre-implantation-like state *in vitro*.

Pluripotency (see Glossary, Box 1) exists in multiple 'flavors', including naïve and primed (Davidson et al., 2015; Nichols and Smith, 2009), which correspond to distinct developmental stages, pre-implantation inner cell mass and post-implantation epiblast, respectively. These two states of pluripotency can be distinguished by several metabolic and molecular features, such as their dependency on signaling pathways and their transcriptional signatures, including those emanating from transposable elements (Theunissen et al., 2016). Epigenetic characteristics such as the levels and distribution of DNA methylation also define naïve and primed pluripotent states. The X chromosome activity status is another hallmark – and as we will see, a robust one – of the various pluripotent states, with naïve pluripotency being characterized by a pre-XCI state and primed pluripotency by a post-XCI state (Fig. 2). Several methods and culture formulations have been defined to sustain naïve pluripotency *in vitro*, with variable outputs (reviewed by Collier and Rugg-Gunn, 2018). Confusion regarding X chromosome activity in these different settings has arisen from partial assessment of XCI and from using inappropriate criteria (i.e. *XIST* expression). In addition, some culture conditions are successful in inducing several features of naïve pluripotency but not in resetting the X chromosome status, suggesting that XCR is a late event in the process (Sahakyan et al., 2017b). There is therefore an intimate connection between XCI and naïve pluripotency: assessment of X chromosome activity is a powerful tool to define 'truly' naïve cells and, conversely, naïve PSC are instrumental to study early stages of XCI. So far, two main culture formulations referred to as 5iLA (Theunissen et al., 2016, 2014) and t2iLGö (Takashima et al., 2014) are compatible with a pre-XCI status (Guo et al., 2017; Sahakyan et al., 2017b; Vallot et al., 2017), as defined by bi-allelic expression of X-linked genes. The accumulation of *XIST* on active X chromosomes is another hallmark of pre-XCI status, with the pattern of *XIST* accumulation in those cells being qualitatively similar to that of early embryos and more diffuse compared with post-XCI cells (Fig. 2; Sahakyan et al., 2017b; Vallot et al., 2017). However, the true equivalence of naïve hESCs to *in vivo* pre-implantation stages is questionable, notably as *XIST* is mostly expressed from only one, and rarely two X chromosomes (Sahakyan et al., 2017b; Vallot et al., 2017). Moreover, conflicting results have been obtained regarding other hallmarks of XCI, notably H3K27me3. Although H3K27me3 was found to be enriched on active X chromosomes decorated by *XIST* in some studies (Sahakyan et al., 2017b), others revealed a lack of accumulation of heterochromatin marks (H3K27me3 and H3K9me3) on *XIST*-expressing Xa in naïve cells (Vallot et al., 2017), similar to embryos (Okamoto et al., 2011). Therefore, further analysis is required to fully assess the chromatin landscape of *XIST*-coated active X chromosomes in humans.

The fact that naïve hESCs capture, to some extent, the pre-XCI status offers, in theory, a unique opportunity to assess the initiation of human XCI, which has so far remained elusive. A direct transition from $Xa^{XIST+}Xa^{XIST-}$ to $Xi^{XIST+}Xa^{XIST-}$ has been reported (Guo et al., 2017), whereas another study described an intermediate $Xa^{XIST-}Xa^{XIST-}$ stage (Sahakyan et al., 2017b), which is analogous to the initial observation of $Xa^{XIST-}Xa^{XIST-}$ hESCs. It remains to be determined whether the latter exists in developing embryos. Moreover, differentiation of naïve cells obtained from post-XCI cells results in biased XCI, with the original Xi always inactivated (Sahakyan et al., 2017b). This indicates that a memory of the previous inactivation status is left, again questioning the true naivety of these cells. This issue might not apply to blastocyst-derived naïve hESCs, which are essentially deprived of XCI memory, and in which $Xa^{XIST+}Xa^{XIST+}$ might be stabilized more efficiently (Sahakyan et al., 2017b).

What can we learn from primed hESCs?

Although a cellular model that confidently mimics XCI establishment is still lacking, primed hESCs, in which XCI has already occurred, might still be informative to understand the early stages of XCI. Several pieces of evidence suggest that the post-XCI status of primed hESCs is distinct from that of differentiated cells. The most obvious feature is the instability of the inactive state in primed hESCs, with several hallmarks of XCI being spontaneously lost upon passages, a phenomenon that has never been reported in any other differentiated cells in culture, potentially with the exception of some, but not all, cancer cells (Bar et al., 2019; Chaligné et al., 2015). This erosion of XCI is characterized by disappearance of *XIST* expression and partial reactivation of the Xi (Fig. 2; Mekhoubad et al., 2012; Vallot et al., 2015). The X chromosome that underwent erosion is referred to as eroded X (Xe; see Glossary, Box 1). It appears that not all genes are similarly susceptible to XCI erosion and, in the absence of *XIST*, gene silencing might be more efficiently maintained in some regions compared with others (Bar et al., 2019; Patel et al., 2017; Vallot et al., 2015). The pattern that emerges from independent analyses is that of a core domain flanking the centromere being resistant to XCI erosion, whereas the middle parts of both short and long chromosome arms are more prone to XCI instability (Bar et al., 2019; Patel et al., 2017; Vallot et al., 2015). The reason for this is unclear, but it might be linked to the organization of the Xi chromatin into distinct territories. There is indeed a correlation between susceptibility to erosion and the pattern of histone marks, with regions normally enriched in H3K27me3 being preferentially reactivated over H3K9me3-marked regions (Vallot et al., 2015). This is probably linked to the fact that H3K27me3 is dependent on *XIST* and lost from the Xi in eroded cells, whereas H3K9me3 is maintained (Vallot et al., 2015). In addition, erosion of XCI is characterized by partial promoter CpG demethylation (Nazor et al., 2012; Patel et al., 2017; Shen et al., 2008). Of note, the degree of XCI instability might depend on the culture conditions (personal observations, J-F.O. and C.R.). In summary, although XCI erosion is a culture artefact with seemingly no equivalence and/or relevance in normal development (Bar et al., 2019), it likely reflects peculiar and stage-specific Xi features. In agreement with this hypothesis,

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the chromatin organization of the Xi was found to distinguish pluripotent from differentiated cells (Vallot et al., 2015, 2016). In post-XCI hESCs, H3K9me3 and H3K27me3 chromatin marks are mutually exclusive and anti-correlated (Vallot et al., 2015). Intriguingly, such bimodal organization of the Xi is also observed in immortalized cells on both metaphase (Chadwick and Willard, 2004) and interphase chromosomes (Chadwick, 2007; Nozawa et al., 2013; Vallot et al., 2016). In contrast, there is significant overlap of H3K9me3 and H3K27me3 in primary differentiated cells (Vallot et al., 2015), thus enhancing XCI stability in a synergistic manner. This is in agreement with previous studies that have shown the redundancy of the multiple layers of epigenetic modifications that ensure stable maintenance of XCI (Csankovszki et al., 2001).

Even if a potential impact of cell culture in remodeling the epigenomic landscape of the X chromosome cannot be excluded, these observations may reflect a multistep establishment of the Xi chromatin landscape. Future studies should also address the presence of additional histone marks and variants that are normally enriched on the Xi, such as H4K20me1, H2AK119Ub and macroH2A.

Molecular control of human XCI

Contribution of XIST to human XCI

Xist has been established as the trigger of XCI in mice (Penny et al., 1996). Although it is implicitly acknowledged that *XIST* exerts similar functions in all species, functional proof for this hypothesis is limited. Initial attempts to understand the function of human *XIST* relied on the introduction of *XIST* genomic and inducible cDNA transgenes on autosomes into various cell types of different origin: mouse ESCs, human adult male cancer cell lines and on one of the three chromosomes 21 in iPSCs derived from Down syndrome patients. In all cases, the ectopic *XIST* RNA is able to spread and to induce some level of silencing in *cis*, albeit to a different extent (Chow et al., 2007; Hall et al., 2002; Heard et al., 1999; Kelsey et al., 2015). In transgenic mESCs, silencing of autosomal genes occurs only upon differentiation, and only in a fraction of cells in cell lines with high transgene copy numbers, even if *XIST* is already expressed in undifferentiated cells (Heard et al., 1999). Autosomal integration of a *XIST* genomic or cDNA transgene in human fibrosarcoma cells also leads to silencing of a nearby introduced reporter, and to global chromatin and transcriptional reorganization of the transgenic chromosome (Chow et al., 2007; Hall et al., 2002), in a manner that depends on the integration site (Kelsey et al., 2015). *XIST* cDNA transgene induction in Down syndrome cells efficiently triggers silencing of the entire supernumerary chromosome 21, at least when *XIST* is induced in a pluripotent context, leading to an almost normal disomic level of expression for genes on chromosome 21 (Jiang et al., 2013). More recently, loss-of-function approaches have been undertaken, in which the whole *XIST* sequence or parts of *XIST* have been deleted in post-XCI cells, such as female embryonic or cancer cells (Lee et al., 2019; Lv et al., 2016). All the deletions that result in loss of *XIST* expression lead to some degree of XCR, questioning the idea that *XIST* is dispensable for XCI maintenance (Brown and Willard, 1994). Taken together, these pieces of evidence suggest that human *XIST* is central to XCI, although the ultimate demonstration of *XIST* function in a more physiologically relevant context, such as naïve hESC differentiation, which recapitulates the initiation of XCI, is still lacking.

Analysis of XCI in hESCs has also pointed to the existence of *XIST*-independent silencing events; signs of X chromosome re-inactivation have been detected upon differentiation of eroded hESCs, in the absence of *XIST* re-expression. For example, although hESCs with eroded XCI display two X chromosome transcription territories, only one persists upon differentiation, and this is accompanied by re-silencing of individual X-linked genes (Vallot et al., 2015). Analysis of differentiated derivatives of hPSCs also showed that monoallelic expression of the X chromosome is expanded during differentiation, independently of *XIST* (Bar and Benvenisty, 2019). In all of the above cases, re-silencing of the Xe is only partial (Bar and Benvenisty, 2019; Mekhoubad et al., 2012; Patel et al., 2017; Vallot et al., 2015). Although the exact mechanisms of re-silencing are still elusive, the previous accumulation of *XIST* appears to have left a memory of the inactive state, likely at the chromatin level, that is not sufficient to maintain silencing of some X-linked regions in pluripotent cells, but which could be re-established upon differentiation. It is possible, for example, that some chromatin marks such as H3K9me3, which are left intact on the Xe in eroded cells but confined to specific domains of the X chromosome, would spread to flanking regions in the 2D or 3D space upon pluripotency exit and facilitate local repression. Assessment of the chromatin organization of the Xe in differentiated cells should help resolve this issue.

Uncoupling *XIST* from XCI

Human early embryogenesis, as studied in naïve hPSCs, provides an unprecedented context in which *XIST* expression is uncoupled from regular XCI. This implies that, in contrast to mice, in which *Xist* accumulation systematically triggers XCI, *XIST* silencing activity is tightly regulated in humans. The mechanisms involved are currently unknown, but several, non-mutually exclusive hypotheses can be proposed (Fig. 3).

First of all, it is still unclear whether *XIST*, despite accumulating to some extent in the nucleus of pre-implantation embryos and naïve hESCs, is truly recruited to X chromosomes. Indeed, the pattern of *XIST* accumulation is more dispersed in these contexts compared with post-XCI cells (Sahakyan et al., 2017a; Vallot et al., 2017), which is reminiscent of observations in mouse/human cell hybrids in which *XIST* spreads out on an active human X chromosome (Clemson et al., 1998). However, whether this is because of loose contact with the X chromosome or normal interaction with a less compacted active X chromosome is to be determined. Investigating *XIST* distribution relative to the X chromosome territories should help solve this issue.

Another possible explanation for X chromosome activity in the presence of *XIST* involves the absence of *XIST* effectors, i.e. factors interacting with *XIST* and mediating its silencing activity. Candidate and unbiased biochemical RNA-centric approaches, as well as genetic screens, have, over the years and mostly in mice, led to the identification of a myriad of factors that interact directly with *Xist* and/or contribute to the capacity of *Xist* to induce XCI (Chu et al., 2015; McHugh et al., 2015; Minajigi et al., 2015; Moindrot et al., 2015; Monfort et al., 2015). Among the latter are transcriptional co-repressors (e.g. SPEN), nuclear matrix or compartmentalization proteins (e.g. LBR and HNRNPU) and members of RNA modification complexes (e.g. RBM15 and WTAP), which target *XIST* RNA itself; however, this list is far from complete. Other *XIST* partners, such as HNRNPU, are important for *Xist* RNA localization and association with the X chromosome territory (Hasegawa et al., 2010; Yamada et al., 2015). All these factors contact *Xist* on more or less specific subdomains of the RNA, notably key repeat regions such as the A-repeat, which is indispensable for *Xist* silencing activity (Wutz et al., 2002). Of course, it remains to be determined whether the functions of the murine interactors of *Xist* in the X-inactivation process are conserved in humans. To allow

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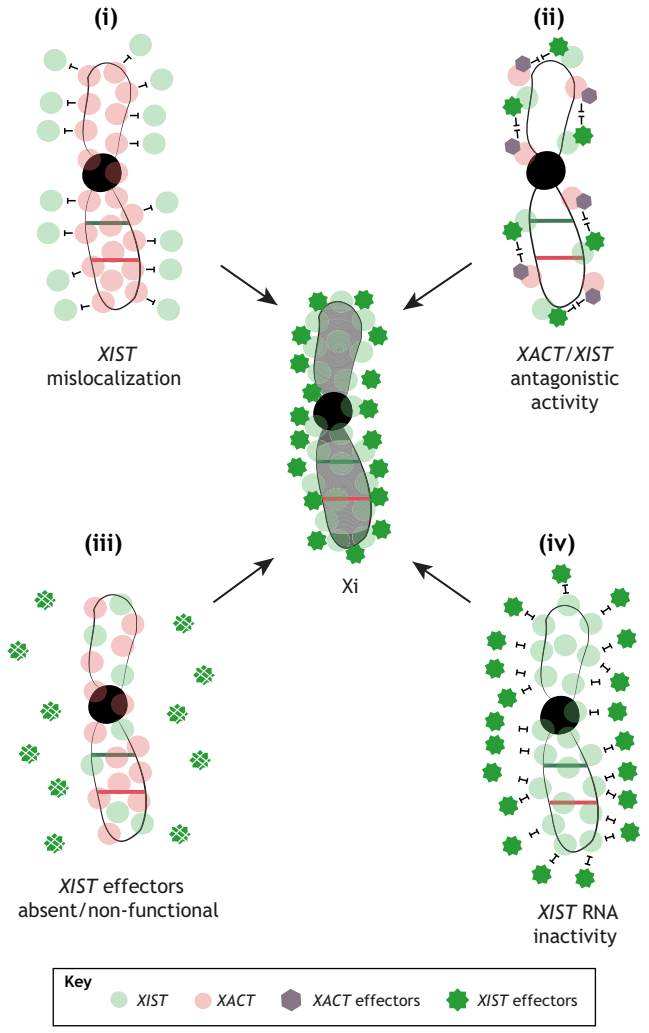

Fig. 3. Different scenarios for uncoupling *XIST* accumulation from XCI during early human development. During pre-implantation human embryonic development, *XIST* coating of X chromosomes does not trigger XCI. Multiple non-exclusive scenarios could explain this human-specific uncoupling. Induction of XCI could be prevented because of improper *XIST* localization/tethering to the X-chromosome (i), which may or may not be *XACT*-dependent. *XIST* activity could also be prevented by the action of *XACT* effectors, which could antagonize *XIST* effectors (ii). Alternatively, *XIST* effectors may not be expressed/active during these stages of development (iii). Finally, *XIST* might not be able to recruit its effectors because of defective processing such as mis-splicing or editing (iv). Whatever the scenario at stake in pre-implantation embryos, *XIST* coating eventually triggers X-chromosome silencing following implantation.

X-chromosome activity in the presence of *XIST*, these XCI effectors may be transiently non-functional, or unable to localize to the X chromosome, possibly because of impaired interaction with *XIST*. This could be owing to specific patterns of post-translational modification of *XIST* partners, post-transcriptional modification of *XIST* RNA or to alternative splicing events that would splice out crucial interacting regions of *XIST*. In this context, it is of interest to emphasize the lack of H3K27me3 enrichment on the X chromosome in early embryos, as *XIST* accumulation and H3K27me3 enrichment have so far been entirely correlated.

In a second scenario, as yet unknown factors might prevent *XIST* from silencing the X chromosome. An interesting candidate is the lncRNA X-active coating transcript (*XACT*; see Glossary, Box 1). *XACT* is an X-linked transcription unit of more than 250 kb, located 50 Mb distal from the *XIST* gene (Fig. 2), which was discovered in humans and is conserved in hominoids, but not in lower apes or in more distantly related species (Vallot et al., 2013). The most striking feature of *XACT* is the nuclear distribution of the *XACT* RNA, which decorates active X chromosomes (Vallot et al., 2013). *XACT* was originally identified in primed PSCs, in which *XACT* enrichment on the Xa mirrors the accumulation of *XIST* on the Xi (Vallot et al., 2013). In embryos, *XACT* expression starts from the four-cell and the eight-cell stage, concomitantly to *XIST* (Petropoulos et al., 2016; Vallot et al., 2017) (Figs 1 and 2). It accumulates on both active X chromosomes, together with *XIST*, until the late blastocyst stage (expanded hatching blastocyst), where its expression varies according to the cell fate: *XACT* expression is maintained in EPI and PE cells, but tends to be shut down in future TE cells, whereas *XIST* remains expressed. *XACT* is also expressed from both X chromosomes together with *XIST* in naïve hESCs (Fig. 2). In both contexts, *XACT* and *XIST* occupy distinct spatial domains and *XACT* could hamper efficient *XIST* spreading and accumulation along the X chromosome, in agreement with its dispersed pattern. Alternatively, *XACT* could prevent the interaction between *XIST* and its effectors.

Regulation of *XIST* expression: the human *XIC*

The distinct dynamics of *XIST* expression during early embryogenesis in humans and mice suggest that its regulatory apparatus has diverged substantially across evolution. However, the study of chromosomal rearrangements in both species pointed to a major contribution of the *XIC*, a domain of the X chromosome encompassing *XIST* and multiple noncoding genes (Fig. 4) (Augui et al., 2011). Several of these elements have been functionally implicated in the XCI process in mice, mostly through the regulation of *Xist* expression. The mouse *Xic* is physically and spatially organized in two topologically associated domains (TADs; see Glossary, Box 1) (Nora et al., 2012), one that contains elements that favor XCI, including *Xist*, and the other containing repressors of *Xist* and/or XCI (Fig. 4). The fact that the *XIST* ON state appears as the default one (Petropoulos et al., 2016; Vallot et al., 2017) raises questions as to the functional conservation of the repressors, at least in the early stages of embryonic development. Among these repressors is *TSIX*, the *XIST* antisense RNA. *Tsix* transcription in mice spans across the entire *Xist* gene and prevents *Xist* upregulation in *cis* (Lee and Lu, 1999). *Tsix* acts, at least in part, by triggering chromatin remodeling across the *Xist* promoter region, in a manner that depends on *Tsix* transcription, but not on *Tsix* RNA (Navarro et al., 2006, 2005; Sado et al., 2005). A *Tsix* ortholog has been described in humans, but similarities to the mouse counterpart in terms of sequence and expression patterns are limited (Migeon et al., 2001, 2002), suggesting that it does not participate in *XIST* regulation. The lack of a *Tsix* equivalent in humans is likely to be linked to the absence of imprinted XCI in this species.

Among the *Xic*-linked *Xist* activators that have been characterized in mice are the two noncoding genes *Jpx* and *Ftx*, which lie 5 and 150 kb, respectively, upstream of *Xist*, within the *Xist* TAD (Fig. 4). Although both contribute to promoting *Xist* accumulation, they do so through distinct mechanisms. *Jpx* RNA controls *Xist* expression in *trans* (Sun et al., 2013; Tian et al., 2010), whereas *Ftx* acts in a transcription-dependent manner to promote *Xist* expression in *cis* (Furlan et al., 2018). In contrast to *Jpx*, *Ftx* RNA is mainly dispensable for XCI initiation (Furlan et al., 2018). Both *Jpx* and *Ftx* have orthologs in humans, and are part of the same *TAD* as *XIST*, but their role has remained elusive. Single cell RNA sequencing

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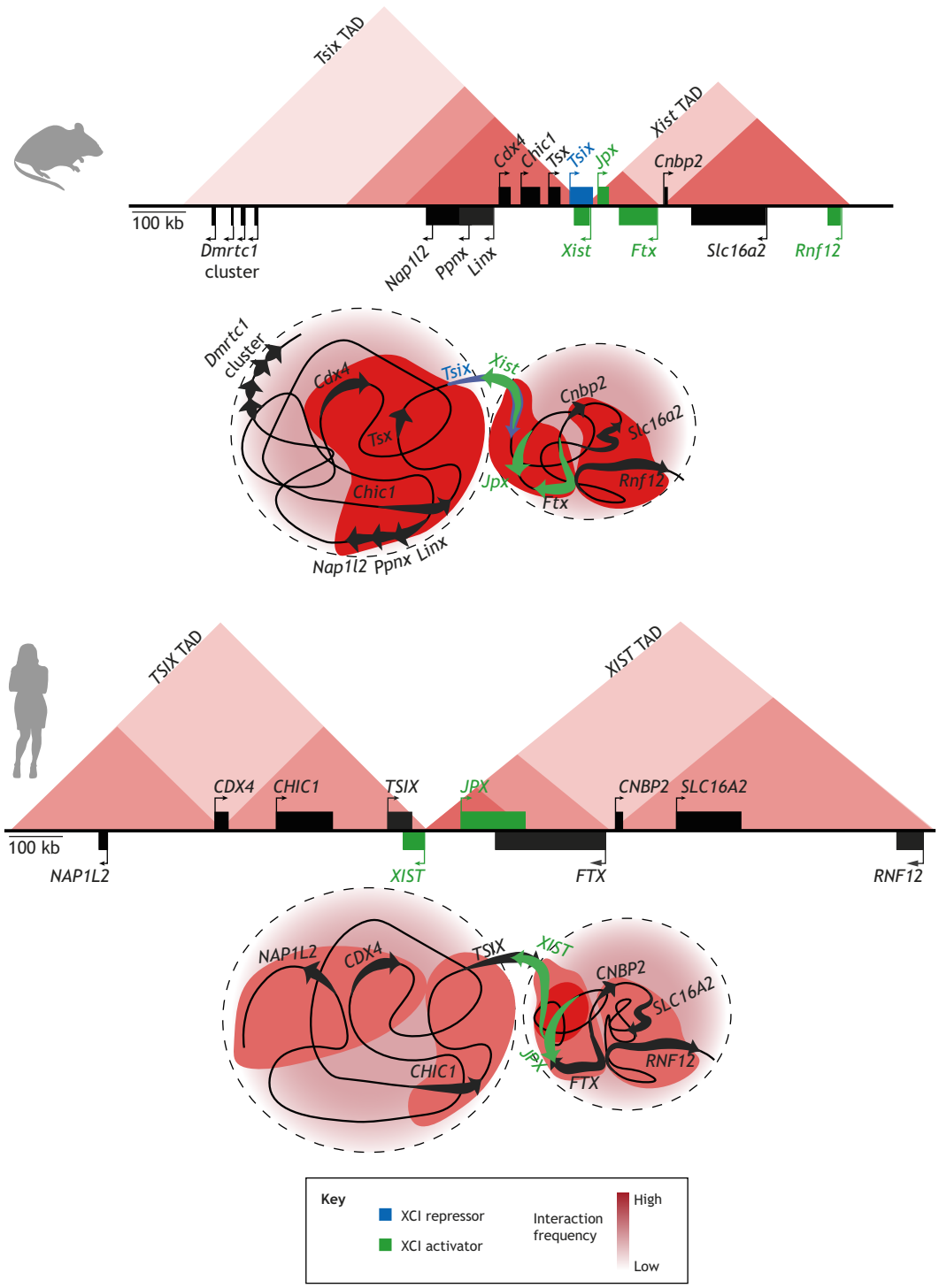

Fig. 4. X-inactivation center in mouse and human. The X-inactivation centers (*Xic*) of mice (top) and humans (bottom) are organized in two conserved topologically associated domains (TAD) that segregate the *cis*-interacting DNA elements within the *XIC*. In this representation, the intensity of the red color correlates with the frequency of the interaction between the connected DNA elements. Positive and negative regulators of XCI are segregated on the chromosome and in space through constrained interactions within the *XIST* and *TSIX* TADs, respectively. Although the overall organization (linear and 3D) is conserved between mouse and human, it is notable that fewer interactions are detected within the human *TSIX* TAD compared with the mouse *Tsix* TAD. This observation could reflect the absence of a *TSIX* repressive regulatory function on *XIST* expression in humans (see text).

(scRNA-seq; see Glossary, Box 1) datasets from human pre-implantation embryos revealed that, within the *XIC*, *JPX* is the earliest gene to be activated, before *XIST* (Rosspopoff et al., 2019 preprint). Functional investigation in naïve and primed hESCs further showed a major contribution for *JPX* in *XIST* expression (Rosspopoff et al., 2019 preprint). However, unlike its mouse counterpart, it is *JPX* transcription, and not RNA, that regulates *XIST* expression in humans (Rosspopoff et al., 2019 preprint). This points to an unanticipated functional plasticity of orthologous lncRNA genes involved in XCI.

Altogether, it appears that, although XCI in humans is as crucially dependent on *XIST* as it is in mice (and likely in all eutherians), the *XIST* regulatory network might have diverged substantially during evolution. There are, however, many questions that remain to be addressed regarding the contribution of conserved and species-specific elements, notably noncoding genes, to XCI in humans.

Conclusion and future perspectives

Fundamental research on human embryos has only begun, yet it has already revealed unanticipated differences in XCI compared with the mouse. There are, however, still many more questions unsolved than answers provided, in particular regarding the timing of XCI initiation and reversion, the precise sequence of molecular events, the elements that control the capacity of *XIST* to induce silencing, and the *XIST* regulatory network. There is no doubt that a combination of models and approaches will be required to progress our understanding of this key epigenetic regulatory process. Mimicking embryo implantation by co-culture or expanding the repertoire of models, for example to study embryo development in a 3D environment, would undeniably be a major asset. In addition, research on XCI will benefit from the improvement of culture conditions that derive and maintain karyotypically stable and truly naïve hESCs. This will allow researchers to decipher the onset of X chromosome silencing, with the important parameter that *in cellulo* studies need, as much as possible, to be validated in embryos. Deciphering common themes and species-specificity of human XCI will impact a plethora of research fields including human embryonic development, stem cell biology, lncRNAs and X-linked diseases. Finally, on a more general note, the study of human XCI will contribute to our knowledge of the plasticity of regulatory networks and mechanisms in evolution, a key aspect to understand the long-sought articulation between genotype and phenotype.

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Competing interests

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