[Epigenetics 4:1, 1-4; January 2009]; ©2009 Landes Bioscience

Point of View

Epigenetic regulation of X-inactivation in human embryonic stem cells

Tamar Dvash and Guoping Fan*

Department of Human Genetics and the Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research; David Geffen School of Medicine; University of California at Los Angeles; Los Angeles, California USA

Abbreviations: XCI, X chromosome inactivation; mESCs, mouse embryonic stem cells; hESCs, human embryonic stem cells; ICM, inner cell mass; IVF, in vitro fertilization; FISH, fluorescent in situ hybridization

Key words: X-inactivation, xist, embryonic stem cells, hESC, ICM, DNA methylation, epigenetics

X chromosome inactivation (XCI) allows dosage compensation of the expression from sex chromosome in mammalian female cells. Although this mechanism is extensively studied in the mouse model organism, the corresponding mechanism during human development is largely unknown. The generation of human embryonic stem cells (hESCs) provides an invaluable tool to address early embryogenesis in humans. Even though hESCs were supposed to shed light on the XCI process in early human embryogenesis, previous studies largely indicated inconsistency in the status of XCI in these cells. Recently, new data suggested that in vitro culture might affect epigenetic mechanisms such as XCI. In this review we will present the existing data regarding XCI variations in hESC as compared to data from the mouse embryo and embryonic stem cells. We will also suggest possible explanations for the conflicting observations in the literature regarding XCI in hESCs.

Introduction

In mammals dosage compensation of the sex chromosome is achieved through X chromosome inactivation (XCI) in the female cells. Our understanding of the initiation and maintenance of XCI in development is mainly based on studies of mouse embryos and embryonic stem cells (mESCs). During XCI in female cells the two X chromosomes are distinguished by a series of genetic and epigenetic processes such as differential transcription, methylation, histone modifications, etc. XCI is initiated in a specific region of the X chromosome named X-inactivation center (Xic). *Xist*, a gene within the Xic, is expressed solely from the inactivated X (Xi) chromosome and is crucial for XCI since its non coding RNA directly interact with and eventually coats, in cis, the X chromosome to be inactivated (reviewed in refs. 1–3). During mouse embryogenesis or in vitro differentiation of female mESCs, *Xist* expression is being negatively regulated by its antisense gene *Tsix* through chromatin modifications.[4,5] Consequently, in the active X chromosome (Xa), expression of *Tsix* prevents the expression of *Xist* and the inactivation process. In human cells, *XIST* is postulated to play an equally important role in the initiation and maintenance of XCI as its counterpart in mouse cells. However, the exact timing and regulation of XCI in human development is still not fully understood.

In the last few years several research groups tried to address the question of XCI in human females with human embryonic stem cells (hESCs). However, the data regarding XCI in hESCs is not as consistent as the reported data for mESCs. In this paper we will describe these findings and try to synthesize different points of views into one framework concerning X chromosome inactivation in female hESCs.

*Correspondence to: Guoping Fan; Department of Human Genetics and the Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research; David Geffen School of Medicine; University of California at Los Angeles; 695 Charles Young Drive South; Los Angeles, California 90095 USA; Tel.: 310.267.0439; Fax: 310.794.5446; Email: gfan@mednet.ucla.edu

Submitted: 09/30/08; Accepted: 11/17/08

Previously published online as an *Epigenetics* E-publication:
http://www.landesbioscience.com/journals/epigenetics/article/7438

X Chromosome Inactivation in the Mouse Embryo and mESCs

XCI is under dynamic developmental control in the female mammalian embryo. In the female zygote both X chromosomes (maternal and paternal) are active. However, upon first cleavage the paternal X chromosome is being inactivated.[6,7] Reactivation of the X chromosome occurs in the blastocyst stage embryo where the paternal X chromosome is reactivated exclusively in the inner cell mass (ICM) cells, whereas in the extra-embryonic tissue the paternal X chromosome remains inactive.[7,8] Both X chromosomes remain active in the epiblast between embryonic days 3.5 to 5.5, then, just before gastrulation XCI is reestablished in the developing mouse embryo in a random pattern.[9] From this stage on, all somatic cells maintain one active and one inactive X chromosome throughout embryonic development and adulthood. In primordial germ cells reactivation of the X chromosome occurs either in E11.5–E13.5 and maintained in the germ cells,[10] or earlier when the PGCs migrate through the hindgut.[11] Female mouse embryonic stem cells, which are derived from the ICM of a blastocyst stage embryo, serve as an authentic in vitro model for the XCI phenomenon in vivo. At the undifferentiated state mESCs show two active X chromosomes, as predicted from studies on the mouse embryos. During in vitro differentiation the random pattern of XCI is recapitulated and maintained as seen in mouse embryos, by *Xist* expression and coating of inactive X (Xi).[12] In fact in mouse development and consequently in mESCs there is correlation between loss of pluripotency (the transition from ICM to gastrulation embryo or undifferentiated mESCs to differentiated cells) and XCI. A recent study suggests a molecular basis for

the coupling of *Xist* regulation and pluripotency. It is shown that in mESCs pluripotency factors such as Oct3/4, Nanog and Sox2 bind *Xist* intron 1. Moreover synergistic binding of these factors to *Xist* intron 1 results in downregulation of *Xist* expression independently of *Tsix* expression.[13] This might provide an explanation for the correlation between the coupled initiation of XCI and differentiation in the mouse.

X Chromosome Inactivation in Human Embryonic Stem Cells

Knowledge regarding XCI in human development is quiet limited and mainly relies on postulations from the mouse, from limited studies of human IVF embryos,[14,15] and recently from several studies with human embryonic stem cells (hESCs). Although human and mouse share essential developmental characteristics major differences between the two species still exist. While XCI in the mouse was shown to be random in the ICM and paternally imprinted in the extra-embryonic tissues, it is believed that in humans XCI is random in both embryo proper and extra-embryonic tissues.[16] Differences also exist in the sequences of the X chromosome inactivation center between mouse and human.

The successful derivation of hESCs from the ICM of a blastocyst embryo in 1998 opens great opportunities to study early developmental events such as XCI in humans.[17] The first study in 2004 suggested that undifferentiated H9 hESCs contain two active X-chromosomes without detectable *XIST* expression.[18] Upon differentiation, H9 hESCs express high levels of *XIST* and exhibit random XCI pattern, consistent with the mouse model system. Nevertheless, the authors reported that sub-clones of H9 cells after drug selection can express *XIST* in an undifferentiated state, suggesting variations of *XIST* gene expression in female hESCs.[18] A surprising result comes from a separate study in 2005 which demonstrated clearly that *XIST* RNA can coat inactive X-chromosomes in a different batch of H9 cells.[19] In addition, differences in *XIST* expression and XCI status in undifferentiated hESCs were reported for different cell lines.[18-21] In 2007 the consortium of international stem cell initiative characterized 26 female hESC lines and found that approximately 50% of cell lines expressed detectable levels of RNA, indicating that *XIST* expression varies among different hESC lines.[21]

More extensive sets of studies came up in early 2008 from three different groups.[22-24] These studies address the discrepancies found in the previous reports. Hall et al.,[23] examined nine lines of NIH approved female hESCs from multiple sources. Their findings suggest that most of the cells examined had undergone XCI prior to differentiation. In their opinion, XCI in undifferentiated hESCs is unexpected according to knowledge from the mouse, therefore this group refers to this type of XCI as "precocious" inactivation. They searched many sublines of female hESCs and found that some undifferentiated H9 cells behave as female mESCs as first reported.[18] They suggest that the rare female hESCs, which do not express *XIST* in an undifferentiated state but are capable of initiating XCI upon differentiation, are "epigenetically naïve" when compared to hESCs that already complete XCI. Thus the presence of XCI in hESC cultures is likely caused by culture adaptation.

Another extensive analysis was done by Silva et al.,[24] who analyzed eleven female hESC lines including H7 and H9 cells as well as seven female HUES hESCs generated by Cowan et al.,[25] Analysis of XCI (by *XIST* RT-PCR and FISH analysis) in these cell lines led them to classify hESCs into the following three subgroups. The class I cells are relatively rare but similar to mESCs, which possess two active X chromosomes and inactivate one of the X chromosomes upon differentiation. The class II cells are prevalent, which consist of hESCs showing XCI in both undifferentiated and differentiated states. The class III cells are those hESCs that no longer express *XIST* under both undifferentiated and differentiated states. By analyzing Cot-1 RNA (high repetitive elements in pre-spliced hnRNA which correlates well with nascent transcription area from a chromosome) in the third subgroup of hESCs, the authors found that one of the X chromosomes is co-localized to Cot-1 holes that are indicative of Xi. This result suggested that in these cells one of the X chromosomes was already inactivated even though no *XIST* expression is detected. Thus, this group suggests that the ground state of undifferentiated female hESCs should have two active X chromosomes without *XIST* expression, which will initiate XCI upon differentiation. The detection of XCI in undifferentiated class II hESCs is due to either partial differentiation or epigenetic drift of class I cells. Furthermore, class II hESCs frequently become class III cells that lose *XIST* expression but maintain XCI under the undifferentiated state in culture, as illustrated from cell state B to C in Figure 1.

In parallel to the above two studies, our group[22] reported an in-depth analysis of XCI in three female hESC lines (HSF6, H7 and H9). Consistent with the previous studies, we also observed high variance of XCI markers (*XIST* FISH, H3K27me3, H4K20me1 and macroH2A1 staining) depending on the passages and the source of the hESCs. Furthermore, we directly observed the loss of XCI markers in subcultures of H9 and HSF6 hESCs during consecutive passaging (switching from B to C state in Fig. 1), suggesting that culture variations directly contribute to differential expression of XCI markers in different batches of cells. We examined factors contributing to culture variations and concluded that stressed culture conditions lead to abnormal nuclear morphology are associated with the loss of *XIST* expression. More importantly, in hESCs that lose *XIST* expression, the *XIST* promoter are biallelically methylated (100% methylation), which is in contrast to 50% or less methylation in control hESCs and female somatic cells. Thus, loss of XCI markers is associated with epigenetic alterations such as methylation mediated-silencing of *XIST* expression.

To look into whether XCI in hESCs is random or non-random, we performed cDNA SNP analysis of hESCs exhibiting XCI and unexpectedly observed monoallelic X-linked gene expression. Our result indicates a non-random XCI pattern in these established female hESCs. Interestingly, we noted that the majority of SNPs in subcultures that lose *XIST* expression (type C cells in Fig. 1) still preserve monoallelic expression for a majority of X-linked genes, indicating that XCI status is largely maintained. This result is in agreement with the findings by Silva et al., that hESCs without *XIST* expression still preserve Xi. However, a portion of the inactivated X alleles were reactivated and a biallelic expression pattern was observed. To systemically determine the molecular changes associated with hESCs that lose *XIST* expression and other XCI markers, we carried out genome-wide gene expression and promoter CpG island methylation analysis in these cells. The levels of reactivation of X-linked genes in *XIST* RNA-negative hESCs are 2-fold of male

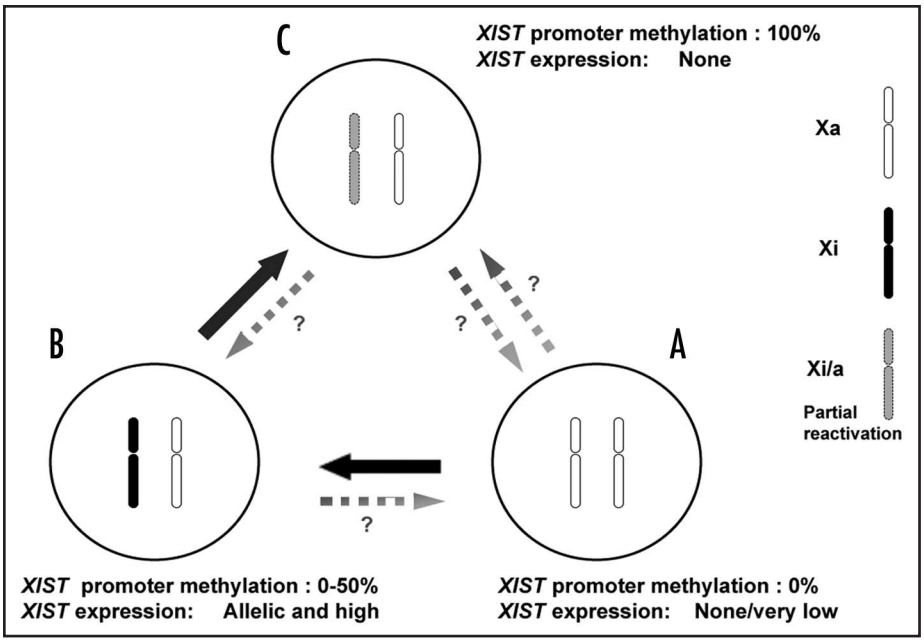

Figure 1. Three states of X-inactivation in cultured female hESCs. According to the recent publications,[22-24] it is postulated that the ground state of hESC is similar to the state of the mouse ICM and mESCs where the two X chromosomes are active (A). However, hESCs tend to acquire XCI even at the undifferentiated state (B). Apparently the maintenance of one inactive and one active X chromosome is also not stable and loss of XCI markers and partial reactivation (Xi/a) of the inactive X chromosome are also observed (C). The three states of XCI can be classified by the combined patterns of methylation on the *XIST* gene promoter and *XIST* RNA expression as annotated. Although not demonstrated currently in the literature, we speculate that these three states of XCI may be reversible by genetic and epigenetic manipulations involving in *XIST* promoter methylation/demethylation.

hESCs and control female hESCs, confirming a disruption of dosage compensation for a subset of X-linked genes. Promoter CpG island demethylation was observed in approximately 12% of the X-linked genes throughout the X chromosome, suggesting that epigenetic alterations on Xi are significant. Interestingly different lines of *XIST* RNA-negative hESCs demonstrate reactivation of different sets of X-linked genes upon losing *XIST* expression, suggesting a stochastic reactivation of a subset of X-linked genes.

Perspectives on the Diversity of XCI in hESCs

Summarizing all the data in the literature, it is clear that there are three states of XCI or *XIST* gene regulation in undifferentiated female hESCs (Fig. 1). Current evidence supports the progressive order of switching the XCI state from A to B and from B to C in culture. Furthermore, these three types of hESCs with different XCI phenotypes can also be annotated by the patterns of *XIST* expression and *XIST* promoter methylation. Most female hESCs resemble type B cells that exhibit XCI and high levels of *XIST* expression with *XIST* promoter methylation between 0–50% in different batches of subcultures.[22] The graded methylation levels of the *XIST* promoter on Xa are consistent with the possibility that DNA methylation of the *XIST* promoter is achieved gradually after XCI is completed in hESCs. When type B cells are converted into C cells in culture, biallelic hyper-methylation of the *XIST* promoter occurs for these hESCs that do not express the detectable level of *XIST* RNA. Finally, although the methylation analysis is not available yet, for type A cells that express no or very low levels of *XIST* RNA, we would predict the *XIST* promoter in these cells are likely unmethylated (as type A cells are the predecessor of type B cells).

One of the intriguing questions concerning XCI regulation in hESCs is about the ground state of XCI in hESCs during the derivation process. It will be important to determine whether varied XCI states in female hESCs are a product of the cell culture or inherent to human ICM cells. In the ICM of mouse embryos and mESCs, both X chromosomes are active; however, the status of XCI in human ICM is still unclear. One should be aware of the developmental differences between mouse and human when predicting human XCI regulation in development using mouse studies.[26,27] A prominent difference for instance is the different time scales of embryogenesis (mouse blastocyst formation occurs at E3.5 whereas human blastocyst formation starts at approximately E4 and ends at E6). We cannot rule out the possibility that the varied time frame in the formation of human blastocyst embryos may contribute to the variation of XCI states in female hESCs during hESC derivation. Indeed, all current analyses of XCI in hESCs are done with established lines at passages >20 that are minimally cultured for more than 3 months in vitro. To firmly understand the ground state of XCI in female hESCs, it may be necessary to characterize the earliest available passages of female hESCs during the derivation process and also correlating it with the XCI status in the human ICM from the IVF blastocyst embryos.

Recent studies also suggest that hESCs are more similar to mouse epiblast stem cells than to mESCs.[28,29] Perhaps hESCs are at a permissive state of exhibiting XCI, which may even give these cells a growth advantage compared to hESCs that have not initiated X-inactivation. Interestingly this phenomenon is similar in a way to another phenomenon commonly observed in mESC culture. During mESC culture one of the X chromosomes is lost quite frequently. The loss of one X chromosome in mESC and inactivation of one of the X chromosome in hESCs upon culture may imply that cells carrying two active X chromosomes might have a growth disadvantage in culture. As a result it is possible that XCI observed upon culture of undifferentiated hESC is a result of a culture "adaptation" that encourages the proliferation of cells with only one active X chromosome.

Lastly, it is also unclear whether the three epi-states of XCI (Fig. 1) in female hESCs would all occur in one direction from A to B to C or actually become reversible through reprogramming. The reprogramming of XCI from mouse fibroblasts back to mESCs state has been demonstrated recently.[34] Therefore, it is theoretically possible that we can reversibly regulate three different XCI states in female hESCs through genetic and epigenetic reprogramming.

Concluding Remarks

Human embryonic stem cells are thought to be the most promising source both for regenerative medicine and for the study of early human embryogenesis. Conventional wisdom would predict that hESCs, by postulation of mouse ESCs, posses two active X chromosomes. XCI would occur upon differentiation as expected from the

mouse studies. Yet, studies described herein project the complexity of XCI in these cells. The three different observed epi-states of XCI in a variety of cells or in the same cell line (e.g., H9 cells) are likely a phenomenon of culture adaptation during the entire course in culture. Therefore, our understanding of XCI in human embryogenesis and in female hESCs will depend on future examination of the ICM of those IVF left-over blastocyst stage embryo or by using the earliest passages of hESCs.

Finally hESCs are considered a major resource for regenerative medicine and as such they should be genetically and epigenetically stable and normal. It is known that maintenance of XCI is crucial for human embryogenesis.[30] Also a significant number of X-linked genes are associated with mental retardation diseases and therefore normal dosage is critical for brain development and function.[31] Besides that, aberrant X-inactivation is correlated with malignancies such as breast cancer.[32,33] We therefore suggest that derivatives of female hESCs, which will eventually be considered for cell transplantation, should be as similar to native human cells as possible in their XCI status.

Acknowledgements

Supported by the California Institute of Regenerative Medicine training grant (T1-00005) to T.D. and comprehensive grant RC1-0111 and NIH RO1 NS044405 to G.F.

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