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X chromosome inactivation: recent advances and a look forward

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X chromosome inactivation, the transcriptional inactivation of one X chromosome in somatic cells of female mammals, has revealed important advances in our understanding of development, epigenetic control, and RNA biology. Most of this knowledge comes from extensive studies in the mouse; however, there are some significant differences when compared to human biology. This is especially true in pluripotent cell types and, over the past few years, a significant amount of work has been dedicated to understanding these differences. This review focuses specifically on recent advances in the mechanism of *Xist* spreading, the role of *Xist* in cancer, the effects of reprogramming on X chromosome inactivation in human induced pluripotent stem cells, and new tools for studying X chromosome inactivation.

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Introduction

The field of X chromosome inactivation (XCI), the process by which one X chromosome in female mammals is transcriptionally inactivated in order to equalize gene expression in males and females, is now in its sixth decade and has produced a substantial understanding of the cell and molecular biology underlying this epigenetic regulation [1,2]. Even though our mechanistic understanding of the events in XCI is quite sophisticated, we are still identifying new players and further refining our understanding as illustrated by recent advances. With the discovery of induced pluripotent stem cells (iPSCs) in 2006 [3], a new subfield of XCI emerged to characterize X chromosome state in these cells and their derivatives. This new technology made it possible to examine the same cells in a somatic context as well as an embryonic-like context to determine changes to the X chromosome during cell fate decisions, providing tools to interrogate reprogramming and pluripotency.

This review will address new mechanistic advances in mouse and human XCI biology, the role of XCI in cancer initiation and progression, and new data on X chromosome state following reprogramming. Finally, it will discuss a new tool that has the ability to mark XCI in individual cells, which may be able to address many outstanding questions in the field. These recent advances and future discoveries in X chromosome biology are certain to aid in the translation of cell based therapies to the clinic (Table 1).

New mechanisms of XCI

Much of the mechanism of X chromosome inactivation has been extensively studied and well characterized, including understanding the role of the antisense inhibitor, *Tsix*, to the proteins recruited to maintain the chromosome-wide inactivation, and the DNA–RNA–protein interactions that maintain X inactivation [4–8]. However, a recent breakthrough was made in understanding how *Xist* is able to spread along the length of the entire chromosome without silencing other chromosomes or active areas of the X chromosome. Engreitz *et al.* using 1054 tiled probes to the 17-kb *Xist* transcript, pulled down unique sequences of genomic DNA bound to *Xist* at five time points during differentiation as *Xist* becomes induced. After ruling out the role of sequence motifs with *Xist*-recruiting ability, they found that the initial DNA sites bound by *Xist* were spatially proximal (based on Hi-C data) to the *Xist* locus [9••]. These results support a model that *Xist* spreads along the length of the chromosome by binding to distal sites that are spatially organized close to the newly transcribed *Xist* RNA. By being able to modify chromatin structure at these regions, *Xist* is able to spread to newly silenced regions of the genome. Furthermore, regions that escape XCI are able to loop out and remain active while still permitting spatial spread of *Xist*. Since much more of the genome escapes XCI in humans compared to mouse, it will be interesting to determine if this mechanism is conserved in humans.

Other work has identified a new long non-coding RNA, *XACT*, specifically in human pluripotent stem cells [10••]. While not expressed in mice, *XACT* coats the active X

Table 1

Understanding mechanisms of X chromosome inactivation can improve all aspects of developing clinical therapies.

Reprogramming	Controlling XCI during reprogramming will allow specific X chromosome states to be achieved in resulting iPSCs. Dual color reporter system may be able to elucidate the timing of reactivation at a fine tuned resolution.
iPSCs	Discoveries, such as *XACT*, show how unique human pluripotent biology is compared to mouse and suggest new ways to control XCI.
Differentiation	Maintaining XCI during differentiation will be crucial to limit the presence of potentially oncogenic cells.
Somatic cells	The more we uncover about different states of XCI in human cells, the better we will be able to determine if *in vitro* cells are replicating the biology of *in vivo* cells.
Transplantation	Monitoring how XCI state changes over long periods of time as well as changes that may occur *in vivo* will be necessary to ensure cells maintain the desired state.

chromosome and, in the absence of *XIST*, coats both chromosomes. Perhaps this reflects a human-specific mechanism by which cells prevent silencing of both X chromosome, instead of, as in mouse, using *TSIX* as an antisense repressor. It is known that the human *TSIX* RNA has significantly less complementarity to human *XIST* than mouse *Tsix* and *Xist*, and its ability to act as an effective suppressor in this way has been questioned [11,12]. This paper begins to shed light on human specific aspects of XCI that may underlie the mechanistic differences between mouse and human.

Finally, two other studies provide additional pieces of the mechanistic puzzle. First is evidence for the role of *Jarid2* in recruiting *PRC2* to *Xist* RNA in helping to mediate inactivation [13•]. The second is the surprising finding that the first intron of *Xist* seems dispensable for *Xist* expression and normal function during XCI in stem cells and during development, despite the fact that the region exhibits strong pluripotency factor binding [14•]. Taken together these mechanistic results illustrate that there is still much to learn about XCI in both humans and mice.

XCI and cancer

The role of the X chromosome in cancer has been well documented but much data is only correlational [15–18]. Recent papers provide genetic and developmental evidence that X chromosome changes in somatic cells can cause cancer. Using human breast cancer as a model, researchers found that half of the sporadic basal-like cancers were characterized by duplication of the active X chromosome and loss of the inactive X chromosome [19]. While these abnormalities did not contribute to global increases of gene expression from the X chromosome, it was associated with overexpression of a subset of genes. In addition, another paper provided evidence that the inactive X chromosomes accumulates more mutations than any other autosome in cancer genomes compared to non-tumorigenic samples [20], suggesting an inability to successfully repair damage. If this inactive X chromosome later becomes active, it could further contribute to genetic mutation load during cancer progression.

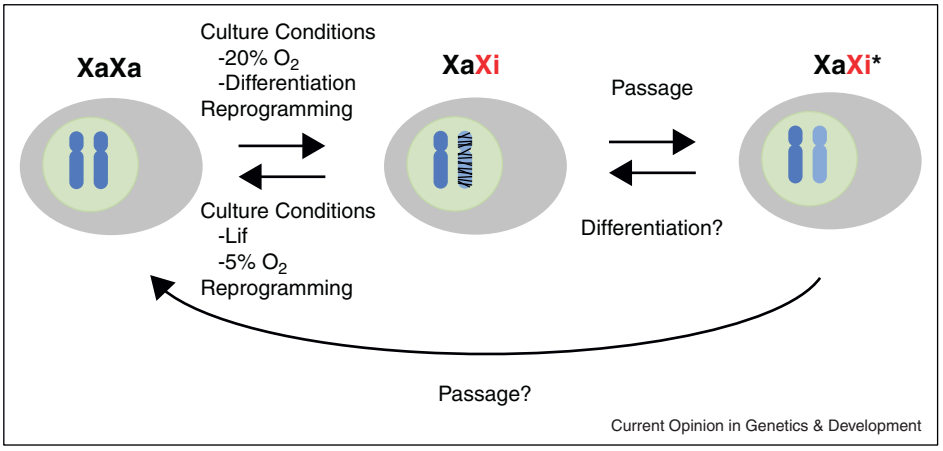
An elegant and convincing study in mouse showed direct evidence that *Xist* loss causes cancer. Researchers conditionally knocked out *Xist in vivo* in mouse hematopoietic stem cells after random X chromosome inactivation had already taken place. A female specific, fully penetrant, lethal blood cancer developed that began killing mice at 1.5 months. After two years, only 10 percent of the mice were still alive and neither homozygous nor heterozygous female mice have escaped the lethal phenotype at the time the research was published [21••]. While this was only demonstrated in one lineage in the mouse, other data suggest that the loss of *XIST* in human iPSCs is strongly correlated with increased expression of X-linked oncogenes [22••]. Interestingly, male iPSCs, compared to female iPSCs, are more homogeneous and do not overexpress these genes suggesting a potential increased risk of tumorigenesis in female stem cells. This is a major hurdle in the clinical translation of female stem cells and will require much more work to understand the different potentials of stem cells with different XCI states (Table 1).

Reprogramming and XCI state

Early mouse studies have revealed simple binaries: pluripotent cell types have two active X chromosomes (XaXa) (extensively reviewed in [2,23]), and somatic cell types have one active and one inactive X chromosome (XaXi) [24]. Differentiation of a mouse pluripotent cell into a somatic cell results in the inactivation of one X chromosome [25]. This is true for both embryonic stem (ES) cells and iPSCs in the mouse with the exception of ES cells derived from the epiblast. Epiblast stem cells (EpiSC) are thought to represent a distinct state of pluripotency, as they cannot contribute to blastocyst chimeras, have variable differentiation bias, and are characterized by an inactive X chromosome [26,27]. However, they can be converted to ES, reactivating the inactive X chromosome in the process [28]. These relationships in mouse have not directly translated to human biology. There is no universal rule governing the X chromosome state in human pluripotent cell types; indeed, a range of states are common (Figure 1). While these differences could be species specific, they may be due to differences in pluripotent state as human ES and mouse EpiSC are similar culture conditions and gene expression and the inactivation of an X chromosome [26,27].

In addition to examining human ES cells, several groups have analyzed iPSCs for X chromosome state and have

Figure 1



Three classes of X chromosome states exist in human pluripotent cells. Three classes of X chromosome state have been shown to exist in human stem cells. The first represents cells with two active X chromosomes which can be achieved by changes in culturing conditions by reprogramming, which occasionally produces cells with two active X chromosomes. The second class of stem cell has an inactive X chromosome, which can also be induced by culturing conditions or due to differentiation. Finally, as many people have shown, XaXi cells can result from the reprogramming process itself. The third class is characterized by partial XCI in that *XIST* is not present but much of the chromosome is still inactivated. Over time, more transcripts across the entire X chromosome can become expressed, known as erosion, and this phenomenon is known to be the result of continued passaging. Questions about the relationship of these three classes still exist. It is unclear whether differentiation can rescue a partially inactivated X chromosome to a fully inactivated one. Additionally it has not been shown whether extensive passaging could render a previously inactivated X chromosome completely reactivated.

generated seemingly conflicting results. Some groups report reactivation of the X chromosome in iPSCs (XaXa) [31–33] while others show that the X chromosome remains inactive (XaXi) [29,34]. Interestingly, there are reports on the variability in XCI (same reprogramming method leading to multiple states; single clone containing cells of different states) suggesting that the variability is biologically, not methodologically, based [33,35]. These differences again raise questions about the suitability of these cell and their byproducts in clinical settings and suggests a need for careful characterization of these cells and their properties (Figure 1).

In spite of these advances, studies have not yet documented an ability to control the X chromosome state in cells, especially iPSCs. However, recent work in this area has provided some exciting insights. A group let by Shinya Yamanaka was able to change culture conditions to affect the outcome of reprogramming. By culturing fibroblasts on SNL feeders, which produce high levels of leukemia inhibitory factor, Tomoda *et al.* were able to produced human iPSCs that were characterized by X chromosome reactivation [36••]. Human iPSCs produced in this manner reactivated *XIST* upon differentiation and iPSCs derived under other conditions and subsequently moved to SNL feeders could be coaxed to reactivate the inactive X chromosome. Interestingly, the SNL feeders provide additional factors other than increased LIF, as rLIF alone only caused biallelic expression of a subset of X chromosome genes compared to those cells grown on the SNL feeders. Supporting their work, many other groups have reported the effects of culture conditions on ES cell XCI state suggesting that different conditions could also control XCI in iPSCs [30•,37,38]. This system provides an exciting opportunity to understand the human biology of XCI changes as a proportion of cells can be forced to switch between XaXa and XaXi states.

Taken together, it is important to determine what constitutes an ideal state of human pluripotent cells, but it is not as easy as deciding on two active X chromosomes or one. How these states are reached is also important: some human iPSCs with two active X chromosomes are due to erosion of XCI and have poor differentiation ability [29], while pluripotent cells can also be converted under defined conditions to replicate the pluripotency state found in mouse ES cells including a reactivated X chromosome [30•]. While XaXa cells are the gold standard in mouse, perhaps cells with two active X chromosomes represent an epigenetic abnormality rather than the ideal state for in human pluripotency. These data suggest that different mechanisms may be involved and perhaps one is preferable to another in generating the ideal state.

Simple tools provide big possibilities

New tools have been developed recently that have aided our understanding of the mechanisms of XCI, especially, as mentioned before, new methods to identify DNA bound to RNA. However a recent paper took a simple approach that is likely to answer fundamental questions

about XCI during development that have yet to been sufficiently studied. Wu *et al.* developed a dual color mouse line by integrating Cre-inducible, fluorescent proteins into the *Hprt1* locus, a locus known to obey XCI, on both X chromosomes [39••]. Using this elegant system, they were able to generate mice in which every single cell was labeled either green or red, reflecting which X chromosome remained active in a given cell. They were able to generate maps of XCI in all the tissues of the body, down to single cell resolution.

This valuable tool opens a number of interesting areas of follow up. While X chromosome reactivation during reprogramming is well known, the precise timing of these events are difficult to study due to the small fraction of cells that eventually become reprogrammed. Using cell lines derived from these mice, one could determine the precise timing of reactivation of the X chromosome in relation to obvious morphological changes or presence of gene expression profile changes. Female germ cell differentiation from stem cells could also benefit from this technology, as they are the only *in vivo* cell type with two active X chromosomes. This type of tool would be extremely useful in a human cell line, where XCI is more variable and less well understood. In the context of reprogramming, it would likely reveal important understanding of the relationship between the three XCI states that exist in human iPSCs (XaXa, XaXi, XaXi*, see Figure 1).

Conclusions: moving toward the clinic

Even after 50 years, the field of XCI is still providing new insights as highlighted by the recent finding of *XACT* in human pluripotent cells. As technologies become more sophisticated and we are better able to profile single cells, we are sure to understand even more about X chromosome biology. As the field moves forward, there are a number of unanswered questions that remain, especially in the human system. Specifically, how will we utilize our knowledge of XCI to impact the future clinical use of stem cells?

Since XCI is a uniquely female biology, it is an important area of study to ensure that patient-specific therapies enter the clinic at similar rates for men and women. As such, there are a number of areas that need to be addressed. First, how do we direct XCI in cell types of interest and how can we ensure that the X chromosome remains inactive? While the mouse has provided incredible insight, many of these studies will need to be conducted in human cell lines to address the human-specific differences. Additionally, what are the *in vivo* consequences of changes in XCI? We know that the mouse hematopoietic system undergoes drastic changes [21••]; are these changes more broadly applicable? Are they relevant in human biology? Initial findings suggest a possible link to cancer in human iPSCs but more work is surely needed [22••]. The X chromosome is full of surprises and if the future of the field is anything like the last few years, it would seem we have much to look forward to.

Acknowledgements

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