Epigenetic stability of repressed states involving the histone variant macroH2A revealed by nuclear transfer to Xenopus oocytes

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Introduction

How various epigenetic mechanisms restrict chromatin plasticity to determine the stability of repressed genes is poorly understood. Nuclear transfer to Xenopus oocytes induces the transcriptional reactivation of previously silenced genes. Recent work suggests that it can be used to analyze the epigenetic stability of repressed states. The notion that the epigenetic state of genes is an important determinant of the efficiency of nuclear reprogramming is supported by the differential reprogramming of given genes from different starting epigenetic configurations. After nuclear transfer, transcription from the inactive X chromosome of post-implantation-derived epiblast stem cells is reactivated. However, the same chromosome is resistant to reactivation when embryonic fibroblasts are used. Here, we discuss different kinds of evidence that link the histone variant macroH2A to the increased stability of repressed states. We focus on developmentally regulated X chromosome inactivation and repression of autosomal pluripotency genes, where macroH2A may help maintain the long-term stability of the differentiated state of somatic cells.

Resistance to Nuclear Reprogramming Revealed by Nuclear Transfer to Xenopus Oocytes

The Xenopus oocyte nuclear transfer system is highly suited to probe the stability of repressed states.1,4 Up to several hundred mammalian somatic nuclei can be directly transplanted into the oocyte’s own nucleus,4 the germinal vesicle (GV) (Fig. 1). Under these conditions, the oocyte imposes a new transcriptional state onto the incoming somatic chromatin. Transcription of previously silenced genes in somatic nuclei is directly induced, after transplantation to an oocyte, in the absence of cell division or DNA synthesis.
The notion that the epigenetic state of genes in somatic nuclei is a critical determinant of reprogramming efficiency is directly supported by Xenopus oocyte nuclear transfer studies. A given gene, *Xenopus laevis* Xist RNA is lost from the Xi following nuclear transfer. Therefore, resistance to this transition to stable silencing during differentiation also occurs in several steps, whereby initial repression is followed by deacetylation and H3K9 methylation, followed by DNA methylation. During X chromosome inactivation (XCI), one of the last events, occurring after silencing is induced, is DNA methylation and the incorporation of the histone variant macroH2A.

Additionally, the acquisition of repressive marks associated with gene repression, including those deposited by Polycomb group proteins, similarly facilitates the establishment of repressed states.

**Resistance to Reprogramming Correlates with the Incorporation of the Histone Variant MacroH2A in the Presence of DNA Methylation**

The sequence of epigenetic events leading to the stable inactivation of the Xi has been particularly well studied. This includes the formation of a nuclear compartment devoid of transcriptional machinery, into which genes are recruited upon their silencing. Initial silencing of genes is followed by other epigenetic changes such as the acquisition of histone marks associated with gene repression, including those deposited by Polycomb group proteins.

**Table 1.** Resistance to Reprogramming Correlates with the Incorporation of the Histone Variant MacroH2A in the Presence of DNA Methylation

| Donor nuclei | Xist RNA | macroH2A | Xenopus oocyte nuclear transfer | Xist RNA | Transcriptional reactivation |
|--------------|----------|----------|--------------------------------|----------|-------------------------------|
| EpiSC        | +        | −        | −                              | −        | +                             |
| ESRA         | +        | −        | −                              | −        | +                             |
| TS           | +        | +        | −                              | −        | −                             |
| MEF          | +        | +        | −                              | −        | −                             |
DNA methylation of Xi genes is a late event of XCI and is known to stabilize the Xi in the somatic cells of developing mouse embryos, because Xi reactivation is seen in DNA methylation-deficient Dnmt1 or Smchd1 mutant embryos. However, Xi reactivation in Dnmt1 mutant embryos is not seen in extraembryonic lineages, where macroH2A is associated with the Xi of extraembryonic cells under normal conditions, reflecting differential regulation of XCI between these lineages.

Treatment of MEFs carrying an X-GFP transgene on the Xi (Xi-GFP) with the 5-methylcytosine analog 5-azacytidine (5-Aza) leads to a 10-fold increase in transgene reactivation. How- ever, this concerns a small proportion of the cells (0.25%), suggesting that additional mechanisms contribute to the stability of the repressed Xi. Indeed, a study showed that 5-Aza treatment in combination with Xist deletion or TSA treatment has a synergistic effect on Xi-GFP transgene reactivation. Overall, these results support the view that DNA methylation is required for stable repression of the Xi in somatic cells, together with other mechanisms. Furthermore, the Xi failed to reactivate from MEFs after nuclear transfer to Xenopus oocytes, while several genes, methylated at their promoters and including lineage specific genes such as MyoD, were reactivated following nuclear transfer. The extreme stability of the Xi after nuclear transfer to Xenopus oocytes further suggests that several mechanisms may be used to confer resistance to nuclear reprogramming.

Resistance toward Xi reactivation could not be explained by differences in DNA methylation before nuclear transfer. Instead, resistance correlated with the presence of the histone variant macro-H2A. It could also be that Xenopus oocyte nuclear transfer conditions reveal differential association and turnover of macro-H2A within the transplanted nuclei (Fig. 3). This reorganization strikingly resembles that occurring upon deletion of Dnmt1 in MEFs. In addition to the Xi, macro-H2A-GFP seems to become enriched at heterochromatic foci, unlike what is seen in the starting nuclei, which show a more uniform nuclear staining (Fig. 3). We think that these heterochromatic foci are pericentric. Pericentric association of macro-H2A has been previously reported. It could also be that Xenopus oocyte nuclear transfer conditions reveal differential association and turnover of macro-H2A within different regions of the transplanted nuclei. Altogether, the continuous association of macro-H2A with heterochromatin after nuclear transfer raises the possibility that it may contribute to resistance toward transcriptional reprogramming.
macroH2A Contributes to the Stability of Repressed States

It is important to stress that the histone variant macroH2A is not required for silencing, nor required for the initiation of XCI and furthermore, in the mouse, mutant embryos are viable, fertile, and their Xi is maintained in a repressed state.\(^2,3\) This does not support the view that macroH2A is critical for the establishment of stable Xi repression. Moreover, macroH2A removal is not sufficient to induce Xi-GFP reactivation.\(^5,31\) However, it was demonstrated previously that depletion of macroH2A by RNAi together with 5-Aza and TSA treatment has a synergistic effect on Xi-GFP reactivation from MEFs, indicating a role of macroH2A in the maintenance of the silenced X chromosome.\(^22,23\) macroH2A seems to also confer resistance toward transcriptional reprogramming, because removal of macroH2A1 and/or macroH2A2 by RNAi in MEFs relieved, partially, the resistance of Xi-GFP to transcriptional reprogramming by Xenopus oocytes.\(^3\) Interestingly, HDAC inhibition under nuclear transfer conditions relieved resistance to the same extent, and also had an additive effect when combined with macroH2A depletion.\(^3\) The overall picture that emerges from these studies is that macroH2A is not required to induce Xi repression, but instead adds another layer of epigenetic repression on top of other silencing mechanisms already in place, in order to ensure the long-term maintenance of silenced states, which restricts nuclear reprogramming.

Implications for the Stability of X Chromosome Inactivation During Development

XCI is a developmentally regulated process and is tightly coupled to the loss of pluripotency.\(^32,33\) The X chromosomes inherited by the gametes are initially active, but the paternal X chromosome quickly becomes inactivated during the first few cell divisions of female mouse embryos (Fig. 4).\(^10\) This imprinted XCI is maintained in the extra-embryonic lineage, where macroH2A associates early with the Xi.\(^21\) In the developing inner cell mass, induction of pluripotency is associated with X reactivation.\(^34\) Hence, ES cells, derived from the inner cell mass (ICM), have two active X chromosomes (Xa). A second round of XCI takes place in the post-implantation epiblast at around stage E5.5. This round of XCI is random; each X chromosome has a 50% chance of becoming inactivated. Importantly, EpiSCs, derived from E5.5-E6.5 epiblasts, express pluripotency genes, have an Xi but do not show macroH2A enrichment on the Xi.\(^3\) This may reflect the in vivo situation in the post-implantation epiblast. We speculate that macroH2A levels increase upon differentiation of epiblast cells after E6.5, because ESRA induces a 4-fold induction of macroH2A1 protein levels.\(^35\) In addition, high induction of macroH2A and its incorporation into the Xi was seen in differentiated EpiSCs.\(^3\) We propose that the Xi of the post-implantation epiblast is inactivated but that its repressed state is not fully stabilized. This may be because the Xi is reactivated in developing primordial germ cells (PGCs) specified from post-implantation epiblast. Hence, long-term, stable inactivation of the Xi may only occur after germ cell lineage specification from the epiblast, when epiblast cells further differentiate. In addition, it has been reported that macroH2A is removed from PGCs during their development.\(^36\) Interestingly, macroH2A is also removed (perhaps actively) from chromatin just after fertilization, during reprogramming in the zygote, but reappears by the morula stage, and this occurs both in fertilized embryos and nuclear transfer embryos.\(^37,38\) Altogether, association of macroH2A with the Xi correlates with its stable and irreversible inactivation during development. Conversely, the removal of macroH2A also correlates with epigenetic reprogramming during embryonic development.

Implications for the Repression of Autosomal Pluripotency Genes

Importantly, macroH2A incorporation in somatic cells is not limited to the Xi. Somatic levels of macroH2A do not differ between male and female cells.\(^39\) macroH2A evolved before XCI and macroH2A variants have been conserved in non-mammalian vertebrates that do not use XCI as a dosage compensation mechanism.\(^10\) As noted above, the levels of macroH2A1 increase upon cellular differentiation and the different macroH2A variants show diverse tissue-specific expression patterns during development.\(^41\) Notably, macroH2A RNAi in MEFs led to enhanced reprogramming efficiencies following nuclear transfer.\(^3\) Despite the lack of obvious defects in developing macroH2A mouse mutants, it may be that, in a similar manner as for the Xi, macroH2A backs-up other epigenetic

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**Figure 3.** macroH2A-GFP reveals a major reorganization of chromatin following transplantation of nuclei into Xenopus oocytes. The nuclei of C2C12 cells expressing macroH2A-GFP were transplanted into Xenopus oocyte GV’s and imaged immediately (0 h) or 3 d after nuclear transfer (72 h).
repression mechanisms already in place to ensure the remarkable stability of repressed states. Hence, loss of macroH2A might be, at least in part, compensated for by other mechanisms. Combined HDAC inhibition and macroH2A depletion also had an additive effect on reprogramming by Xenopus oocytes, indicating that macroH2A may also restrict reprogramming through non-HDAC related pathways. Future areas of research include testing the efficiency of nuclear reprogramming in the absence of macroH2A by other procedures, such as cell fusion, nuclear transfer to eggs and induced pluripotency. We conclude that macroH2A shapes chromatin to confer stability to transcriptional states in somatic cells.

Concluding Remarks

Histone variants, and especially macroH2A variants, offer unparalleled means to alter chromatin plasticity and structure. While many epigenetic mechanisms are used to induce and maintain the repressed state of genes, histone variants such as macroH2A may help to reinforce the mechanisms already in place to confer increased long-term stability of repressed states. If this were the case, one would expect the loss of macroH2A to be associated with decreased stability of the differentiated state, for which evidence is accumulating. The Xenopus oocyte system is uniquely suited to address the mechanisms regulating epigenetic memory and in particular those that restrict reprogramming and confer stability to repressed states.

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