Commentary

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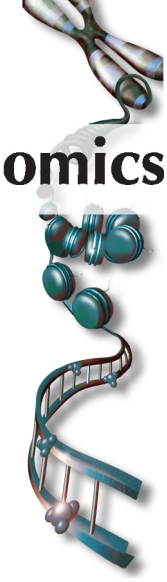
Epigenomics



Naive pluripotent stem cells as a model for studying human developmental epigenomics: opportunities and limitations

Peter J Rugg-Gunn*,[1]

[1]Epigenetics Programme, Babraham Institute, Babraham, Cambridge, CB22 3AT, UK

* Author for correspondence: peter.rugg-gunn@babraham.ac.uk

"An important, outstanding challenge is to evaluate whether naive hESCs are a distinct and stable state, or whether they are a response to the strong signaling conditions that are used to maintain naive hESCs in culture."

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Next year will mark the 20th anniversary since the discovery of human embryonic stem cells (hESCs) and the demonstration that these remarkable cells can differentiate into all lineages of the body [1]. Impressive progress since then has delivered hESC-derived therapies to the clinic, established hESCs as a cellular platform for drug screening, toxicology assays and disease modeling, and advanced our understanding of human developmental biology.

Although derived from epiblast cells of the preimplantation human embryo, the biological properties of hESCs more closely reflect the early postimplantation embryo that is formed several days later in development [2–5]. This distinction in timing is important because it helps to evaluate the characteristics of hESCs by comparing with their equivalent cells *in vivo*. Classifying the current hESC lines as similar to postimplantation cells also opens up an obvious need to capture hESCs in an alternative state that recapitulates the preimplantation embryo.

Several reports of preimplantation-like human stem cells, broadly termed 'naive' hESCs, have raised the hope that deriving such a cell type is achievable [6–11]. These recent additions to the stem cell hierarchy are particularly exciting for developmental epigeneticists. Access to an *in vitro* model that reproduces the epigenetic state of preimplantation epiblast cells would enable new opportunities to study the underlying mechanisms of many key epigenetic processes, such as the regulation of X-chromosome inactivation in humans.

As with all novel cell types, there are on-going discussions about how to evaluate this new cell state. The task is complicated further because different cell culture conditions are used to derive and sustain naive hESCs, resulting in a spectrum of cell states and associated difficulties when comparing between studies. Developmental strategies differ between species, and so expectations and extrapolations from other organisms, such as the mouse, might be misleading when applied to human studies.

To address these challenges, proposed criteria to define the naive state of hESCs have established a common framework for researchers to evaluate their own cells against. The criteria include transcriptional profiles, metabolic activity, cell-surface protein expression and signaling responses [3,12,13]. Reassuringly, naive hESC-associated pluripotency factors are detected in human and primate preimplantation epiblast cells, providing support that naive hESC lines maintained in certain conditions can recapitulate the gene expression program of epiblast cells *in vivo* [2,14,15].

Importantly, several epigenomic hallmarks are also used to categorize naive hESCs, such as the presence of two active X-chromosomes in female cells, and global DNA hypomethylation [3,13]. These defining epigenetic characteristics satisfy expectations based on the available human embryo data, and provide exciting opportunities for further study. However, there are also important considerations about what these epigenetic hallmarks actually indicate and how we can interpret them, which I will discuss below.

Future Medicine

DNA hypomethylation: capturing the preimplantation epigenome or shaped by culture conditions?

An important, outstanding challenge is to evaluate whether naive hESCs are a distinct and stable state, or whether they are a response to the strong signaling conditions that are used to maintain naive hESCs in culture. For example, the addition of a MEK1/2 inhibitor (a universal component of naive embryonic stem cell [ESC] media) to conventional mouse and human ESCs leads to a rapid, global loss of DNA methylation through the downregulation of UHRF1 and DNA methyltransferases [16]. This occurs independently of an initial cell-state change. These observations lead to the question of whether the hypomethylated epigenome reflects the biology of the preimplantation human embryo and is an informative readout of the naive state, or alternatively, is it a direct effect of MEK1/2 inhibition that simply mimics our expectations? Comparing the methylomes between naive hESCs and human embryos reveals similar levels of low, global DNA methylation [11,13,17]. However, a closer examination of methylation levels at CpG sites between naive hESCs and human embryos showed that the methylomes are actually quite different; naive hESCs have fairly uniform and low levels across the genome, whereas the embryo retains regions with higher levels of DNA methylation [17]. Although an important first step, one caveat here is that this comparison used data from whole embryos, of which the pluripotent epiblast cells are a relative minority compared with the extraembryonic cells. Additional embryo methylation datasets, preferably from single cells of defined lineage, are required for a more detailed comparison.

The naive hESC methylation profiles fit with a model in which the DNA methylation machinery is continually suppressed. It is important to remember that epiblast cells in the embryo are only transient, and if epiblast cells were maintained with inhibited DNA methylation activity then they too would probably display uniformly low levels of methylation across the genome. There are important implications of this however, for example, the methylation marks at imprinted gene control regions are erased in naive hESCs [13,17]. This contrasts with the human preimplantation embryo where methylated imprints are intact, despite the strong demethylating environment [18]. Indeed, initial experiments suggest that imprints are relatively resistant to demethylation compared with other regions in the genome when hESCs are challenged with MEK1/2 inhibition, and prolonged inhibitor treatment is required to erase the imprints (unpublished observation).

An important, unresolved question is whether the erasure of DNA methylation during human embryo development is triggered, in part, through the suppression of MEK1/2 activity. In particular, it would be interesting to observe whether UHRF1 and DNA methyltransferase levels in the embryo are affected by MEK1/2 inhibitor treatment. Addressing this gap would help give biological context to the hypomethylated state of naive hESCs. It is also important to remember that the loss of DNA methylation by itself through the deletion of DNA methyltransferases is not sufficient to induce the activation of naive-associated genes or the acquisition of other naive state properties [19]. Whether DNA methylation needs to be removed for cells to transition to a naive state is unknown.

The difference in DNA methylation stability between hESCs and the embryo might not be restricted to human cells, as two recent reports showed that naive mouse ESCs lose their imprints when cultured in media containing MEK1/2 inhibitor [20,21]. These studies contrast with several previous reports that demonstrated imprint stability, and the reasons for the discrepant findings are not obvious. Loss of imprints in mouse ESCs might be associated with defective developmental potential [20,21], although this needs to be examined more systematically and also in the context of naive hESC differentiation.

Taken together, DNA hypomethylation is a hallmark of preimplantation epiblast cells and is an appropriate biomarker to define naive-state hESCs. The erasure of imprints is troubling as this leads to gene dysregulation, and might also indicate that other regions of the genome are vulnerable to aberrant demethylation. Imprint erasure is not entirely unexpected, however, given the prolonged growth in strong demethylating conditions. Additional research is needed on DNA methylation in human embryos to provide a more precise comparison with hESCs and to understand how demethylation is controlled and also protected against. Knowing whether mechanisms are conserved in the embryo will enable a careful assessment of whether DNA hypomethylation in naive hESCs is caused simply by exposure to signaling inhibitors, or is an accurate response to stable cell-state change.

X-chromosome activation is a defining property of naive hESCs

Another defining characteristic of naive hESCs is the presence of two active X chromosomes in female cells [13,22,23]. This discovery is particularly exciting because it presents a major difference from conventional hESCs, which typically have one active and one inactive X-chromosome (reflecting their postimplantation status). Moreover,

naive hESCs recapitulate closely the pre-X-inactivation state of the human embryo [22] and, therefore, provide a tractable cell culture model to study the process of X-chromosome inactivation in humans.

The human embryo ensures X-chromosome dosage compensation using mechanisms distinct from the mouse, potentially through the competition of opposing long noncoding RNAs, *XIST* and *XACT* [14,22]. The presence of two active X chromosomes is stable in naive hESCs, and most cells co-express *XIST* and *XACT*. Interestingly, the proportion of cells with biallelic *XIST* transcription varies between cell lines and over passage [22,23], and it is unclear why this expression pattern should be unstable or potentially detrimental.

One X-chromosome is inactivated upon naive hESC differentiation, although curiously the choice of which chromosome is inactivated appears to be nonrandom [13,23]. Notably, the two studies that showed this observation used naive hESCs that were reprogrammed from conventional hESCs, and so this unanticipated observation could be due to an epigenetic 'memory' of the previously active chromosome. It will be interesting to see if naive hESCs that are derived directly from an embryo (i.e., without an epigenetic memory of conventional hESCs) show random X inactivation, and also whether there are differences in chromosome skewing between cells that express *XIST* from two versus one chromosome. As an interesting alternative hypothesis, Payer and colleagues proposed that differences in the developmental regulation of X-chromosome inactivation between mouse and human could explain why naive hESCs seem to lack the capability to enforce random inactivation [24].

The loss of DNA methylation in somatic cells is sufficient to trigger the activation of individual genes on a previous silenced X chromosome [25]. So is it possible that the two active X chromosomes, a hallmark of naive hESCs, are simply a response to DNA hypomethylation? Here, the evidence suggests not. First, the loss of DNA methylation is rapid and precedes X-chromosome reactivation by several days (unpublished observations). Second, X-chromosome reactivation occurs uniformly and robustly, which is not predicted to occur if it was a haphazard, secondary event following DNA hypomethylation. Third, there is a good similarity between naive hESCs and preinactivation-state epiblast cells in terms of X-chromosome transcript levels and also histone modifications. Overall, my opinion is that the presence of two active X chromosomes is one of the best biomarkers for naive hESCs and should be widely adopted in characterizing hESC states.

Conclusion & future perspective

The discovery of a new cell state can capture the attention of researchers across fields and open up new research directions. A careful assessment of any new state is required to infer accurately the potential opportunities and limitations of the new system. Keeping the biological relevance at the forefront, and benchmarking key properties to corresponding cells of the embryo, will also help to critically evaluate cell phenotype. The collective studies on hESCs have shown that a spectrum of pluripotent states is possible depending on the growth conditions used, and it is therefore increasingly important to clearly define cell states using agreed and established criteria. This information will aid others in selecting the most suitable system for their particular research question.

Naive hESCs provide the only cellular model to investigate preimplantation human biology and are already being used to deliver important new insights into gene regulation. The study of these cells will facilitate major advances in developmental epigenetics. In turn, a better understanding of DNA methylation erasure and X-chromosome inactivation during human embryo development is required to interpret some of the unanticipated traits of naive hESCs and to assess whether these features reflect aberrant regulation or human-specific differences. In addition, several epigenomic hallmarks of naive hESCs are still untested *in vivo*, such as changes in chromatin organization and accessibility, and are a priority for future study. The next few years will deliver exciting molecular details of human embryology and provide a blueprint for the development of our own species.

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