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Human Embryonic Stem Cells as a Model for Studying Epigenetic Regulation During Early Development

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KEY WORDS
human embryonic stem cells, epigenetics, genomic imprinting, methylation

ABBREVIATIONS
hESCs human embryonic stem cells
mESCs mouse embryonic stem cells
SNP single nucleotide polymorphism
DMR differentially methylated region
BWS Beckwith-Wiedemann Syndrome
PRC2 polycomb repressive complex 2

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ABSTRACT
In order to exploit the exceptional potential of human embryonic stem cells (hESCs) in cell-replacement therapies, the genetic and epigenetic factors controlling early human development must be better defined. Limitations in human embryonic material restrict the scale of studies that can be performed, and therefore an in vitro model in which to study epigenetic regulation in human preimplantation cell types would be desirable. hESCs could provide such a model, but since they are derived from a stage in mammalian development when the genome is undergoing global epigenetic remodelling, it is unclear whether their epigenetic status would be stable or subject to variation. Herein, we discuss recent work that examines allele-specific imprinted gene expression and methylation patterns, thereby demonstrating that hESCs maintain a substantial degree of epigenetic stability during culture. Therefore, we suggest that hESCs could provide a model for studying epigenetic regulation during the early stages of human cellular pluripotency and differentiation. Furthermore, we propose specific experiments using such a model to address important questions pertaining to epigenetic mechanisms of certain human disorders.

Human embryonic stem cells (hESCs) are derived from the inner cell mass of the preimplantation stage human blastocyst. Their unique ability to self-renew in culture whilst retaining the ability to differentiate into a large number of somatic cell types confers upon hESCs an exceptional potential for cell-replacement therapies. Currently, much research is focused on differentiating hESCs towards clinically useful cell types, such as cardiomyocytes or dopaminergic neurons. Whereas many of the genetic factors accompanying lineage development in hESCs are known, the epigenetic changes remain poorly defined. Understanding how epigenetic regulation occurs in early human development could expedite our progress towards generating clinically useful cells. However, the practical and ethical limitations in obtaining human embryonic material restrict the scale of studies that can be performed. Therefore, hESCs could potentially provide an in vitro model for studying epigenetic regulation in early human development. However, this challenge is complicated further because hESCs are derived from a stage in mammalian development when the genome is undergoing global epigenetic remodelling. Therefore it is unclear whether the epigenetic status of hESCs would be stable or subject to variation upon differentiation. Because this fundamental issue of epigenetic stability must be addressed in order to determine whether hESCs could make a reproducible in vitro model, we recently undertook an epigenetic analysis of these cells. We used imprinted genes, whose regulation is parent-of-origin dependent, as an indication of epigenetic stability because their allele-specific expression and methylation patterns in mouse embryonic stem cells (mESCs) have been shown to be vulnerable to perturbation upon culture.

HESCs were cultured from middle passage (p40-p65) to high passage (p66-p155) under standard conditions. Confirmation of their undifferentiated state at all passages was routinely achieved by immunohistochemistry for typical hESC markers (SSEA1\textsuperscript{\textregistered}, OCT4\textsuperscript{\textregistered}, TRA-1-60\textsuperscript{\textregistered}; Fig. 1), and pluripotency was demonstrated by in vitro expression of markers characteristic of each of the three germ layers (ectoderm, beta-tubulin III; endoderm, alpha-fetoprotein; mesoderm, myosin; (Fig. 1) after differentiation. In order to distinguish mRNA transcripts from each parental allele we identified single nucleotide polymorphisms (SNPs) that differed between the two parental alleles of six imprinted genes. Interestingly, the three imprinted genes that are typically expressed from the paternally-inherited allele (IGF2, IPN, KCNQ1OT1) showed expression from only one allele in all hESC samples (undifferentiated and differentiated), which is consistent with the maintenance of normal imprinting of these genes. The three imprinted genes that are characteristically expressed from the maternally-inherited allele (H19, SLC22A18,
NESP55/GNA5 also generally showed expression from one allele only, but there was more variation in their expression than for the paternal expressed genes. For example, H19 was expressed from one allele in most hESC lines studied, but upon prolonged culture of one cell line, expression from the previously silent H19 allele was detected. After further passage, H19 eventually became equally expressed from both alleles in this cell line, which resulted in a measurable increase in H19 RNA compared to lower passage cells. The effect of increased H19 expression on hESC behavior is currently under investigation, although the cells appear morphologically normal (our unpublished observations).

Another maternally expressed imprinted gene, SLC22A18, showed predominant expression from one allele, although there was consistently some expression (~25%) from the ‘minor’ allele. Since the proportion of SLC22A18 minor allele expression stayed constant over a long period of culture (over 100 passages), we suggest that this is not an example of epigenetic instability as observed for H19, but rather an expression phenotype inherent to the cells. In humans, low levels of expression from the paternal allele have previously been reported for this gene.13 The third maternally expressed gene studied, NESP55, generally showed expression from one allele, although some expression (~20%) from the minor allele was observed in two out of five samples.

It is notable that the highest levels of expression detected from the minor allele were shown by imprinted genes characterized by maternal expression (i.e., repression of the paternal allele). Conversely, imprinted genes characterized by paternal expression appeared to maintain strict repression of the maternal allele. This indication that repression on the paternal chromosome might be less ‘stringent’ may thereby suggest differences in the epigenetic states of the maternally and paternally repressed chromosome homologues.

We also studied methylation patterns at the three key imprint control regions responsible for regulating many of the imprinted genes discussed above. Changes in methylation of these sequences are associated with loss of imprinting over large domains,14–16 and in humans, such changes are associated with the etiology of numerous epigenetic disorders.17 Our analysis revealed that there were normal patterns of methylation in each of the three imprint control regions investigated, which is indicative of normal epigenetic regulation at these key regions. Interestingly, from current understanding of H19 regulation we would have expected loss of methylation at the H19/IGF2 imprint control region on the paternal allele in the hESC line that expressed H19 from both chromosomes at high passage. We found instead that at the H19/IGF2 imprint control region as well as the H19 promoter, the normal differentially methylated pattern persisted despite biallelic expression. This suggests that factors other than DNA methylation could be involved in regulating this imprinted region in hESCs. Further examination of this hypothesis would not only yield important mechanistic information about the epigenetic regulation of the H19/IGF2 region, but also could provide insight into the interactions between various epigenetic mechanisms capable of regulating imprinted gene expression during early human development. Furthermore, loss of imprinting despite maintenance of methylation has implications for the diagnosis and understanding of disease mechanisms. Other potential epigenetic regulatory mechanisms in addition to DNA methylation include alterations in histone modifications (possibly mediated by Polycomb group proteins), as well as noncoding RNA-mediated mechanisms or other trans-acting factors. Each of these possibilities will now be briefly discussed in turn (Fig. 2).

Selected amino acids on the amino-terminal tails of histones can be post-translationally modified by acetylation, methylation, phosphorylation and ubiquitination to create a localized chromatin conformation that can influence gene expression.18 Imprinted regions are known to have specific and distinct histone modifications that are characteristic for each of the two parental alleles.19–23 For example, the maternal promoter of H19 is hyperacetylated and the paternal one is hypoacetylated at histone H4. Consequently, the region surrounding the maternal H19 allele is transcriptionally accessible and the region surrounding the paternal allele is repressive.19,20 Accordingly, one hypothesis for the loss of H19 paternal repression that we observed during prolonged culture of one cell line could be the acquisition of active chromatin marks on the paternal allele. Studies to resolve this are currently in progress.

Modifications of histones during development are known to involve Polycomb group proteins, which maintain long-term gene silencing by creating a transcriptionally repressive environment. For instance, during development, the Polycomb Repressive Complex 2 (PRC2) is responsible for initiating epigenetically repressed domains by methylating Lysine 27 of histone H3.24 This complex contains several components, including Eed, Ezh2 and Suz12, which are all
The de-repression of allele plays such a role in human development in hESCs as a model for studying epigenetic regulation during early development.

Figure 2. Potential epigenetic mechanisms by which imprinted gene expression could be regulated in hESCs. These include DNA methylation of imprint control regions; histone modifications, including those mediated by polycomb protein complexes; RNA-mediated repression; and other trans-acting factors [see text]. These mechanisms, acting together or independently, could account for the observed complexity of imprinted gene regulation during early mammalian development.

Noncoding RNA-mediated mechanisms also can act to repress gene expression through a number of different pathways, such as transcriptional cleavage of mRNA transcripts by RNA-interference, inhibition of translation, and transcriptional gene silencing by directing repressive complexes to specific DNA regions. Noncoding RNAs have been suggested to play a role in regulating imprinted gene expression, however, currently, there is no evidence of this mechanism being actively involved in H19 regulation.

An alternative mechanism that might explain our observations involves other trans-acting factors. One candidate factor is the CCCTC-binding protein, CTCF, which adheres to the unmethylated maternal allele, enabling downstream enhancers to interact with and transcribe the downstream H19 gene rather than the upstream IGF2 gene. Aberrant binding of CTCF to the methylated paternal H19 allele could result in activation of this allele. However, published evidence that CTCF does not bind when this region is methylated makes this possibility seem unlikely. Mutation of bases within the CTCF binding sites that do not affect methylation would also prevent binding of this factor but we did not detect any such change upon sequencing the appropriate regions. It remains to be seen whether CTCF is binding to the methylated paternal H19 allele in our cells. However, we did not detect any change in IGF2 expression in the samples that were biallelic for H19, suggesting that normal CTCF binding is probably unaffected. Nevertheless, we cannot rule out a role for other trans-acting repressive factors, such as loss of methyl-binding domain proteins within this region.

In view of the severity of human diseases (including some potentially associated with fertility treatments and certain cancers) that are related to epigenetic stability, a deeper understanding of human epigenetic mechanisms during early development is essential. Our study has shown that genomic imprinting is generally stable in hESCs, at least at the regions studied. This stability could be a reflection of robust derivation and culture environment, or alternatively, an indication of the substantial epigenetic stability of human blastocysts and the pluripotent cells derived from them. Of course, it is possible that other areas of the hESC genome not yet studied are adversely affected by in vitro culture. Indeed, recent work by others has suggested that methylation patterns in gene-associated CpG islands and in ribosomal repeat regions in hESCs can be variable in culture (Allegrucci C, Young L, personal communication). It is interesting to speculate that imprinted regions maybe less vulnerable to culture-based perturbations than compared to other areas of the genome. However, our observations that imprinted genes and the control regions studied so far are epigenetically stable is encouraging. This will allow workers in this field to use hESCs as a model to investigate epigenetic changes during the early stages of human cellular pluripotency and differentiation. Embryonic stem cells are an opportune model in which to study such epigenetic changes because it is likely that there is considerable reprogramming of their genome during their differentiation. Studies carried out on mESCs have shown that the undifferentiated state is epigenetically marked by histone modifications of active chromatin, and that within 24 hours of in vitro differentiation these marks are erased, the epigenome then becoming reprogrammed by specific repressive modifications. These observations are consistent with the hypothesis that stem cell differentiation is accompanied by a restriction in the set of genes that can be expressed. So far, most of the studies examining epigenetic changes in development have been carried out on mESCs. However, differences between human and mouse embryonic stem cells, such as gene expression profiles, gene regulatory mechanisms, genomic imprint stability, and possibly x-chromosome inactivation, provides a compelling case for complementary epigenetic studies of hESCs.

Determination of a more precise epigenetic profile of pluripotency, much like the 'stemness' signature of stem cell gene expression will provide useful insight into the functional relationship between epigenotype and cellular phenotype during development. More importantly, the subsequent changes associated with the first steps of differentiation into the various lineages need to be accurately mapped. Combining this epigenetic information with our existing understanding of genetic factors involved in hESC differentiation will expedite our progress towards generating safe, clinically useful cell types from them.
References


