Human Molecular Genetics, 2007, Vol. 16, Review Issue 2 **R243–R251**
doi:10.1093/hmg/ddm245

Status of genomic imprinting in human embryonic stem cells as revealed by a large cohort of independently derived and maintained lines

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Received August 9, 2007; Revised August 9, 2007; Accepted August 22, 2007

Investigation of the epigenetic stability of human embryonic stem cells (hESCs) is a crucial step for their use in cell-replacement therapies, as well as for assessing whether hESCs model epigenetic regulation in human pre-implantation cell types. To address these issues, we have examined the expression of imprinted genes in a previous study and more recently in 46 individual hESC lines as part of the International Stem Cell Initiative. Our results show that nearly all hESC lines examined possessed a substantial degree of epigenetic stability, despite differences in genetic background and in their derivation and initial propagation conditions. However, some hESCs did show loss of allele-specific expression, which could have implications for hESC differentiation and epigenetic stability (both *in vitro* and after clinical transplantation). A benefit of our and other recent studies of genomic imprinting in hESCs was the identification of imprinted genes that provide a useful indication of epigenetic stability. *SNRPN*, *IPW* and *KCNQ1OT1* were highly stable and thus appeared insensitive to perturbation; in contrast, *H19*, *IGF2* and *MEG3* were more variable and thus could potentially provide a sensitive indication of epigenetic status. In this review, we examine the differences between imprinted genes in their susceptibility to perturbation and discuss the potential molecular basis for these differences. This examination provides insight into the regulation of genomic imprinting in hESCs and the corresponding peri-implantation stages of human development.

INTRODUCTION

The key properties of self-renewal and pluripotency confer upon human embryonic stem cells (hESCs) considerable promise for regenerative medicine and for modeling early human development (1,2). Although much progress has been made recently in elucidating the epigenetic mechanisms that regulate these properties in embryonic stem (ES) cells (reviewed in 3), there are numerous questions that remain to be addressed. These include whether the same epigenetic mechanisms are used during early mammalian embryonic and stem cell development and how stable is the epigenetic status of pluripotent embryonic tissues upon prolonged *in vitro* culture as stem cells. Both of these questions are relevant not only to our basic understanding of development, but also to address safety concerns over assisted reproductive technologies (ART) and the use of stem cell-derived differentiated tissues in cell-replacement therapies.

This review will discuss recent work by ourselves and others that aim to address these questions. In particular, a recent global study of genomic imprinting stability in hESCs has revealed new insights into the epigenetic status of these cells (4), thus identifying common regulatory elements that appear particularly sensitive to *in vitro* perturbation in human pre-implantation cell types.

GENOMIC IMPRINTING

Genomic imprinting is an epigenetic process resulting in parent-of-origin specific preferential (monoallelic) expression. The majority of the identified imprinted genes (~80%) are physically linked in clusters, which is thought to facilitate

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their coordinate regulation (5), although a number of apparently isolated individual imprinted genes have also been identified (6,7). Imprinted gene clusters are often very large (several megabases in size) and there is usually a high conservation in cluster structure between mice and humans. Adding to the complexity of coordinate control, the same cluster often contains maternally expressed genes, paternally expressed genes and non-imprinted genes (Fig. 1) (5,8). Within many clusters, imprinting centers (ICs) have been identified; these elements regulate allele-specific expression of numerous genes within a large region.

The nature of genomic imprinting is that both parental chromosomes are present within the same diploid, somatic nucleus, and yet the transcriptional machinery of the cell is able to identify which chromosome should be expressed and which repressed. Extensive studies of genomic imprinting have revealed that this differential parent-of-origin specific expression occurs because imprinted genes and ICs carry distinguishing epigenetic marks, such as DNA methylation and histone modifications, on their controlling regions. Thus, transcriptional regulation of imprinted genes is predominantly epigenetic, making genomic imprinting an excellent model for studying epigenetic regulation.

Most DNA methylation occurs within differentially methylated regions (DMRs), which are CpG-rich sequences located within ICs with the methylation status distinguishing the two parentally inherited alleles. All ICs are germline DMRs (also called primary marks), in which the allele-specific DNA methylation is established in either the male or the female gamete, and is subsequently maintained throughout development. In contrast, other DMRs (sometimes referred to as somatic DMRs) have parental-allele-specific methylation that is established during embryonic development (secondary marks). The hierarchical relationship between epigenetic regulators is considered necessary to establish and maintain correct allele-specific imprinting (Fig. 2). Deregulation of genomic imprinting is associated with pre-natal lethality in mice and with numerous human pathologies, ranging from behavioral disorders to cancer (9–14).

CULTURE-INDUCED PERTURBATION OF GENOMIC IMPRINTING

Non-human embryos

Detrimental effects on stability of genomic imprinting caused by *in vitro* culture of embryos have now been reported in a number of species. Sasaki *et al*. (15) were the first to show in mice that *in vitro* fertilization and embryo culture results in biallelic expression of the imprinted gene *H19* in extraembryonic tissues. Subsequently, Khosla *et al*. demonstrated that fetuses generated after pre-implantation culture in serum-containing medium had gained methylation at the maternally inherited *H19* DMR, which was correlated with decreased *H19* expression (16). In addition, data from the Bartolomei group showed that when early mouse embryos were cultured in Whitten's medium (non-serum-containing), 63% of them become biallelic for *H19*, when compared to 14% of those cultured in potassium-containing simplex optimized medium (KSOM) supplemented with amino acids, and 6% of those developing as *in vivo* controls (17). Other imprinted genes examined, *Snrpn* and *Peg3*, were monoallelically expressed in the same blastocysts (17). In mid-gestation embryos, biallelic *H19* expression had persisted in placental tissues, but expression was often monoallelic in embryonic tissues (17). Therefore, it was possible that at the blastocyst stage, only the trophectoderm was biallelic for *H19* and that cells within the inner cell mass (ICM) were epigenetically stable. However, methylation analysis of immunosurgically isolated ICMs showed that there was a similar loss of methylation as observed for the entire blastocyst (17). Taken together, these data suggest that *in vitro* culture can alter imprinted gene expression and methylation in mouse embryos.

Human embryos

Infertile couples have been benefiting from ART, such as *in vitro* fertilization and intracytoplasmic sperm injection, for nearly 30 years, and these currently account for 1–3% of all births in developed countries (13). To improve ART efficiency rates, recent practice trends have been to increase the use of intracytoplasmic sperm injection, as well as to institute the use of *in vitro* oocyte maturation and to prolong embryo culture to the blastocyst stage (13). However, detrimental effects caused by *in vitro* culture and manipulation of mouse embryos have been well documented and therefore the possibility that similar perturbations may be occurring during ART had to be considered.

Case-controlled cohort studies from a number of countries have shown a statistically significant increase in the frequency of epigenetic disorders in ART children when compared to matched controls (18–21). For example, the average risk of developing Beckwith-Wiedemann syndrome (BWS) appears to be 4–6-fold higher after ART than in children conceived without ART. Of course, this increased prevalence may be due to other non-ART related effects, such as factors causing the infertility or other factors involved in the treatment of infertility, such as hormonal administration. There is some circumstantial evidence for such confounded effects: Ludwig *et al*. (22) reported that the longer the infertility of the couple, the higher the chance of a child with Angelman syndrome (AS). However, all these studies are complicated by the difficulty in assessing proper controls, such as having *fertile* couples undergo ART. Such control data may eventually become available as a result of fertile couples using pre-implantation genetic diagnosis (PGD) to select embryos free of recessive genetic disorders that the couples carry. These embryos are cultured *in vitro* (as for other ART procedures) before PGD and implantation of selected embryos into the mother. In most instances, the couples enlisted for PGD will already have conceived a child without the use of ART, thereby demonstrating their fertility. Hence, examination of PGD-originated children will thus reveal whether infertility is a contributory factor to the increased incidence of epigenetic disorders in ART children. Overall, the incidence of imprinting disorders is rare, even after ART, and is too low to justify screening all ART offspring (18–21). Nonetheless, sufficient evidence now exists for some link, however poorly understood, between *in vitro* embryo culture in mice (or ART in humans) and epigenetic abnormalities to compel further

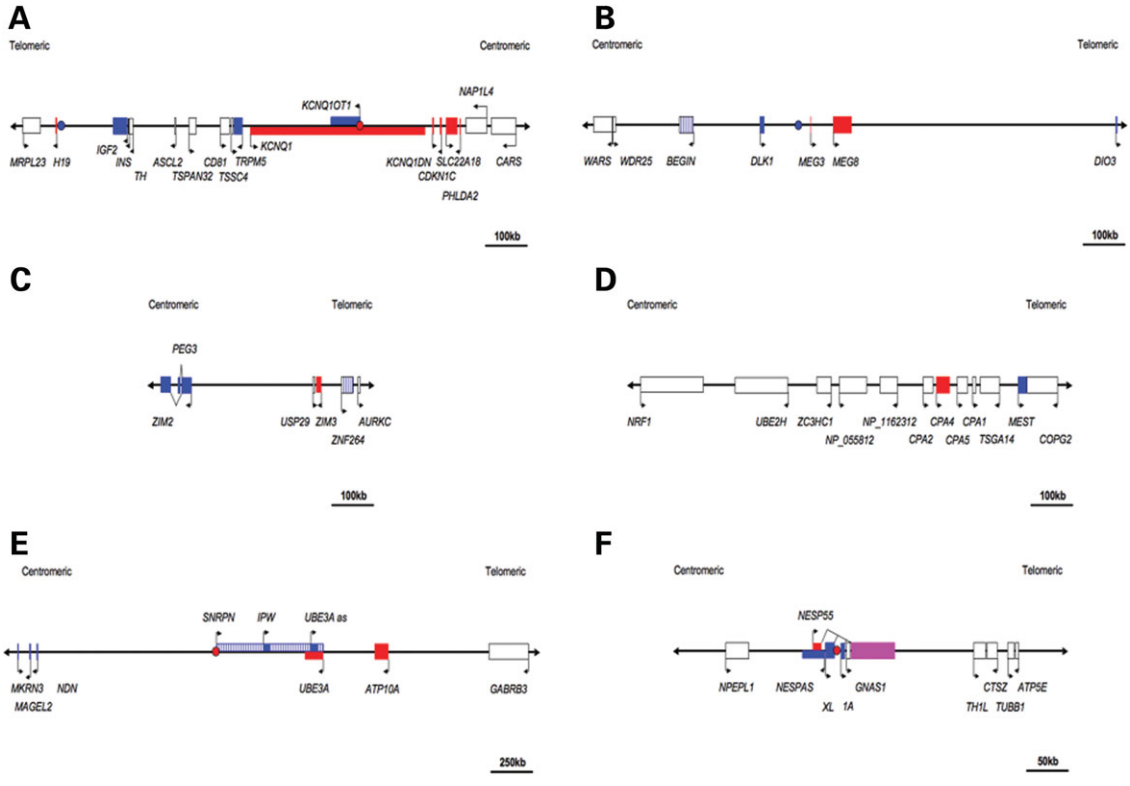

Figure 1. Scale maps showing the location and transcriptional information of genes within six human imprinted gene regions relevant to this review. (**A**) 11p15.5; (**B**) 14q32; (**C**) 19q13.4; (**D**) 7q32; (**E**) 15q11-q12; (**F**) 20q13. Genes colored in blue are paternally expressed; genes colored in red are maternally expressed; *GNAS1* (F) is colored purple as the transcript can be paternally or maternally expressed depending on the initiating exon. *BEGIN* and *ZNF264* are shaded blue as they have been shown to be paternally expressed in other species but no information is available for humans. *SNRPN* (E) is shaded blue because it consists of a complex and not fully characterized paternally expressed transcript containing numerous smaller transcripts (not shown). Filled circles represent DMRs: blue, paternally methylated; red, maternally methylated.

research to determine the mechanisms by which *in vitro* culture effects epigenetic stability in human blastocysts.

Embryonic stem cells

Since ES cells are derived from a period in mammalian development characterized by global epigenetic remodeling (23,24), it was not clear whether their imprinted gene expression and methylation patterns would be stable or subject to variation upon derivation and subsequent culture.

Direct evidence demonstrating that mouse embryonic stem cells (mESCs) can have altered imprinted gene expression came from Feil *et al*. (25), who detected biallelic expression and hypomethylation of the imprinted gene *Zrsr1* in mESCs. Furthermore, they found that in four mESC lines examined, imprinted gene expression was unstable, with variable expression from both parental alleles (26). Analysis of parentally inherited methylation suggested that mESCs can have appropriate epigenetic regulation at early passages, but even a short period in culture is sufficient to cause loss of methylation imprints in some cells (26). Importantly, these epigenetic perturbations persisted through post-implantation development, resulting in aberrant imprinted gene expression in the ES cell-derived fetus (26). Subsequently, a study by Humpherys *et al*. (27) concluded that expression levels of *H19* and *Mest* vary widely between individual mESC subclones. Such variability is likely to reflect epigenetic changes that occur during *in vitro* culture among sister cells derived from a single cell, which is consistent with the notion that the epigenetic state of ES cells is prone to instability (27). Therefore, imprinted gene expression and methylation patterns appear to be variable upon derivation and subsequent propagation of at least some mESCs.

Our own initial characterization of hESCs, as well as the data from two additional studies, showed that they possess a substantial degree of genomic imprinting stability in culture, in contrast to the mouse (28–30). Monoallelic imprinted gene expression was detected not only in undifferentiated hESCs, but this stability was retained after differentiation (28,30). The only loss of imprint stability reported in three studies was the appearance of biallelic *H19* expression in one subline of hESCs at high-passage number (28). Intriguingly, this loss of stability was independent of a change in allele-specific DNA methylation (28). These three studies were limited, however, by relatively small number of cell lines examined (8 in all) (28–30). Moreover, many of those lines were derived and maintained in similar culture conditions, making it difficult to generalize about the epigenetic status exhibited by hESC lines derived and maintained in other conditions. To provide a definitive assessment of

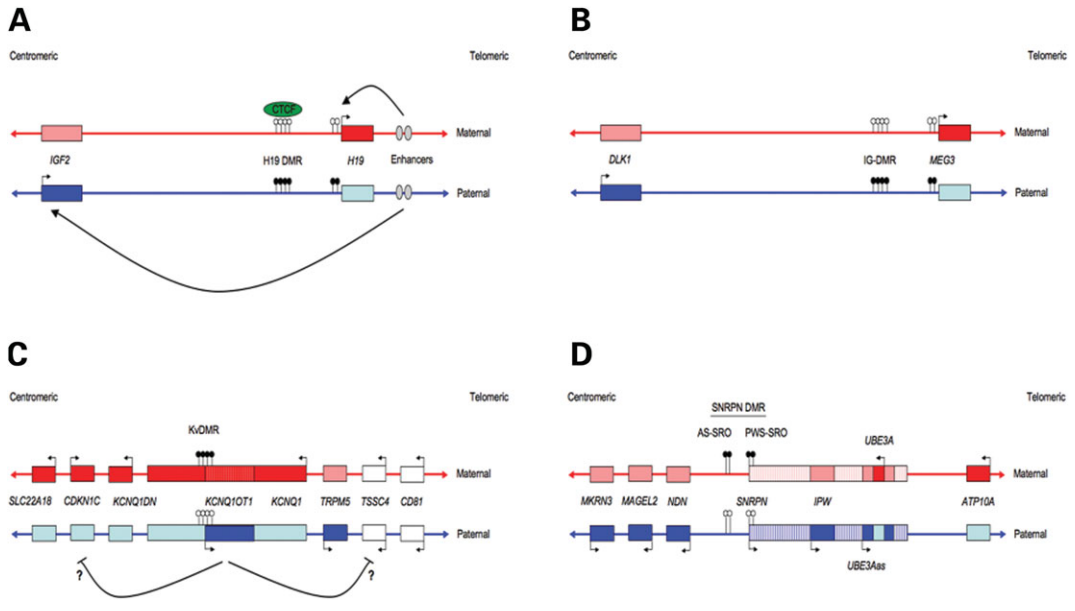

Figure 2. Schematic diagrams (not to scale) showing the position and methylation status of four DMRs in humans that are relevant to this review. Maternally inherited alleles are shown as red, paternally inherited alleles are blue. DMRs are indicated by contiguous 'lollipops'; filled, methylated; open, unmethylated. Non-imprinted genes are colored white. (**A**) The *H19* DMR is a germline-acquired paternally methylated region that acts as a chromatin insulator [binding CTCF and thus blocking enhancer-initiated *IGF2* transcription (curved arrows) when unmethylated on the maternal allele]. In addition, the hypermethylated paternal DMR directs post-fertilization silencing of *H19 in cis*, probably by spreading methylation to the *H19* promoter (64–66). (**B**) The IG-DMR is a germline-acquired paternally methylated region. Removal of the IG-DMR from the maternally inherited allele results in a maternal to paternal epigenotype switch, whereas removal from the paternally inherited allele has no effect (12). The *MEG3* promoter acquires post-fertilization methylation on the paternally inherited allele. (**C**) The KvDMR is a germline-acquired maternally methylated region. On the paternally inherited allele *KCNQ1OT1* is expressed and is likely to initiate domain-wide chromatin repression (blunt, curved arrows), leading to the inactivation of genes *in cis* (35,36). (**D**) The *SNRPN* DMR is a germline-acquired maternally methylated region composed of two distinct functional components, the PWS-SRO, which appears to be required for maintenance of the paternal epigenotype in somatic cells (9,67), and the AS-SRO, which appears to be required for establishment of the maternal epigenotype during oogenesis (68). How the PWS-SRO and AS-SRO function in establishing and maintaining imprinted gene expression across the domain is still unclear, although recent observations show that there is allele-specific transcription factor binding to certain regions within the *SNRPN* promoter, which may provide some clues to the mechanisms involved (69).

genomic imprinting stability, we (PJR-G and RAP) have recently participated in the International Stem Cell Initiative, which enabled us to examine allele-specific expression of imprinted genes in 46 independently derived hESC lines. The results of this initiative have recently been published (4), and here we expand upon its genomic imprinting aspects, discussing how these data, together with previous studies, contribute to our current understanding of epigenetic regulation in early human development.

EXAMINATION OF IMPRINTED GENE EXPRESSION IN A LARGE COHORT OF HUMAN EMBRYONIC STEM CELL LINES

We examined the expression of 10 different imprinted genes, which had distinguishing parental single nucleotide polymorphisms (SNPs) in DNA and RNA samples from 46 hESC lines, provided by participants in the International Stem Cell Initiative (ISCI). We found that many imprinted genes examined were monoallelically (i.e. stably) expressed in the majority of mRNA samples obtained from these hESC lines (4). For example, in all samples, monoallelic expression of the paternally expressed genes *IPW*, *SNRPN*, *KCNQ1OT1* and *PEG3* was detected. In addition, the majority (37/52) of hESC samples showed monoallelic expression of *IGF2*. Some variations were observed, however, for example TE03 hESC line samples provided by one laboratory expressed *IGF2* biallelically, but in contrast, TE03 samples from another laboratory showed relatively normal *IGF2* expression. Intriguingly, array analysis showed that these same respective samples had contrasting *XIST* expression (4), implying that there could be widespread epigenetic differences between the two TE03 sublines. Nevertheless, the disparity of allele-specific *IGF2* expression between TE03 samples suggests that the epigenetic regulation of this gene is dependent on the culture conditions, rather than being an inherent property of each cell line. Other examples of variable *IGF2* expression included the CCTL-9 hESC line, which initially expressed *IGF2* monoallelically, but after further passaging, the same cell line was biallelic for *IGF2*. This suggests that epigenetic mechanisms regulating *IGF2* expression are sensitive to culture-based perturbations in CCTL-9 cells. Overall, allele-specific *IGF2* expression appears to be relatively stable, although some differences were observed between cell lines, differentiation status and passage number.

The paternally expressed imprinted gene, *MEST*, showed biallelic expression in half of the samples (20/38), with the remaining showing monoallelic expression (nine samples) or partial-allelic expression (nine samples) (monoallelic defined as 0–14% minor allele contribution to total gene expression, partial-allelic as 15–29% minor allele contribution and

biallelic as 30–50% minor allele contribution). Allele-specific expression of *MEST* did not differ from this pattern when a particular cell line was cultured in separate laboratories, suggesting that *MEST* allele-specific expression is inherent to each particular cell line and not dependent on the culture conditions within the individual laboratory. However, this may not provide an accurate reflection of the potential for epigenetic perturbation in hESCs, as there is currently no evidence that the variable *MEST* expression in hESCs is any different from the variation observed in human blastocysts (31).

The majority of maternally expressed imprinted genes examined also exhibited monoallelic expression. For example, monoallelic *H19* expression was detected in all samples except one (53/54), suggesting that *H19* allele-specific expression is generally stable in hESCs, at least at the passage numbers examined in the recent study (4), in contrast to our previous findings for *H19* in a subline of H9 hESCs (28).

Monoallelic expression of the maternally expressed *GNAS*-associated transcript *NESP55* was detected in all samples except one (11/12). Similarly, allele-specific expression analysis of the maternally expressed gene *MEG3* (also known as *GTL2*) revealed monoallelic expression in nine samples and biallelic expression in three samples. All three samples exhibiting biallelic expression were from the same hESC line, HES-4, which was cultured in two separate laboratories. This suggests that the imprint stability of *MEG3* was also inherent to the individual cell line, rather than strictly dependent on the culture conditions.

In contrast, monoallelic expression of the maternally expressed gene *SLC22A18* was detected in only one sample, with partial-allelic expression in six and biallelic expression in 16 samples. Genomic imprinting of *SLC22A18* in humans is tissue specific (32,33), and this gene does not appear to be consistently expressed in an allele-specific pattern in hESCs.

These findings indicate that for the imprinted genes examined, epigenetic instability is a rare occurrence in hESCs, and in those cases may reflect either an inherent property of the gene itself during early development, or of the particular hESC line. This general stability across numerous hESC lines is striking, considering that most of the cell lines have been derived in different laboratories, often using different techniques for ICM isolation and propagation. Moreover, the different hESC lines were cultured for various durations before the onset of the study, and each cell line was cultured according to the protocols of the individual participating laboratory prior to being included in the ISCI study. Although most samples provided for the ISCI analysis were ultimately cultured in identical cell culture medium, this transition occurred relatively late in the culture history of each hESC line. Thus, any epigenetic changes that may have occurred before transfer to the ISCI standard conditions would likely have been maintained by the cells. As no consistent epigenetic changes were consistently detected in any of the studies to date of imprinted gene expression in hESCs, it appears that epigenetic stability is an inherent property of most hESCs.

Why should hESCs possess such epigenetic stability? Given the maintenance of monoallelic expression of most imprinted genes during normal *in vivo* development, it is reasonable to conclude that human peri-implantation embryos have robust mechanisms to maintain imprinted gene expression. Although it is possible that epigenetic changes can occur during *in vitro* culture of human blastocysts, the frequency of such epimutations appears low (19–21,34), thus reinforcing the concept of general epigenetic stability at this stage of human development.

There could be multiple, complementary, stage-dependent epigenetic mechanisms regulating genomic imprinting in hESCs. Evidence for such complementarity in hESCs is provided by the observation that monoallelic imprinted gene expression often persists in the absence of normal differential methylation at the regulatory DMR. For example, despite loss of its IC methylation (KvDMR), *KCNQ1OT1* was monoallelically expressed in the CCTL-14 and HUES-17 cell lines (PJR-G, unpublished observations). Thus, it is likely that there are epigenetic mechanisms other than DNA methylation that maintain allele-specific repression in hESCs. Some mESCs have allele-specific histone modifications at the KvDMR (35,36). In mouse extraembryonic tissues, such histone modifications are thought to maintain imprinted gene expression in the absence of DNA methylation (35,36). It is therefore possible that hESCs also possess allele-specific histone modifications, and that these are sufficient to maintain monoallelic *KCNQ1OT1* expression even if DNA methylation is perturbed. It would be interesting to investigate allele-specific KvDMR histone modifications in hESCs and thereby ascertain whether these modifications alone are able to account for the maintenance of monoallelic *KCNQ1OT1* expression in the hESC lines that are devoid of KvDMR methylation. However, if hESCs have multiple, overlapping mechanisms to maintain imprinted gene expression, could this render genomic imprinting *too stable* for use as an indicator of their overall epigenetic status?

IMPRINTED GENE EXPRESSION AS AN INDICATOR OF EPIGENETIC STATUS IN HUMAN EMBRYONIC STEM CELLS

A benefit of the recent studies of imprinted gene expression in hESCs is the identification of genes that can potentially provide a useful indication of epigenetic stability. *SNRPN*, *IPW* and *KCNQ1OT1* show highly stable monoallelic expression patterns; in contrast, *H19*, *IGF2* and *MEG3* were more variable and could therefore provide a more sensitive indication of epigenetic status. Why imprinted gene expression was variable for these particular three genes is currently unclear, as there is no obvious pattern (e.g. favoring de-repression of maternally or paternally expressed genes). However, from the cumulative data provided by all the studies to date, we suggest three possible explanations for the observed patterns of allele-specific imprinted gene expression in hESCs. First, the developmental onset of transcription may influence imprinted gene expression (Table 1); secondly, a particular imprinted gene's expression may differ depending on whether it is regulated by maternally or paternally inherited methylation (Table 1); and thirdly, the pattern of imprinted gene expression may depend on whether the gene provides a growth advantage to hESCs.

Table 1. Summary of imprinted gene expression in human embryonic stem cells

	Parentally inherited expression	Timing of gene activation during development[a]	Imprint instability in hESCs[b]
Regulated by paternally inherited methylation			
H19	Maternal	Peri-implantation (h)	Variable
IGF2	Paternal	Pre-implantation (h)	Unstable
MEG3	Maternal	Pre-implantation (m)	Variable
Regulated by maternally inherited methylation			
SNRPN	Paternal	Pre-implantation (h)	Stable
IPW	Paternal	Pre-implantation (h)	Stable
SLC22A18	Maternal	N.D.	Unstable
MAGEL2	Paternal	N.D.	Stable
KCNQ1OT1	Paternal	Pre-implantation (m)	Stable
KCNQ1	Maternal	Post-implantation (h)	Stable
PEG3	Paternal	Peri-implantation (m)	Stable
MEST	Paternal	Pre-implantation (h)	Unstable
NESP55	Maternal	N.D.	Variable
PEG10	Paternal	N.D.	Stable

[a]Where reported, expression data for human embryos is used (h), otherwise mouse expression data is used (m). N.D., expression not determined in embryo.
[b]Definition of terms used to characterize stability of imprinted gene expression. Stable: imprinted gene is always monoallelically expressed; variable: expression is monoallelic in the majority of hESC lines but biallelic expression is occasionally detected; unstable: frequently shows biallelic expression.
References: (4,17,28–31,35,38,70,71).

Timing of imprinted gene activation during development

The two imprinted genes studied on chromosome 15, *SNRPN* and *IPW*, were always monoallelically expressed, thus demonstrating marked epigenetic stability. Imprinted gene expression within the orthologous region in mESCs is also very stable (37). The reason for such regionalized stability is unclear, but the observations clearly suggest that this imprinted domain is epigenetically robust in ES cells. In contrast to many other imprinted genes, *SNRPN* and *IPW* are highly expressed in undifferentiated hESCs (data not shown) (30). Furthermore, *SNRPN* is monoallelically expressed from the eight-cell stage onwards in human embryos (38), suggesting that the epigenetic mechanisms regulating *SNRPN* imprinting are likely to be already well established at the developmental stage when hESCs are derived. Nevertheless, such stability in hESCs during culture renders *SNRPN* and *IPW* imprinted gene expression an insensitive indicator of the overall epigenetic status of hESCs.

Another imprinted gene always monoallelically expressed in hESCs was *KCNQ1OT1*. In mouse development, this gene is expressed exclusively from the paternally inherited allele in late morulae (35), and this transcription is likely to be critical for establishing epigenetic regulation at the KvDMR domain (35,36). Although *KCNQ1OT1* expression has not been studied in early human development, the clear evolutionary conservation between the mouse and human KvDMR domains suggests that the human domain is similarly regulated. Therefore, by the stage of human development when hESCs are derived, it is likely that allele-specific chromatin modifications at the KvDMR have already been established. Thus, epigenetic regulation of this domain is likely to be already established in human blastocysts and this pattern would be expected to persist in the hESCs derived therefrom. In sum, allele-specific *KCNQ1OT1* expression may also be an insensitive indicator of overall epigenetic status of hESCs due to the stability of this domain in hESCs.

Although *H19* and *IGF2* were generally monoallelically expressed in hESCs, some samples showed variable allele-specific expression. Imprinted gene expression and methylation patterns within the *H19–IGF2* imprinted domain are commonly altered in mouse embryos and mESCs upon *in vitro* culture (15,17,26,27,39). When compared to *SNRPN* (described above), *H19* and *IGF2* are expressed at low levels in undifferentiated hESCs (data not shown) (30). Furthermore, the initiation of monoallelic expression of *H19* and *IGF2* occurs at a later stage of mammalian development when compared with *SNRPN* (40–42). During mouse development, the timing of transcriptional activation within the *H19–Igf2* region seems to occur at the peri-implantation stage. Accordingly, epigenetic regulation of the *H19–IGF2* domain may not be fully established at the time of ES cell derivation. Therefore, allele-specific expression of *H19* and *IGF2* may be sensitive indicators of epigenetic perturbations in ES cells, and thus represent a useful index of the overall epigenetic status of hESCs in culture.

In any case it is clear that the *in vitro* culture environment can alter *H19* and *IGF2* imprinted gene expression. Thus, as for mouse ESCs and embryos (16,17,39), culture conditions can have a significant effect on the epigenetic status of hESCs. During future assessment and refinement of hESC culture conditions, such effects of *in vitro* environment should be considered. In sum, imprinted gene expression of *H19* and *IGF2* appears to be useful indicators of the epigenetic stability of hESCs, and thus for retention of their developmental normality during derivation and subsequent culture.

Are paternally methylated ICs more easily perturbed in culture than maternally methylated ICs?

Gene-specific differences in epigenetic stability could also arise due to the asymmetrical epigenetic development of parental pronuclei immediately after fertilization, when the paternal genome is specifically and rapidly demethylated (43–48). Whereas the majority of imprinted genes examined in this study are regulated by ICs containing maternally inherited methylation; *H19*, *IGF2* and *MEG3* are regulated by paternally inherited methylation (Fig. 2). Although the *H19* DMR and IG-DMR (the respective ICs for these genes) appear protected from this event (49,50), it is possible that some of their methylation may be lost and thus predispose these regions to culture-based perturbations. Furthermore, methylation of secondary DMRs at *H19* and *MEG3* appears to be required for the long-term imprint stability of these genes. Such epigenetic stabilization is likely to occur just prior to or during implantation, and therefore could be absent or perturbable upon ES cell derivation. Further examination of the molecular basis for differences between imprinted genes in their susceptibility to perturbation may provide insight into the regulation of genomic imprinting in hESCs

and the corresponding peri-implantation stage of human development.

Although allele-specific expression of *H19* and *IGF2* was generally stable in most hESC cells, some variation was observed between lines. For example, the lines CCTL-9, CCTL-14, HUES-17 and TE03 consistently expressed *IGF2* from both alleles. Why these particular lines appear to be more sensitive to epigenetic perturbation within the *H19–IGF2* domain when compared with other hESC lines is unclear. There is no published evidence to provide insight into why, for example, HUES-17 appears to be more epigenetically unstable that HUES-5, as the lines were derived and propagated in the same laboratory using identical reagents (51). One possible explanation for this difference is that the epigenetic variation is a reflection of differences between individual *in vitro* fertilization-generated human pre-implantation blastocysts. This could render some hESCs more susceptible to epigenetic perturbations that other hESC lines. Interestingly, hESCs have been derived from slightly different stages of human development (52,53). Therefore, it is possible that some hESCs lines were established from human blastocysts just prior to, or during, the acquisition of promoter methylation within the *H19–IGF2* domain, and thus are likely to be prone to epigenetic perturbations. In contrast, those hESC lines derived from a slightly later stage of human development, when the epigenetic regulation within the *H19–IGF2* domain may be more established, are likely to be less prone to epigenetic perturbations at this region, but an assessment of this hypothesis would require further studies of hESCs derived at different developmental stages.

A selective growth advantage in human embryonic stem cells

Loss of imprint stability could lead to a growth advantage of hESCs, thereby increasing the likelihood of its persistence and detection. This hypothesis could explain the apparent gene-specific differences in imprint stability. It is possible that loss of imprint stability in hESCs is generally stochastic and can occur with similar frequency for different imprinted genes, but only those genes whose upregulation confers a growth advantage would be detected. For example, a recent report showed that IGF2 may be an important survival factor for hESCs (54); therefore, hESCs with increased *IGF2* expression (caused by upregulation of transcription and/or loss of imprint stability to biallelic expression) may have a selective advantage in culture. This could explain the observed higher incidence of biallelic *IGF2* expression when compared with other imprinted genes in hESCs (4). This hypothesis could be tested by comparing the survival behavior of hESCs that are either monoallelic or biallelic for *IGF2*.

Consequences of loss of imprinted gene stability in human embryonic stem cells

Loss of stable imprinted gene expression, leading to biallelic expression, changes the dosage of the corresponding gene products and the relative biochemical activities of the pathways they mediate. Depending on the gene in question, this increase in expression may result in changes in hESC behavior and/or differentiation. The developmental consequences of altered expression patterns of imprinted genes in hESCs are currently unknown. Many imprinted genes are involved in tissue differentiation during development and there is some evidence that overexpression of certain imprinted genes in ES cells may result in their biased differentiation towards specific tissues (55). Therefore, particular hESC lines may be better suited to differentiation along specific cell lineages than others as a consequence of their epigenetically determined patterns of gene expression.

Finally, biallelic expression of *H19*, *IGF2*, *MEST* and *MEG3* has been correlated with various human pathologies, including cancers (56–62). The obvious concern is using differentiated hESCs that aberrantly express one or more of these genes in cell replacement therapies. Therefore, certain hESCs lines, such as those exhibiting monoallelic gene expression after differentiation, may be better suited to therapeutic use than hESC lines that show variable expression. It is nevertheless reassuring that mESCs with variable imprinted gene expression contribute to normal development *in vitro* in chimeric mice (27,63). Similarly, hESCs with variable imprinted gene expression may be capable of normal tissue function when transplanted and integrated into an existing organ in cell-based therapies. A systematic comparison of the growth and differentiation behavior between hESC lines that show altered allele-specific expression should address this issue.

CONCLUSIONS

Although allele-specific expression of the imprinted genes described here was generally stable, some variation was observed and this may have consequences for hESC differentiation and for their use in cell replacement therapy. As a number of imprinted genes are involved in early differentiation, subtle differences in their expression status could be responsible for the varying efficiencies of differentiation observed between hESC lines. Of further importance is that biallelic expression of *H19*, *IGF2*, *MEST* and *MEG3* has all been correlated with various human pathologies, including cancers. Whether hESCs that aberrantly express one or more imprinted genes would behave normally after transplantation, or whether they would cause problems such as proliferative abnormalities is uncertain, but clearly needs to be addressed.

ACKNOWLEDGEMENTS

We are grateful to the participants in the International Stem Cell Initiative, who provided the samples analyzed therein, and to the UK Stem Cell Bank for processing and distributing the samples for our analysis in that study. The International Stem Cell Initiative was led by Prof. Peter Andrews, University of Sheffield.

FUNDING

The International Stem Cell Initiative was sponsored and supported by the International Stem Cell Forum, with additional support from the UK Medical Research Council. P.J.R.-G. was supported by a MRC Studentship, A.C.F.-S. was

supported by BBSRC, MRC and EU-FP6 and R.A.P. was supported by a MRC International Appointments Initiative grant.

Conflict of Interests statement. None declared.

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