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Extra View

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.[1,2] 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.[3] Currently, much research is focused on differentiating hESCs towards clinically useful cell types, such as cardiomyoctyes[4] or dopaminergic neurons.[5] Whereas many of the genetic factors accompanying lineage development in hESCs are known,[6] 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 restricts 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 remodeling.[7,8] Therefore it is unclear whether the epigenetic status of hESCs would be stable or subject to variation upon their derivation and subsequent culture. Because this fundamental issue of epigenetic stability must be addressed in order to determine whether hESCs would make a reproducible in vitro model, we recently undertook an epigenetic analysis of these cells.[9] 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.[10,11]

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[12] (SSEA1^{-ve}, OCT4^{+ve}, SSEA4^{+ve}, TRA-1-60^{+ve}; (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*, *IPW*, *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*,

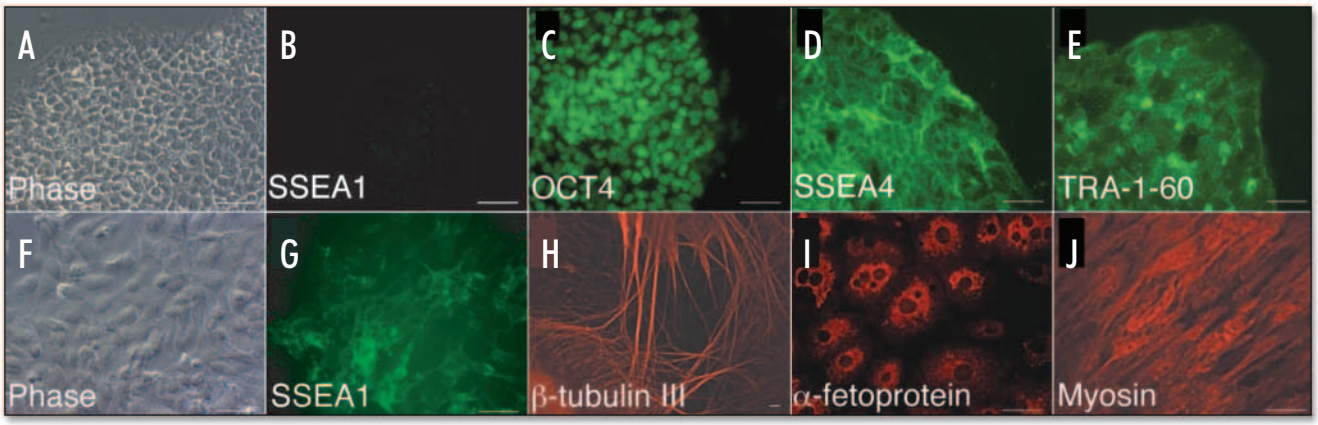

Figure 1. Images of hESCs when (A–E) undifferentiated and (F–J) after 20 days of differentiation. (A) Phase image showing a hESC colony; (B) the same colony is negative for SSEA1 staining demonstrating the absence of differentiated cells; (C) OCT4 (different colony); (D) SSEA4; (E) TRA-1-60; (F) phase image of differentiated cells; (G) the same differentiated hESCs show SSEA1 staining; (H) β-tubulin III as a marker for ectoderm lineage; (I) α-fetoprotein, endoderm; (J) myosin, mesoderm. Scale bars represent 50 μM.

NESP55/GNAS) also generally showed expression from one allele only, but there was more variation in their expression than for the paternally 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

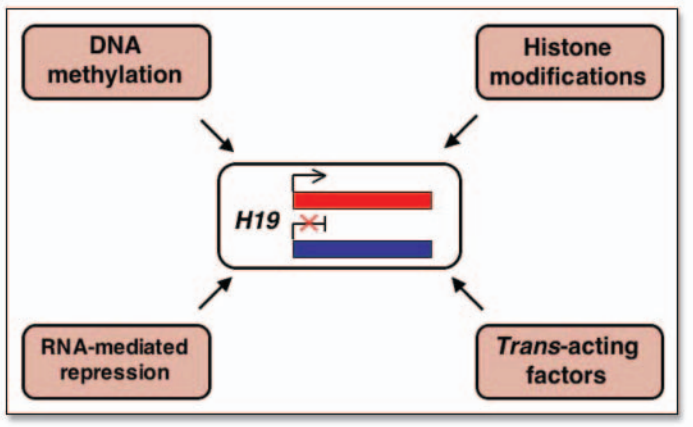

Figure 2. Potential epigenetic mechanisms by which imprinted gene expression could be regulated in hESCs. These include DNA methylation of imprint control regions; histone modifications, including those mediated by polycomb protein complexes; RNA-mediated repression; and other trans-acting factors (see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

essential for murine embryonic development as shown by their individual knockout phenotypes.[25-27] Interestingly, the mouse null mutation for the gene encoding *Eed* resulted in the de-repression of a subset of paternally repressed alleles.[28] Furthermore, this loss of imprinting did not involve specific changes in DNA methylation of the corresponding regulatory regions, implying that *Eed* acts downstream or even independently of DNA methylation. A possible mechanism for this was shown recently in mESCs, which exhibited allele-specific association of PRC2 components at specific imprinted genes within the KvDMR imprint control region.[23] This binding could potentially regulate localized repressive histone methylation marks. However, it remains to be determined whether *Eed* plays such a role in paternal-origin specific allelic activity. Also, since *H19* was not examined in the previous study of mouse development,[28] there is no basis for predicting whether our observed loss of paternal *H19* repression in hESCs could be partly caused by a change in *EED*. It has been suggested that loss of *EED* function in human development could be responsible for certain human epigenetic disorders, such as Beckwith-Wiedemann Syndrome (BWS).[28] Modifying *EED* in hESCs through loss and gain of function experiments[29,30] could provide researchers with a human in vitro model for testing this hypothesis.

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,[31] 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.[32,33] Mutation of bases within the CTCF binding sites that do not affect methylation would also prevent binding of this factor[34] 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[9] 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 epigenome 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.[35,36] 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.[37] 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,[38] gene regulatory mechanisms,[39] genomic imprint stability,[9] and possibly x-chromosome inactivation,[40] 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[41] 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

1. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. Science 1998; 282:1145-7.
2. Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: Somatic differentiation in vitro. Nat Biotechnol 2000; 18:399-404.
3. Bradley JA, Bolton EM, Pedersen RA. Stem cell medicine encounters the immune system. Nat Rev Immunol 2002; 2:859-71.
4. Passier R, Oostwaard DW, Snapper J, Kloots J, Hassink RJ, Kuijk E, Roelen B, de la Riviere AB, Mummery C. Increased cardiomyocyte differentiation from human embryonic stem cells in serum-free cultures. Stem Cells 2005; 23:772-80.
5. Yan Y, Yang D, Zarnowska ED, Du Z, Werbel B, Valliere C, Pearce RA, Thomson JA, Zhang SC. Directed differentiation of dopaminergic neuronal subtypes from human embryonic stem cells. Stem Cells 2005; 23:781-90.
6. Brandenberger R, Wei H, Zhang S, Lei S, Murage J, Fisk GJ, Li Y, Xu C, Fang R, Guegler K, Rao MS, Mandalam R, Lebkowski J, Stanton LW. Transcriptome characterization elucidates signaling networks that control human ES cell growth and differentiation. Nat Biotechnol 2004; 22:707-16.
7. Reik W, Dean W, Walter J. Epigenetic reprogramming in mammalian development. Science 2001; 293:1089-93.
8. Li E. Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet 2002; 3:662-73.
9. Rugg-Gunn PJ, Ferguson-Smith AC, Pedersen RA. Epigenetic status of human embryonic stem cells. Nat Genet 2005; 37:585-7.
10. Dean W, Bowden L, Aitchison A, Klose J, Moore T, Meneses JJ, Reik W, Feil R. Altered imprinted gene methylation and expression in completely ES cell-derived mouse fetuses: Association with aberrant phenotypes. Development 1998; 125:2273-82.
11. Humpherys D, Eggan K, Akutsu H, Hochedlinger K, Rideout IIIrd WM, Biniszkiewicz D, Yanagimachi R, Jaenisch R. Epigenetic instability in ES cells and cloned mice. Science 2001; 293:95-7.
12. Henderson JK, Draper JS, Baillie HS, Fishel S, Thomson JA, Moore H, Andrews PW. Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. Stem Cells 2002; 20:329-37.
13. Dao D, Frank D, Qian N, O'Keefe D, Vosatka RJ, Walsh CP, Tycko B. *IMPT1*, an imprinted gene similar to polyspecific transporter and multi-drug resistance genes. Hum Mol Genet 1998; 7:597-608.
14. Thorvaldsen JL, Duran KL, Bartolomei MS. Deletion of the H19 differentially methylated domain results in loss of imprinted expression of H19 and Igf2. Genes Dev 1998; 12:3693-702.
15. Fitzpatrick GV, Soloway PD, Higgins MJ. Regional loss of imprinting and growth deficiency in mice with a targeted deletion of KvDMR1. Nat Genet 2002; 32:426-31.
16. Bielinska B, Blaydes SM, Buiting K, Yang T, Krajewska-Walasek M, Horsthemke B, Brannan CI. De novo deletions of SNRPN exon 1 in early human and mouse embryos result in a paternal to maternal imprint switch. Nat Genet 2000; 25:74-8.
17. Paulsen M, Ferguson-Smith AC. DNA methylation in genomic imprinting, development, and disease. J Pathol 2001; 195:97-110.
18. Turner BM. Cellular memory and the histone code. Cell 2002; 111:285-91.
19. Pedone PV, Pikaart MJ, Cerrato F, Vernucci M, Ungaro P, Bruni CB, Riccio A. Role of histone acetylation and DNA methylation in the maintenance of the imprinted expression of the *H19* and *Igf2* genes. FEBS Lett 1999; 458:45-50.
20. Grandjean V, O'Neill L, Sado T, Turner B, Ferguson-Smith A. Relationship between DNA methylation, histone H4 acetylation and gene expression in the mouse imprinted Igf2-H19 domain. FEBS Lett 2001; 488:165-9.
21. Gregory RI, Randall TE, Johnson CA, Khosla S, Hatada I, O'Neill LP, Turner BM, Feil R. DNA methylation is linked to deacetylation of histone H3, but not H4, on the imprinted genes *Snrpn* and *U2af1-rs1*. Mol Cell Biol 2001; 21:5426-36.
22. Lewis A, Mitsuya K, Umlauf D, Smith P, Dean W, Walter J, Higgins M, Feil R, Reik W. Imprinting on distal chromosome 7 in the placenta involves repressive histone methylation independent of DNA methylation. Nat Genet 2004; 36:1291-5.
23. Umlauf D, Goto Y, Cao R, Cerqueira F, Wagschal A, Zhang Y, Feil R. Imprinting along the Kcnq1 domain on mouse chromosome 7 involves repressive histone methylation and recruitment of Polycomb group complexes. Nat Genet 2004; 36:1296-300.
24. Cao R, Zhang Y. The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. Curr Opin Genet Dev 2004; 14:155-64.
25. Faust C, Schumacher A, Holdener B, Magnuson T. The eed mutation disrupts anterior mesoderm production in mice. Development 1995; 121:273-85.
26. O'Carroll D, Erhardt S, Pagani M, Barton SC, Surani MA, Jenuwein T. The polycomb-group gene *Ezh2* is required for early mouse development. Mol Cell Biol 2001; 21:4330-6.
27. Pasini D, Bracken AP, Jensen MR, Denchi EL, Helin K. Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. Embo J 2004; 23:4061-71.
28. Mager J, Montgomery ND, de Villena FP, Magnuson T. Genome imprinting regulated by the mouse Polycomb group protein Eed. Nat Genet 2003; 33:502-7.
29. Vallier L, Rugg-Gunn PJ, Bouhon IA, Andersson FK, Sadler AJ, Pedersen RA. Enhancing and diminishing gene function in human embryonic stem cells. Stem Cells 2004; 22:2-11.
30. Zwaka TP, Thomson JA. Homologous recombination in human embryonic stem cells. Nat Biotechnol 2003; 21:319-21.
31. Sleutels F, Zwart R, Barlow DP. The noncoding Air RNA is required for silencing autosomal imprinted genes. Nature 2002; 415:810-3.
32. Bell AC, Felsenfeld G. Methylation of a CTCF-dependent boundary controls imprinted expression of the *Igf2* gene. Nature 2000; 405:482-5.
33. Hark AT, Schoenherr CJ, Katz DJ, Ingram RS, Levorse JM, Tilghman SM. CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. Nature 2000; 405:486-9.
34. Engel N, West AG, Felsenfeld G, Bartolomei MS. Antagonism between DNA hypermethylation and enhancer-blocking activity at the H19 DMD is uncovered by CpG mutations. Nat Genet 2004; 36:883-8.
35. Lee JH, Hart SR, Skalnik DG. Histone deacetylase activity is required for embryonic stem cell differentiation. Genesis 2004; 38:32-8.
36. Perry P, Sauer S, Billon N, Richardson WD, Spivakov M, Warnes G, Livesey FJ, Merkenschlager M, Fisher AG, Azuara V. A dynamic switch in the replication timing of key regulator genes in embryonic stem cells upon neural induction. Cell Cycle 2004; 3:1645-50.
37. Surani MA. Reprogramming of genome function through epigenetic inheritance. Nature 2001; 414:122-8.
38. Ginis I, Luo Y, Miura T, Thies S, Brandenberger R, Gerecht-Nir S, Amit M, Hoke A, Carpenter MK, Itskovitz-Eldor J, Rao MS. Differences between human and mouse embryonic stem cells. Dev Biol 2004; 269:360-80.
39. Rao M. Conserved and divergent paths that regulate self-renewal in mouse and human embryonic stem cells. Dev Biol 2004; 275:269-86.
40. Migeon B. Nonrandom X chromosome inactivation in mammalian cells. Cytogenet Cell Genet 1998; 80:142-8.
41. Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC, Melton DA. "Stemness": Transcriptional profiling of embryonic and adult stem cells. Science 2002; 298:597-600.