Published in final edited form as:

Mol Biotechnol. 2009 March ; 41(3): 201–207. doi:10.1007/s12033-008-9118-8.

Differential Expression of Epigenetic Modulators During Human Embryonic Stem Cell Differentiation

Sharla M. O. Phipps¹, William K. Love¹, Troy E. Mott¹, Lucy G. Andrews¹, and Trygve O. Tollefsbol^{1,2,3,4,✉}

¹ Department of Biology, University of Alabama at Birmingham, 175 Campbell Hall, 1300 University Boulevard, Birmingham, AL 35294-1170, USA

² Center for Aging, University of Alabama at Birmingham, Birmingham, AL 35294, USA

³ Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL 35294, USA

⁴ Clinical Nutrition Research Center, University of Alabama at Birmingham, Birmingham, AL 35294, USA

Abstract

Although the progression of aging and the diseases associated with it are extensively studied, little is known about the initiation of the aging process. Telomerase is down-regulated early in embryonic differentiation, thereby contributing to telomeric attrition and aging. The mechanisms underlying this inhibition remain elusive, but epigenetic studies in differentiating human embryonic stem (hES) cells could give clues about how and when DNA methylation and histone deacetylation work together to contribute to the inactivation of *hTERT*, the catalytic subunit of telomerase, at the onset of the aging process. We have confirmed the differentiation status of cultured hES colonies with morphological assessment and immunohistochemical stainings for pluripotent stem cells. In hES cells with varying degrees of differentiation, we have shown a stronger association between hES differentiation and expression of the epigenetic regulators DNMT3A and DNMT3B than between genetic modulators of differentiation such as c-MYC. We also propose a new model system for analyses of stem cell regions, which are differentially down-regulating the expression of *hTERT* and the actions of epigenetic modulators such as the DNMTs and histone methyltransferases.

Keywords

Aging; Epigenetics; Telomerase; Stem cells; DNMTs

Introduction

Stem cells, generally defined as progenitor cells able to differentiate into multiple lineages, have self-renewal ability and are able to repopulate the tissue of origin following transplant into a damaged recipient [1]. In addition to their use in transplantation therapy, stem cells are being used to observe genetic responses to environmental cues leading to cellular commitment to a differentiated phenotype as well as the onset of aging.

Embryonic stem (ES) cells are pluripotent cells surgically extracted from the inner cell mass of blastocysts. More specifically, human embryonic stem (hES) cells are highly proliferative, give rise to all tissue lineages, remain undifferentiated under stringent culture conditions with fibroblast growth factors and feeder cells, and express telomerase activity three to six times

greater than that expressed by MDA cells, an immortal human breast carcinoma cell line [2, 3].

The study of differentiated embryoid bodies (EBs) can reveal information about the early steps of differentiation. EBs are formed when ES cells are allowed to aggregate while grown in suspension or in media containing methyl cellulose. The formation of EBs involves the spontaneous differentiation of ES cells into various lineages and is used as a tool to study the expression of developmental markers and pluripotent lineage potential of mammalian cells. EBs are not immortal as evidenced by the fact that they are telomerase-negative and senesce from telomeric attrition after approximately 40 population doublings [4]. Recent studies have shown a global demethylation that occurs as cells transition from ES cells to EBs [5]. These studies also indicated that embryoid-derived cultures were telomerase-negative due to hypomethylation of the promoter of *hTERT*, the catalytic subunit of telomerase. These results suggest that epigenetic processes such as DNA methylation are involved in the transition of ES cells into EBs. While the general rule is that hypermethylation of a gene promoter contributes to the silencing of the downstream gene, the opposite seems to be true for a few genes [6]. One gene in particular, *hTERT*, is well known not only as an epigenetically regulated gene [7–10], but also a gene that becomes deactivated upon hypomethylation of its promoter [11,12]. In other words, it is possible that hypomethylation of the *hTERT* promoter in normal ES cells could lead to the silencing of telomerase, as was indirectly investigated in this study. Moreover, global trends of demethylation do not necessarily influence gene-specific effects of hyper- or hypomethylation that regulate gene transcription [13].

We have previously studied how DNA methylation and histone acetylation are involved in inactivating *hTERT* during early development using as a model embryonic human teratocarcinoma (HT) cells induced to differentiate with *all-trans* retinoic acid (ATRA) [10, 14]. These pluripotent HT cells are derived from nonseminomatous germ-cell tumors and have the potential to differentiate into somatic and extraembryonic tissues [15]. ATRA-induced morphological changes toward a more differentiated phenotype, decreased the growth rate of these cells, and reduced telomerase activity [10,14]. Bisulfite genomic sequencing revealed a gradual accumulation of methylation of CpG sites within the *hTERT* promoter [14]. Although this study indicated that hypermethylation of the *hTERT* promoter region led to the silencing of the downstream gene, these findings illustrate that DNA methylation plays a key role in the down-regulation of telomerase activity as cells differentiate, whether it be a hypomethylation or hypermethylation causing the change in expression.

hES cells express a number of markers that allow for characterization and separation based on differentiation status. One commonly used marker of hES cellular differentiation is alkaline phosphatase (AP) [16]. AP is highly expressed in hES cells and not expressed as highly in differentiated cells, making it an excellent means to determine differentiation status. Another marker used to assess differentiation is the cell surface glycoprotein fibronectin (FN) [14]. FN is involved in such processes as embryogenesis, cell migration, and cell adhesion, and is not expressed until the cells have begun to be committed to a certain lineage [17].

This study investigates the expression of genetic and epigenetic-mediated regulation of differentiation and *hTERT* expression during the course of normal cellular differentiation. To the best of our knowledge, the method employed in this study represents the first time that the interior of hES cells has been viewed via immunohistochemical staining and demonstrates a strong correlation between *hTERT* expression and DNMT3A/3B expression.

Methods

Cell Culture

Mitomycin C-treated mouse embryonic feeder fibroblasts (Chemicon International, Inc, Billerica, MA) were plated and grown on a thin layer of 0.1% gelatin (2×10^5 feeders per well of a 6-well plate). hES cells were obtained from Dr. Douglas Melton's laboratory (Harvard University) and cultured according to the protocol outlined at the WiCell Research Institute (http://www.wicell.org/index.php?option=com_content&task=section&id=9&Itemid=256). Cells were grown in hES DMEM/F12 supplemented with Knockout Serum Replacement, 1 mM L-glutamine, 0.1 mM β -mercaptoethanol, 1% nonessential amino acids, and 40 ng/mL basic fibroblast growth factor. In order to maintain cells in the undifferentiated state, hES colonies were grown on a layer of mitomycin C-inactivated mouse feeder cells and passaged once per week with Collagenase dispersion (Sigma-Aldrich, St. Louis, MO). In order to induce spontaneous differentiation, cells were grown without passaging for an additional 14 days after the expiration of the feeder layer.

Immunohistochemistry

ES colonies were harvested, fixed with 10% neutral-buffered formalin (Electron Microscopy Sciences, Hatfield, PA), processed with HistoGel (Richard-Allan Scientific, Kalamazoo, MI), and embedded in paraffin blocks. Sections were cut at 5 μ m each and processed with standard deparaffinization and antigen retrieval (BioGenex, San Ramon, CA). Endogenous peroxidase was quenched with 3% H_2O_2 . All primary antibodies were diluted 1:50. Antigens were localized with the RTU Vectastain Universal ABC Kit (Vector Laboratories, Inc, Burlingame, CA). Stains were allowed to develop for 1 min with the Stable liquid DAB Substrate/Chromogen system (Innovex, Parsippany, NJ). Sections were counterstained with Mayer's Hematoxylin (Sigma-Aldrich, St. Louis, MO), mounted in Aquamount (Vector Laboratories, Inc, Burlingame, CA), and photographed with the SPOT RT3 Digital Camera (Diagnostic Instruments, Sterling Heights, MI). Depicted photographs are representative of triplicate determinations.

Results

Stem Cell Differentiation Occurs Along a Characteristic Pathway

The compact colonies that are characteristic of undifferentiated stem cell growth begin differentiating at the periphery, eliminating colony boundaries, and flattening the formerly spherical colony shape. Cells around the periphery become elongated and almost neuronal in appearance and seem to merge with the underlying monolayer of mouse-feeder cells (Fig. 1), but the center of the colony remains partially raised. When colonies are fully differentiated, they form a characteristic clearing in the region where the center of the colony originated. In harvesting the differentiated colonies for analysis, it is impossible to separate the mouse feeders from the flattened stem cell growth. Consequently, mouse feeders are seen in a flat layer along the periphery of harvested differentiated sections. Figure 1 also shows hematoxylin and eosin staining of harvested stem cell colonies in the undifferentiated and fully differentiated state.

Differentiation Status Confirmed via Immunohistochemistry

The expression of FN is barely detectable in undifferentiated hES cells (Fig. 2), but is readily observed in both mouse-feeder cells and differentiated hES cells (right panel). An elevated level of AP also serves as a marker of undifferentiated hES cells and clear differences can be observed in the immunohistochemical stains of Fig. 2.

Positive hTERT Staining Only Seen in Undifferentiated hES Cells

hES colonies cultured on mouse-feeder fibroblasts with proper supplements in the media, which appeared undifferentiated by visual inspection maintained high levels of hTERT expression (Fig. 3). The only hTERT protein detectable in differentiated cultures was due to cross-reactivity of the hTERT rabbit monoclonal antibody (Epitomics) with the mTERT expressed in the feeder layer. The differences in expression patterns between mTERT and hTERT explain the manifestation of mTERT in the differentiated mouse-fibroblast cells. As early as 14 days into induced differentiation, *hTERT* is observed to be locked in the silenced state in the hES cells (Fig. 3).

De Novo DNMT Expression is Most Strongly Linked to Differentiation Status

DNMT1 staining was found to decrease during staining of hES cells (Fig. 3). Indirect immunostaining of undifferentiated stem cell sections showed a number of positive cell nuclei stained for DNMT3A/3B, but this staining was virtually absent in all sections of differentiated hES cultures observed (Fig. 3). We investigated the levels of dimethylated H3-K9 in the hES cells and observed comparable levels of positive staining in cells that are differentiated and undifferentiated (Fig. 4).

Genetic Markers of Cellular Maturity Examined in hES Cultures

In Fig. 4, sections exhibit c-MYC staining that disappears when the cells differentiate, but it is interesting to note that the lack of c-MYC expression also corresponds to absence of de novo methyltransferase activity.

Discussion

Substantial evidence indicates the fundamental role played by DNA methyltransferases (DNMTs) in gene regulation during development and differentiation. The major maintenance methyltransferase, DNMT1, shows a preference for hemimethylated DNA and appears to function in both differentiated and undifferentiated hES colonies (Fig. 3). The de novo methyltransferases DNMT3A and 3B are critical for early development and may have specific targets to regulate certain functional pathways during differentiation [18].

It has been shown that telomerase has a stabilizing effect on DNMT1 [19]. This stabilization may serve to maintain cells in a replicative state and, if observed in a variety of cell types, could partially explain the global decrease in DNA methylation, which occurs after the loss of telomerase activity as cells and tissues differentiate and age. In addition, the cluster of abundant CpG sites in the *hTERT* promoter region makes it a likely candidate for regulation by DNA methylation.

The genetic markers of cellular maturity examined in hES cultures are integrated with the regulation of telomerase during the course of normal cellular differentiation. The oncoprotein c-MYC has an activating effect on telomerase because of the two E-boxes in the *hTERT* core promoter [20,21]. Levels of c-MYC decrease and levels of the c-MYC-agonist MAD1 increase as cells differentiate. It is theorized that methylation of the E-boxes prevents c-MYC from binding and allows the MAD1-mediated repression of telomerase activity [22]. Be this as it may, we have a stronger correlation between hES differentiation and expression of the epigenetic regulators DNMT3A and DNMT3B than between genetic modulators of differentiation such as c-MYC.

Recent advances in stem cell and developmental biology provide exciting opportunities for advancement in the development of alternative disease treatments and regenerative medicine. The prospect of being able to reverse growth of fully differentiated cells along developmental

lineages and revert them back to totipotent, self-renewing “stem” cells could eliminate the moral, political, and ethical controversy surrounding stem cell research. The established significance of the de novo methyltransferases in early development associates them in processes such as nuclear reprogramming and somatic cell nuclear transfer. Epigenetic reprogramming of cells is dependent on the competence of the donor cell type and expression of a specific cadre of epigenetic regulators because less-differentiated cells are ultimately more successful in producing pluripotent cells. Consequently, the ability to reproduce a totipotent phenotype hinges on our ability to fully understand the processes that establish and maintain the undifferentiated state.

This study provides a novel view of the process of stem cell differentiation and illustrates a striking difference between levels of expression of the epigenetic modulators, the DNMTs. Because of the close interaction between epigenetic processes and cellular differentiation, we cannot rule out the possibility that other mechanisms besides DNA methylation serve to lock *hTERT* in the silenced state as cells initiate the aging process. The striking correlation between the lack of DNMT3A/3B expression and the *hTERT* expression, however, should lead to further mechanistic studies concerning the de novo methyltransferases and differentiation.

Utilizing the methodology for harvesting and processing hES cells during the course of differentiation, future studies could investigate the compartments of the stem cell colonies in which hTERT is being silenced. In the core of partially differentiated stem cells, hTERT should be readily detectable as the differentiation process has not yet reached the center. The fully differentiated periphery of stem cell colonies would have no telomerase activity and should show staining patterns similar to those observed in the harvested, differentiated colonies in this study. The region in which telomerase is in the process of being silenced may yield a wealth of information concerning the factors involved in cellular maturation. Confocal microscopy and double labeling experiments with nick-translated probes against the *hTERT* promoter (methylated, acetylated, forms, etc.) and antibodies against DNMTs, histone deacetylases, and histone methyltransferases would allow for the elucidation of the time course of telomerase silencing and the relative importance of these epigenetic modulators in this process.

Acknowledgements

The authors wish to extend sincere thanks to Hsu-Kun Wang in the Department of Biochemistry and Molecular Genetics at the University of Alabama at Birmingham for superb technical assistance and thank the Melton laboratory at Harvard University for the supply of human embryonic stem cells. This study was supported in part with grants from the Susan G. Komen for the Cure and the National Cancer Institute (R01 CA129415) as well as the Glenn Foundation for Medical Research.

References

1. Verfaillie CM, Pera MF, Lansdorp PM. Stem cells: Hype and reality. *Hematology* 2002;369–391.10.1182/asheducation-2002.1.369 [PubMed: 12446433]
2. Sperger JM, Chen X, Draper JS, et al. Gene expression patterns in human embryonic stem cells and human pluripotent germ cell tumors. *Proceedings of the National Academy of Sciences of the United States of America* 2003;100:13350–13355.10.1073/pnas.2235735100 [PubMed: 14595015]
3. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. *Science* 1998;5391:1145–1147.10.1126/science.282.5391.1145 [PubMed: 9804556]
4. Shambloott MJ, Axelman J, Littlefield JW, et al. Human embryonic germ cell derivatives express a broad range of developmentally distinct markers and proliferate extensively in vitro. *Proceedings of the National Academy of Sciences of the United States of America* 2001;98:113–118.10.1073/pnas.021537998 [PubMed: 11134532]
5. Kremenskoy M, Kremenska Y, Ohgane J, et al. Genome-wide analysis of DNA methylation status of CpG islands in embryoid bodies, teratomas, and fetuses. *Biochemical and Biophysical Research Communications* 2003;311:884–890.10.1016/j.bbrc.2003.10.078 [PubMed: 14623263]

6. Mukhopadhyay NK, Gordon GJ, Maulik G, Doerre G, Liu BC, Bueno R, et al. Histone deacetylation is directly involved in desilencing the expression of the catalytic subunit of telomerase in normal lung fibroblast. *Journal of Cellular and Molecular Medicine* 2005;9:662–669.10.1111/j.1582–4934.2005.tb00496.x [PubMed: 16202213]
7. Guilleret I, Yan P, Grange F, Braunschweig R, Bosman FT, Benhattar J. Hypermethylation of the human telomerase catalytic subunit (*hTERT*) gene correlates with telomerase activity. *International Journal of Cancer* 2000;101:335–341.10.1002/ijc.10593
8. Dessain SK, Yu H, Reddel RR, Beijersbergen RL, Weinberg RA. Methylation of the human telomerase gene CpG island. *Cancer Research* 2000;60:537–541. [PubMed: 10676632]
9. Devereux TR, Horikawa I, Anna CH, Annab LA, Afshari CA, Barrett JC. DNA methylation analysis of the promoter region of the human telomerase reverse transcriptase (*hTERT*) gene. *Cancer Research* 1999;59:6087–6090. [PubMed: 10626795]
10. Lopatina NG, Poole JC, Saldanha SN, Hansen NJ, Key JS, Pita MA, et al. Control mechanisms in the regulation of telomerase reverse transcriptase expression in differentiating human teratocarcinoma cells. *Biochemical and Biophysical Research Communications* 2003;306:650–659.10.1016/S0006-291X(03)01033-7 [PubMed: 12810068]
11. Guilleret I, Benhattar J. Demethylation of the human telomerase catalytic subunit (*hTERT*) gene promoter reduced *hTERT* expression and telomerase activity and shortened telomeres. *Experimental Cell Research* 2003;289:326–334.10.1016/S0014-4827(03)00281-7 [PubMed: 14499633]
12. Kitagawa Y, Kyo S, Takakura M, Kanaya T, Koshida K, Namiki M, et al. Demethylating reagent 5-azacytidine inhibits telomerase activity in human prostate cancer cells through transcriptional repression of *hTERT*. *Clinical Cancer Research* 2000;6:2868–2875. [PubMed: 10914736]
13. Lai SR, Phipps SM, Liu L, Andrews LG, Tollefsbol TO. Epigenetic control of telomerase and modes of telomere maintenance in aging and abnormal systems. *Frontiers in Bioscience* 2005;10:1779–1796.10.2741/1661 [PubMed: 15769667]
14. Liu L, Saldanha SN, Pate MS, Andrews LG, Tollefsbol TO. Epigenetic regulation of human telomerase reverse transcriptase promoter activity during cellular differentiation. *Genes Chromosomes and Cancer* 2004;41:26–37.10.1002/gcc.20058 [PubMed: 15236314]
15. Damjanov I, Horvat B, Gibas Z. Retinoic acid-induced differentiation of the developmentally pluripotent human germ cell tumor-derived cell line, NCCIT. *Laboratory Investigation* 1993;68:220–232. [PubMed: 7680083]
16. O'Connor MD, Kardel MD, Iosfina I, Youssef D, Lu M, Li MM, et al. Alkaline phosphatase-positive colony formation is a sensitive, specific, and quantitative indicator of undifferentiated human embryonic stem cells. *Stem Cells (Dayton, Ohio)* 2008;26:1109–1116.10.1634/stemcells.2007-0801
17. Goessler UR, Bugert P, Bieback K, Stern-Straeter J, Bran G, Hörmann K, et al. Integrin expression in stem cells from bone marrow and adipose tissue during chondrogenic differentiation. *International Journal of Molecular Medicine* 2008;21:271–279. [PubMed: 18288373]
18. Hsieh CL. In vivo activity of murine de novo methyl-transferases, *Dnmt3a* and *Dnmt3b*. *Molecular and Cellular Biology* 1999;19:8211–8218. [PubMed: 10567546]
19. Young JI, Sedivy JM, Smith JR. Telomerase expression in normal human fibroblasts stabilizes DNA 5-methylcytosine transferase I. *The Journal of Biological Chemistry* 2003;278:19904–19908.10.1074/jbc.M301685200 [PubMed: 12665523]
20. Poole J, Andrews L, Tollefsbol T. Activity, function, and gene regulation of the catalytic subunit of telomerase (*hTERT*). *Gene* 2001;269:1–12.10.1016/S0378-1119(01)00440-1 [PubMed: 11376932]
21. Liu L, Lai S, Andrews LG, Tollefsbol TO. Genetic and epigenetic modulation of telomerase activity in development and disease. *Gene* 2004;340:1–10.10.1016/j.gene.2004.06.011 [PubMed: 15556289]
22. Tollefsbol T, Andrews L. Mechanisms for telomerase gene control in aging cells and tumorigenesis. *Medical Hypotheses* 2001;56:630–637.10.1054/mehy.2000.1241 [PubMed: 11399111]

Fig. 1.

Phase contrast photography and hematoxylin and eosin staining of differentiating hES colonies. Colonies were induced to differentiate by growing them without routine passaging for an additional 2 weeks after the mouse feeders expired (a total of approximately 1 month). Phase contrast photographs were taken at 200 $\times$ and 100 $\times$ of undifferentiated and differentiating stem cell colonies, respectively. Hematoxylin and eosin staining of harvested hES colony sections were taken at 200 $\times$ magnification

Fig. 2. Molecular evidence of hES differentiation status. Immunohistochemistry staining for the glycoprotein FN that is only expressed after cells have committed to differentiation. Confirmation of the differentiation status of hES colonies with immunohistochemical staining for the marker AP. Photographs were taken at 200× magnification. *Arrows* indicate examples of staining for FN or AP. Insets are provided for close-up viewing of stains

Fig. 3. hTERT and DNMT immunohistochemistry. Photographs taken at 200 $\times$ illustrate the expression of the catalytic subunit of telomerase, hTERT. hTERT is only visible in differentiated sections in the mouse-feeder layer because of cross-reactivity of the antibody. The undifferentiated section displays abundant hTERT staining throughout the entire colony, both periphery and core. Staining for DNMT1 displays a decrease but not total loss of expression, whereas staining for DNMT3a/3b does indicate a drastic loss of expression. *Arrows* indicate nuclear staining examples

Fig. 4. Dimethylated histone 3 lysine 9 (H3-K9) and c-Myc immunohistochemistry. Photographs were taken at 200 $\times$ magnification. Staining for dimethyl-H3-K9 illustrates a clear increase in the level of histone methylation in differentiated cells. There is positive c-MYC staining in the undifferentiated section, but not in the differentiated section. *Arrows* indicate staining examples of nuclei. The graphical representation at the bottom shows the relative expression of the three epigenetic proteins, DNMT1, DNMT3a/3b, and dimethyl-H3-K9