BMB
reports

Epigenetic modification of retinoic acid-treated human embryonic stem cells

Hyun Sub Cheong[1], Han Chul Lee[2], Byung Lae Park[1], Hyemin Kim[3], Mi Jin Jang[3], Yong Mahn Han[3], Seun-Young Kim[2], Yong Sung Kim[2,] & Hyoung Doo Shin[1,4,*]*

[1]Department of Genetic Epidemiology, SNP Genetics, Inc., Seoul 153-803, [2]Medical Genomics Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, [3]Department of Biological Sciences and Center for Stem Cell Differentiation, Korean Advanced Institute of Science and Technology, Daejeon 305-701, [4]Department of Life Science, Sogang University, Seoul 121-742, Korea

Epigenetic modification of the genome through DNA methylation is the key to maintaining the differentiated state of human embryonic stem cells (hESCs), and it must be reset during differentiation by retinoic acid (RA) treatment. A genome-wide methylation/gene expression assay was performed in order to identify epigenetic modifications of RA-treated hESCs. Between undifferentiated and RA-treated hESCs, 166 differentially methylated CpG sites and 2,013 differentially expressed genes were discovered. Combined analysis of methylation and expression data revealed that 19 genes (*STAP2, VAMP8, C10orf26, WFIKKN1, ELF3, C1QTNF6, C10orf10, MRGPRF, ARSE, LSAMP, CENTD3, LDB2, POU5F1, GSPT2, THY1, ZNF574, MSX1, SCMH1,* and *RARB*) were highly correlated with each other. The results provided in this study will facilitate future investigations into the interplay between DNA methylation and gene expression through further functional and biological studies. [BMB reports 2010; 43(12): 830-835]

INTRODUCTION

Human embryonic stem cells (hESCs) are unique in their ability to maintain pluripotence. This property makes hESCs leading candidates for use in cell therapy and in studies on early human development. Retinoic acid (RA), the most potent natural form of vitamin A, plays an important role in mediating the growth and differentiation of both normal and transformed cells (1, 2). It is essential for many diverse biological functions including growth, vision, reproduction, embryonic development, differentiation of epithelial tissues, and immune responses (2). *In vitro*, RA induces differentiation of hESCs into a number of specific cell types.

Differentiation of a specific cell type involves the establishment of a precise epigenetic profile composed of genome-wide epigenetic modifications such as DNA methylation and histone modification. Since epigenetic modifications in gene areas regulate transcriptional activity, the epigenetic profile of the cell reflects the transcriptome, at least partially (3-5).

hESCs have been investigated using multiple techniques, including gene expression profiling, mitochondrial sequencing, immunocytochemistry, genotyping, functional assays, and DNA methylation assay (6-10). DNA methylation of the genome is the key to maintaining the differentiated state of hESCs (11, 12), and it must be reset during differentiation by RA treatment.

Differences between hESC lines with respect to gene expression profiles have been investigated before (13), and it has also been demonstrated that hESCs have unique DNA methylation profiles compared to other cell types, including embryonic germ cells, trophoblast stem cells, and several adult stem cell populations (8, 14). Key regulators of development such as Oct4 and NANOG are also controlled by epigenetic mechanisms (15, 16). However, a whole-genomic correlation study on DNA methylation and gene expression has not been reported.

The present study utilized DNA methylation and gene expression assays to generate whole-genomic methylation and gene expression profiles for both undifferentiated hESCs and RA-treated hESCs. These results provide valuable information that can be used to identify differentially methylated CpG sites and differentially expressed genes.

RESULTS

We applied a comprehensive DNA methylation profiling approach to assess the epigenetic states of three hESC lines (CHA3-hES, CHA4-hES, and SNUhES3) as well as their epigenetic modifications after RA treatment. A whole-genome DNA methylation assay method was used to analyze the methylation status of 27,578 CpG sites selected from more than

*Corresponding authors. Hyoung Doo Shin, Tel: 82-2-705-8615; Fax: 82-2-2026-4299; E-mail: hdshin@sogang.ac.kr; Yong Sung Kim, Tel: 82-42-879-8110; Fax: 82-42-879-8119; E-mail: yongsung@kribb.re.kr
DOI 10.5483/BMBRep.2010.43.12.830

Received 18 June 2010, Accepted 25 October 2010

Keywords: DNA methylation, Epigenetic modification, Gene expression, Human embryonic stem cell, Retinoic acid

14,000 well-annotated genes. We measured the overall methylation levels after RA treatment. We found that the average methylation level in the RA-treated hESCs (29.5%) was greater than in the undifferentiated hESCs (27.1%). The lower methylation level we obtained for the undifferentiated hESCs was expected since global hypomethylation has been reported often in embryonic stem cells (17).

To discover which CpG sites contribute the most to the epigenetic modification of hESCs by RA treatment, we compared the DNA methylation patterns between undifferentiated hESCs and RA-treated hESCs. This analysis produced a list of 166 CpG sites from 151 genes that significantly contribute to the separation of the two groups. Among them, the top 100 CpG sites, based on $|\Delta\beta|$, are shown in Fig. 1A. We then clustered all of the samples based on their relative methylation levels at these 166 CpG sites (data not shown). Three hESC lines were correctly aggregated into the two other major clusters, which comprise undifferentiated hESC lines and RA-treated cells.

We also investigated three hESC lines for differential expression of genes upon RA treatment. A total of 9,736 distinct genes (23% of the RefList) passed the expression criteria of a Detection Score $\geq$0.99. Among them, 2,013 genes were differentially expressed. We observed that 1,003 genes were upregulated ($>$1.5-fold) and 1,010 genes were downregulated ($<$0.66-fold). The extreme differences observed between undifferentiated hESCs and RA-treated hESCs are shown in Fig. 1B.

Combined analysis of methylation and expression data revealed that 19 genes (*STAP2, VAMP8, C10orf26, WFIKKN1, ELF3, C1QTNF6, C10orf10, MRGPRF, ARSE, LSAMP, CENTD3, LDB2, POU5F1, GSPT2, THY1, ZNF574, MSX1, SCMH1,* and *RARB*) were highly correlated with each other (Pearson correlation coefficient $\geq$0.8) (Supplementary Table 1).

To validate the methylation status of the highly correlated genes, we selected two genes (*CENTD3* and *MSX1*) and performed bisulfate sequencing. Bisulfate sequencing of 400-500 bp including Illumina probe position revealed hypermethylation (36.7% and 19.6%) in SNUhES3 cells after RA treatment that was consistent with the genome-wide DNA methylation (Supplementary Fig. 1).

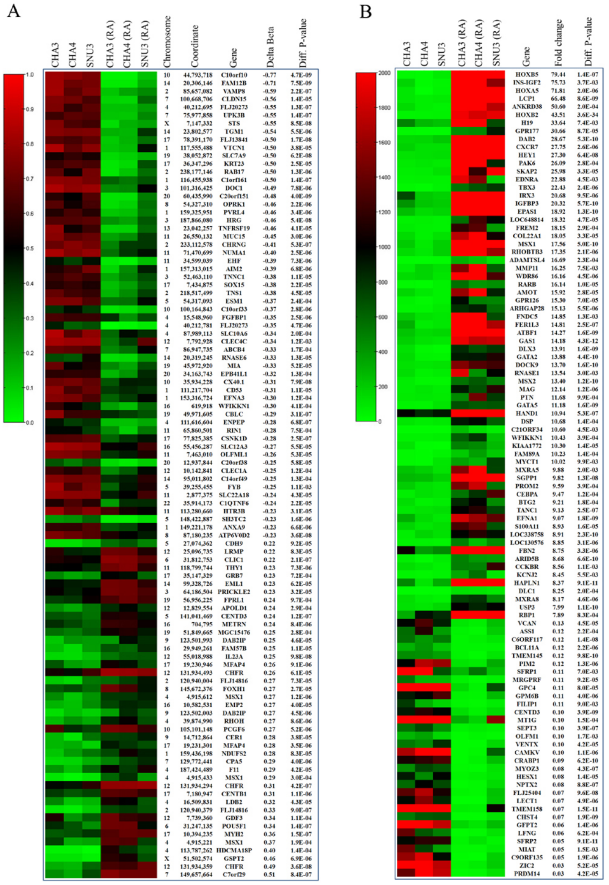

Fig. 1. Heatmaps of differential methylation and gene expression assays. (A) The 100 most differentially methylated CpG sites in undifferentiated hESCs and RA-treated hESCs. Sample IDs and their average beta values (methylation levels) are shown. Chromosome, coordinate, related gene name, delta beta value, and Diff. P values of each CpG site are also presented. (B) The 100 most differentially expressed genes in undifferentiated hESCs and RA-treated hESCs. Sample IDs and their average signal value (expression level) are shown. Gene name, fold change values, and Diff. P values are also presented.

DISCUSSION

Despite their differences in origin, different sample preparation methods, and karyotypes, three hESC lines were correctly aggregated into two other major clusters, which comprise undifferentiated hESC lines and RA-treated cells. This suggests that the three hESC lines share a common epigenetic signature, which is likely linked to embryonic stem (ES) cell-specific properties such as self-renewal and pluripotence.

CpGs on the *C10orf10, FAM12B, VAMP8, CLDN15,* and *FLJ20273* genes were the most hypomethylated, whereas *C7orf29, CHFR, GSPT2, HDCMA18P,* and *MSX1* were the most hypermethylated after RA treatment. Of these genes, methylation of *CHFR* is known to be associated with silencing of *CHFR* expression in various types of cancer (18), and *CHFR* is also known as a tumor suppressor (19). This means that cells in which *CHFR* was epigenetically inactivated constituted differentiated hESCs.

In order to define the relationship between methylation and expression of genes, we performed gene expression profiling to compare both methylation status and gene expression levels. Among the differentially methylated genes, *HOXB5, INS-IGF2, HOXA5, LCP1,* and *ANKRD38* were the most highly upregulated, whereas *PRDM14, ZIC2, C9orf135, MIAT,* and *SFRP2* were downregulated after RA treatment. Among these genes, *HOXA5* was found to be rapidly induced within mouse ES cells as a result of RA treatment (20). In addition, knock-

down of *PRDM14* by siRNA induced expression of early differentiation marker genes (21). These previous studies were remarkably consistent with our findings.

Combined analysis revealed that 19 genes were highly correlated with each other. Among them, we identified seven genes (*STAP2, VAMP8, C10orf26, WFIKKN1, ELF3, C1QTNF6* and *C10orf10*) that were hypomethylated and upregulated upon RA treatment. STAP2 is a signal-transducing adaptor molecule that binds to STAT3 and STAT5, resulting in regulation of integrin-mediated T-cell adhesion through protein degradation of focal adhesion kinase (22). VAMP8 is a member of the vesicle associated membrane protein (VAMP) family and is required for activation-induced degranulation of mature human mast cells (23).

MRGPRF, ARSE, LSAMP, CENTD3, LDB2, POU5F1, GSPT2, THY1, and *ZNF574* were identified as hypermethylated and downregulated (Supplementary Table 1). Among these nine genes, *POU5F1* (also known as *Oct4*) is a transcription factor previously shown to be expressed only in pluripotent cells of the embryo where it promotes differentiation when downregulated (24-26). RA-induced differentiation of a human embryonic carcinoma cell line into neurons is also accompanied by sequential DNA methylation of the promoter regions of *POU5F1* (27). *THY1*, which plays a critical role in maintaining the undifferentiated status of ES cells, was also hypermethylated and downregulated (correlation P value = 0.02) (28, 29). The mechanism of *THY1* gene inactivation due to hypermethylation has been previously determined (30, 31).

Three genes (*MSX1, SCMH1*, and *RARB*) did not fit in the standard paradigm of extensive methylation being correlated with gene silencing. In previous studies, upregulation of *RARB* was reported in RA-treated embryonic stem cells and cancer cells (32, 33). In this study, *RARB* was also hypermethylated and upregulated in RA-treated hESCs (Supplementary Table 1). This methylation, unlike the common epigenetic paradigm, shows positive correlation between the methylation of two upstream CpG sites and gene expression. Our results indicate that methylation of the upstream CpG sites in these hESC lines was correlated with an increase in *RARB* expression. The data further suggest that methylation of CpG sites is required for a cell to express high levels of *RARB* when induced by RA. There is also additional evidence that DNA hypermethylation in specific regions (promoter or genebody) can lead to upregulation of transcription (34, 35).

In our combined analysis, CpG sites of 11 genes were located in the promoter region while others were in the coding region. The relationship between promoter methylation and gene expression is well-known. Recent studies have found that gene-body methylation in differentially expressed genes is a consistent phenomenon throughout the human genome (36-38). Although we have no functional evidence, the change in DNA methylation levels in the promoter and coding regions of the gene can alter expression levels.

In summary, we presented genome-wide DNA methylation and gene expression profiles of hESCs upon RA treatment. To our knowledge, this is the first time such research has been reported. The results provided in this study will facilitate investigations into the interplay between DNA methylation and gene expression through further functional and biological studies.

MATERIALS AND METHODS

Human embryonic stem cell culture, RNA, and DNA extraction

Three hESC lines (CHA3-hES, CHA4-hES, and SNUhES3) were analyzed for this study by following Human Subjects Institutional Review Board approved protocols (Supplementary Table 2). The hESCs were maintained on Mitomycin C (Sigma, St. Louis, MO, USA)-treated STO (ATCC CRL-1503) feeders (39). Prior to being treated with retinoic acid (RA), hESCs were transferred onto Matrigel® (BD Biosciences, Bedford, MA, USA)-coated culture dishes in STO-conditioned medium (CM), as described previously (40). After 2 d of feeder-free culture, 50 μM RA was applied to hESCs for 5 d. When treated with RA under stem-cell conditions, CHA4-hES cells displayed a drastic morphological change as a differentiated state, and Western blot analysis also showed that expression of OCT4 protein was dramatically reduced (Supplementary Fig. 2). Thus, hESCs could be differentiated by treatment with RA for 5 d (26). To extract total RNA from the control and RA-treated hESCs, TRIzol (Invitrogen) was used according to the manufacturer's protocol. DNA was extracted from the hESCs using a Qiagen DNeasy kit (Qiagen, Hilden, Germany) in preparation for analysis on bead arrays.

Whole-genome DNA methylation assay

Of the three hESC lines, two cell lines (CHA4-hES and SNUhES3) were run in quadruplicate and one (CHA3-hES) in singlet. One microgram of genomic DNA from each sample was bisulfite converted using a EZ DNA methylation kit (Zymo Research, Orange, CA, USA), and 200 ng of the converted DNA was used for amplification. Amplified DNA was hybridized to the HumanMethylation27 BeadChip (Illumina, San Diego, CA, USA), and the arrays were imaged using a BeadArray™ Reader (Illumina). Image processing and intensity data extraction were performed according to Illumina's instructions. Each methylation signal was used to compute a "Beta" value (β), which is a quantitative measure of DNA methylation ranging from 0 for completely unmethylated cytosines to 1 for completely methylated cytosines (8).

Whole-genome gene expression assay

All three cell lines were run in triplicate. RNA isolated from the hESC lines was used for gene expression analysis using the Human-6 Whole-Genome Expression BeadChip (Illumina). Biotin-labeled cRNA was produced by means of a linear amplification kit (Ambion, Austin, TX, USA) using 300 ng of quality-checked total RNA as an input. Chip hybridizations, washing, Cy3-streptavidin staining, and scanning were performed

on a BeadArray™ Reader (Illumina) platform using reagents and by following protocols supplied by the manufacturer. The "Detection Score" was used to determine expression.

Differential DNA methylation/gene expression analysis

Differential DNA methylation and gene expression analysis were performed with the Methylation and Gene Expression Modules in Illumina's BeadStudio software. The Illumina data were normalized using the background and quantile functions for DNA methylation and gene expression, respectively. We identified CpG sites/genes that were differentially methylated/expressed in RA-treated hESCs using the t-test error model implemented in BeadStudio. The methylation/expression difference score (Diff. Score) takes into account background noise and sample variability (41).

In order to identify differentially methylated CpG sites between control hESCs and RA-treated hESCs, we performed a t-test on the difference in mean methylation level between the two groups. We selected sites with a Diff. Score $>$ 20 (P value $<$ 0.01) and with an additional filter of mean $|\Delta\beta| > 0.17$, the estimated error in β (42). This resulted in a list of 166 sites, the top 100 of which, based on $|\Delta\beta|$, were chosen to provide a readable list in Fig. 1A. For differential gene expression data analysis, "signal" values below the detection limit were arbitrarily set to the level of threshold detection in order to avoid nonsense values for expression ratios. Significantly differentially expressed genes had a fold change of at least 50% with a Diff. Score $>$ 20 (P value $<$ 0.01). This resulted in a list of 2,013 differentially expressed genes. Among them, the top 100 genes, based on the fold change, are shown in Fig. 1B.

Combined analysis of differentially methylated/expressed data

Differentially methylated CpG sites (n = 166) and differentially expressed genes (n = 2,013) were combined based on gene name represented in both data sets. Pearson correlation coefficients were calculated between the expression signal values and methylation β values. We collected significantly correlated sites/genes with a Pearson correlation coefficient $>$0.8 (P value $<$ 0.05) (Supplementary Table 1).

Bisulfite sequencing

Genomic DNA (1 μg) of SNUhES3 was modified by sodium bisulfite using a EZ DNA Methylation kit (ZYMO Research) according to the manufacturer's instructions. For amplification of bisulfite-modified DNA, we used the MethPrimer program (43) to design the forward and reverse primer sets of two genes (*CENTD3* and *MSX1*), including Illumina probe position. Bisulfite-modified DNA (1 μl) was amplified in a 20 μl volume containing primers. Primer information is available in Supplemental Table 3. Samples were heated to 95°C for 12 min and then subjected to 35 cycles of denaturation at 95°C for 45 s, annealing for 45 s, extension at 72°C for 60 s, and then incubation at 72°C for 10 min and cooling to 4°C. The PCR products were visualized on a 1% agarose gel by ethidium bromide staining, purified from the gel using a Qiagen Gel Extraction kit, and cloned using pGEM-T Easy Vector (Promega). Ten clones were randomly chosen for sequencing. Complete bisulfite conversion was assured when $<$0.01% of the cytosines in non-CG dinucleotides in the final sequence were not converted.

Acknowledgements

This research was supported by the Bio R&D program through the National Research Foundation of Korea, funded by the Ministry of Education, Science and Technology (2007-2004101).

REFERENCES

1. Chambon, P. (1996) A decade of molecular biology of retinoic acid receptors. *Faseb J.* **10**, 940-954.
2. Soprano, D. R., Teets, B. W. and Soprano, K. J. (2007) Role of retinoic acid in the differentiation of embryonal carcinoma and embryonic stem cells. *Vitam. Horm.* **75**, 69-95.
3. Lewis, J. D., Meehan, R. R., Henzel, W. J., Maurer-Fogy, I., Jeppesen, P., Klein, F. and Bird, A. (1992) Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA. *Cell* **69**, 905-914.
4. Lee, J. H. and Skalnik, D. G. (2002) CpG-binding protein is a nuclear matrix- and euchromatin-associated protein localized to nuclear speckles containing human trithorax. Identification of nuclear matrix targeting signals. *J. Biol. Chem.* **277**, 42259-42267.
5. Tate, P. H. and Bird, A. P. (1993) Effects of DNA methylation on DNA-binding proteins and gene expression. *Curr. Opin. Genet. Dev.* **3**, 226-231.
6. Adewumi, O., Aflatoonian, B., Ahrlund-Richter, L., Amit, M., Andrews, P. W., Beighton, G., Bello, P. A., Benvenisty, N., Berry, L. S., Bevan, S., Blum, B., Brooking, J., Chen, K. G., Choo, A. B., Churchill, G. A., Corbel, M., Damjanov, I., Draper, J. S., Dvorak, P., Emanuelsson, K., Fleck, R. A., Ford, A., Gertow, K., Gertsenstein, M., Gokhale, P. J., Hamilton, R. S., Hampl, A., Healy, L. E., Hovatta, O., Hyllner, J., Imreh, M. P., Itskovitz-Eldor, J., Jackson, J., Johnson, J. L., Jones, M., Kee, K., King, B. L., Knowles, B. B., Lako, M., Lebrin, F., Mallon, B. S., Manning, D., Mayshar, Y., McKay, R. D., Michalska, A. E., Mikkola, M., Mileikovsky, M., Minger, S. L., Moore, H. D., Mummery, C. L., Nagy, A., Nakatsuji, N., O'Brien, C. M., Oh, S. K., Olsson, C., Otonkoski, T., Park, K. Y., Passier, R., Patel, H., Patel, M., Pedersen, R., Pera, M. F., Piekarczyk, M. S., Pera, R. A., Reubinoff, B. E., Robins, A. J., Rossant, J., Rugg-Gunn, P., Schulz, T. C., Semb, H., Sherrer, E. S., Siemen, H., Stacey, G. N., Stojkovic, M., Suemori, H., Szatkiewicz, J., Turetsky, T., Tuuri, T., van den Brink, S., Vintersten, K., Vuoristo, S., Ward, D., Weaver, T. A., Young, L. A. and Zhang, W. (2007) Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nat. Biotechnol.* **25**, 803-816.
7. Andrews, P. W., Benvenisty, N., McKay, R., Pera, M. F., Rossant, J., Semb, H. and Stacey, G. N. (2005) The

International Stem Cell Initiative: toward benchmarks for human embryonic stem cell research. *Nat. Biotechnol.* **23**, 795-797.
8. Bibikova, M., Chudin, E., Wu, B., Zhou, L., Garcia, E. W., Liu, Y., Shin, S., Plaia, T. W., Auerbach, J. M., Arking, D. E., Gonzalez, R., Crook, J., Davidson, B., Schulz, T. C., Robins, A., Khanna, A., Sartipy, P., Hyllner, J., Vanguri, P., Savant-Bhonsale, S., Smith, A. K., Chakravarti, A., Maitra, A., Rao, M., Barker, D. L., Loring, J. F. and Fan, J. B. (2006) Human embryonic stem cells have a unique epigenetic signature. *Genome Res.* **16**, 1075-1083.
9. Liu, Y., Shin, S., Zeng, X., Zhan, M., Gonzalez, R., Mueller, F. J., Schwartz, C. M., Xue, H., Li, H., Baker, S. C., Chudin, E., Barker, D. L., McDaniel, T. K., Oeser, S., Loring, J. F., Mattson, M. P. and Rao, M. S. (2006) Genome wide profiling of human embryonic stem cells (hESCs), their derivatives and embryonal carcinoma cells to develop base profiles of U.S. Federal government approved hESC lines. *BMC Dev. Biol.* **6**, 20.
10. Loring, J. F. and Rao, M. S. (2006) Establishing standards for the characterization of human embryonic stem cell lines. *Stem Cells* **24**, 145-150.
11. Brunner, A. L., Johnson, D. S., Kim, S. W., Valouev, A., Reddy, T. E., Neff, N. F., Anton, E., Medina, C., Nguyen, L., Chiao, E., Oyolu, C. B., Schroth, G. P., Absher, D. M., Baker, J. C. and Myers, R. M. (2009) Distinct DNA methylation patterns characterize differentiated human embryonic stem cells and developing human fetal liver. *Genome Res.* **19**, 1044-1056.
12. Rodolfa, K., Di Giorgio, F. P. and Sullivan, S. (2007) Defined reprogramming: a vehicle for changing the differentiated state. *Differentiation* **75**, 577-579.
13. Abeyta, M. J., Clark, A. T., Rodriguez, R. T., Bodnar, M. S., Pera, R. A. and Firpo, M. T. (2004) Unique gene expression signatures of independently-derived human embryonic stem cell lines. *Hum. Mol. Genet.* **13**, 601-608.
14. Shiota, K., Kogo, Y., Ohgane, J., Imamura, T., Urano, A., Nishino, K., Tanaka, S. and Hattori, N. (2002) Epigenetic marks by DNA methylation specific to stem, germ and somatic cells in mice. *Genes Cells* **7**, 961-969.
15. Hattori, N., Nishino, K., Ko, Y. G., Hattori, N., Ohgane, J., Tanaka, S. and Shiota, K. (2004) Epigenetic control of mouse Oct-4 gene expression in embryonic stem cells and trophoblast stem cells. *J. Biol. Chem.* **279**, 17063-17069.
16. Hattori, N., Imao, Y., Nishino, K., Hattori, N., Ohgane, J., Yagi, S., Tanaka, S. and Shiota, K. (2007) Epigenetic regulation of Nanog gene in embryonic stem and trophoblast stem cells. *Genes Cells* **12**, 387-396.
17. Zvetkova, I., Apedaile, A., Ramsahoye, B., Mermoud, J. E., Crompton, L. A., John, R., Feil, R. and Brockdorff, N. (2005) Global hypomethylation of the genome in XX embryonic stem cells. *Nat. Genet.* **37**, 1274-1279.
18. Toyota, M., Sasaki, Y., Satoh, A., Ogi, K., Kikuchi, T., Suzuki, H., Mita, H., Tanaka, N., Itoh, F., Issa, J. P., Jair, K. W., Schuebel, K. E., Imai, K. and Tokino, T. (2003) Epigenetic inactivation of CHFR in human tumors. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 7818-7823.
19. Yu, X., Minter-Dykhouse, K., Malureanu, L., Zhao, W. M., Zhang, D., Merkle, C. J., Ward, I. M., Saya, H., Fang, G., van Deursen, J. and Chen, J. (2005) Chfr is required for tumor suppression and Aurora A regulation. *Nat. Genet.* **37**, 401-406.
20. Sauter, C. N., McDermid, R. L., Weinberg, A. L., Greco, T. L., Xu, X., Murdoch, F. E. and Fritsch, M. K. (2005) Differentiation of murine embryonic stem cells induces progesterone receptor gene expression. *Exp. Cell Res.* **311**, 251-264.
21. Tsuneyoshi, N., Sumi, T., Onda, H., Nojima, H., Nakatsuji, N. and Suemori, H. (2008) PRDM14 suppresses expression of differentiation marker genes in human embryonic stem cells. *Biochem. Biophys. Res. Commun.* **367**, 899-905.
22. Sekine, Y., Tsuji, S., Ikeda, O., Sugiyma, K., Oritani, K., Shimoda, K., Muromoto, R., Ohbayashi, N., Yoshimura, A. and Matsuda, T. (2007) Signal-transducing adaptor protein-2 regulates integrin-mediated T cell adhesion through protein degradation of focal adhesion kinase. *J. Immunol.* **179**, 2397-2407.
23. Sander, L. E., Frank, S. P., Bolat, S., Blank, U., Galli, T., Bigalke, H., Bischoff, S. C. and Lorentz, A. (2008) Vesicle associated membrane protein (VAMP)-7 and VAMP-8, but not VAMP-2 or VAMP-3, are required for activation-induced degranulation of mature human mast cells. *Eur. J. Immunol.* **38**, 855-863.
24. Niwa, H., Miyazaki, J. and Smith, A. G. (2000) Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat. Genet.* **24**, 372-376.
25. Pesce, M., Gross, M. K. and Scholer, H. R. (1998) In line with our ancestors: Oct-4 and the mammalian germ. *Bioessays.* **20**, 722-732.
26. Yeo, S., Jeong, S., Kim, J., Han, J. S., Han, Y. M. and Kang, Y. K. (2007) Characterization of DNA methylation change in stem cell marker genes during differentiation of human embryonic stem cells. *Biochem. Biophys. Res. Commun.* **359**, 536-542.
27. Deb-Rinker, P., Ly, D., Jezierski, A., Sikorska, M. and Walker, P. R. (2005) Sequential DNA methylation of the Nanog and Oct-4 upstream regions in human NT2 cells during neuronal differentiation. *J. Biol. Chem.* **280**, 6257-6260.
28. Wang, J., Rao, S., Chu, J., Shen, X., Levasseur, D. N., Theunissen, T. W. and Orkin, S. H. (2006) A protein interaction network for pluripotency of embryonic stem cells. *Nature* **444**, 364-368.
29. Boyer, L. A., Lee, T. I., Cole, M. F., Johnstone, S. E., Levine, S. S., Zucker, J. P., Guenther, M. G., Kumar, R. M., Murray, H. L., Jenner, R. G., Gifford, D. K., Melton, D. A., Jaenisch, R. and Young, R. A. (2005) Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* **122**, 947-956.
30. Lung, H. L., Bangarusamy, D. K., Xie, D., Cheung, A. K., Cheng, Y., Kumaran, M. K., Miller, L., Liu, E. T., Guan, X. Y., Sham, J. S., Fang, Y., Li, L., Wang, N., Protopopov, A. I., Zabarovsky, E. R., Tsao, S. W., Stanbridge, E. J. and Lung, M. L. (2005) THY1 is a candidate tumour suppressor gene with decreased expression in metastatic nasopharyngeal carcinoma. *Oncogene* **24**, 6525-6532.
31. Sanders, Y. Y., Pardo, A., Selman, M., Nuovo, G. J.,

Tollefsbol, T. O., Siegal, G. P. and Hagood, J. S. (2008) Thy-1 promoter hypermethylation: a novel epigenetic pathogenic mechanism in pulmonary fibrosis. *Am. J. Respir. Cell Mol. Biol.* **39**, 610-618.
32. Shen, S., van den Brink, C. E., Kruijer, W. and van der Saag, P. T. (1992) Embryonic stem cells stably transfected with mRAR beta 2-lacZ exhibit specific expression in chimeric embryos. *Int. J. Dev. Biol.* **36**, 465-476.
33. Qiu, H., Lotan, R., Lippman, S. M. and Xu, X. C. (2000) Lack of correlation between expression of retinoic acid receptor-beta and loss of heterozygosity on chromosome band 3p24 in esophageal cancer. *Genes. Chromosomes. Cancer* **28**, 196-202.
34. De Larco, J. E., Wuertz, B. R., Yee, D., Rickert, B. L. and Furcht, L. T. (2003) Atypical methylation of the interleukin-8 gene correlates strongly with the metastatic potential of breast carcinoma cells. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 13988-13993.
35. Kelavkar, U. P., Harya, N. S., Hutzley, J., Bacich, D. J., Monzon, F. A., Chandran, U., Dhir, R. and O'Keefe, D. S. (2007) DNA methylation paradigm shift: 15-lipoxygenase-1 upregulation in prostatic intraepithelial neoplasia and prostate cancer by atypical promoter hypermethylation. *Prostaglandins Other Lipid. Mediat.* **82**, 185-197.
36. Ball, M. P., Li, J. B., Gao, Y., Lee, J. H., LeProust, E. M., Park, I. H., Xie, B., Daley, G. Q. and Church, G. M. (2009) Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. *Nat. Biotechnol.* **27**, 361-368.
37. Hellman, A. and Chess, A. (2007) Gene body-specific methylation on the active X chromosome. *Science* **315**, 1141-1143.
38. Lee, T. F., Zhai, J. and Meyers, B. C. (2010) Conservation and divergence in eukaryotic DNA methylation. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 9027-9028.
39. Oh, S. K., Kim, H. S., Ahn, H. J., Seol, H. W., Kim, Y. Y., Park, Y. B., Yoon, C. J., Kim, D. W., Kim, S. H. and Moon, S. Y. (2005) Derivation and characterization of new human embryonic stem cell lines: SNUhES1, SNUhES2, and SNUhES3. *Stem. Cells* **23**, 211-219.
40. Xu, C., Inokuma, M. S., Denham, J., Golds, K., Kundu, P., Gold, J. D. and Carpenter, M. K. (2001) Feeder-free growth of undifferentiated human embryonic stem cells. *Nat. Biotechnol.* **19**, 971-974.
41. Chudin, E., Kruglyak, S., Baker, S. C., Oeser, S., Barker, D. and McDaniel, T. K. (2006) A model of technical variation of microarray signals. *J. Comput. Biol.* **13**, 996-1003.
42. Bibikova, M., Lin, Z., Zhou, L., Chudin, E., Garcia, E. W., Wu, B., Doucet, D., Thomas, N. J., Wang, Y., Vollmer, E., Goldmann, T., Seifart, C., Jiang, W., Barker, D. L., Chee, M. S., Floros, J. and Fan, J. B. (2006) High-throughput DNA methylation profiling using universal bead arrays. *Genome Res.* **16**, 383-393.
43. Lapointe, J., Li, C., Higgins, J. P., van de Rijn, M., Bair, E., Montgomery, K., Ferrari, M., Egevad, L., Rayford, W., Bergerheim, U., Ekman, P., DeMarzo, A. M., Tibshirani, R., Botstein, D., Brown, P. O., Brooks, J. D. and Pollack, J. R. (2004) Gene expression profiling identifies clinically relevant subtypes of prostate cancer. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 811-816.