in the 3′-region downstream from the TSS are involved in gene regulation.

In this study, we demonstrated T-DMR plurality to be involved in tissue-specific gene expression. DNA methylation regulates not only in gene expression, but also in other gene functions; therefore, T-DMRs identified by D-REAM could provide investigative insight into the roles of genome-wide DNA methylation. We conclude that T-DMR profiles are tissue specific and facilitate tissue identification by reflecting tissue-specific gene functions.

Methods

Mice and genomic DNA extraction

Male mice (C57BL/6NCrj, 12- to 13-wk-old mice for liver, cerebrum, and kidney; 6-wk-old for spleen) were euthanized after fasting for 16 h. Tissue samples were collected and frozen at −80°C until use. The samples (<20 mg) were thawed, homogenized, and incubated with 300 µL of lysis solution (10 mM Tris-HCl at pH 8.0, 5 mM EDTA, 200 mM NaCl, 0.2% SDS, and 200 µg/mL proteinase K) at 55°C for 30 min. Samples were extracted with a phenol/chloroform/isoamyl alcohol (PCI) mixture, incubated with RNase for 30 min, and re-extracted with PCI. DNA was precipitated with ethanol and dissolved in 20 µL of Tris-EDTA (TE) buffer (pH 8.0).

Combined bisulfite restriction analysis (COBRA) and bisulfite sequencing

Genomic DNA was digested with PstI. Digested DNA (3 µg) was denatured with 0.3 N NaOH. Sodium metabisulfite (pH 5.0) and hydroquinone were added to a final concentration of 2.0 M and 0.5 mM, respectively. The reaction mixture was incubated in the dark at 55°C for 16 h. DNA was purified using the Wizard DNA Clean-up System (Promega KK), treated with 0.3 M NaOH at 37°C for 15 min, and precipitated with ethanol. It was then dissolved in 20 µL of TE buffer (pH 8.0) and used in a concentration range of 1/100 to 1/20 for PCR analysis with Immolase Taq DNA polymerase (Bioline). During the bisulfite reaction, unmethylated CpGs are converted to TpGs, while methylated CpGs remain intact. For restriction mapping, 10% of the PCR product was digested with HpyCH4IV at 37°C overnight and electrophoresed with the undigested product (control) on a 1% agarose gel. The CpG methylation status within the HpyCH4IV restriction sites was assessed according to the proportion of cleaved fragments. For bisulfite sequencing, 50% of the PCR product was gel-extracted and subcloned into the pGEM-T easy vector (Promega KK). A minimum of 10 clones was sequenced, and the methylation status of individual CpGs was determined.

D-REAM

Genomic DNA (5 µg) was digested with HpyCH4IV (New England BioLabs) overnight. The digestion was monitored by gel electrophoresis. Digested DNA was recovered by ethanol precipitation following extraction with PCI and chloroform, and was dissolved in TE buffer (pH 8.0). Fifty nanograms of the DNA sample were ligated to the R-adaptor pair (Supplemental Table S2) using T4 DNA ligase (New England BioLabs). Following treatment with the Klenow fragment, the DNA was digested with TaqI at 65°C for at least 1 h and purified using a Microspin S-300 HR column (GE Healthcare UK Ltd.). DNA samples were then ligated to the N-adaptor pair (Supplemental Table S2) and purified using the Wizard SV Gel and PCR Clean-up System (Promega KK). PCR was performed using Immolase Taq DNA polymerase and the R18 and N18 primers in the presence of dUTP under the following conditions: denaturation at 95°C for 7 min and 20 cycles, each cycle comprising 95°C for 30 sec, 62°C for 30 sec, and 72°C for 2 min. A total of 10 µg of amplified DNA was used for microarray analysis. When NotI was used as the first methylation sensitive restriction enzyme, we used the R-adaptor pair for NotI instead of that for HpyCH4IV.

Microarray analysis was conducted using the GeneChip System (Affymetrix), and all procedures were performed according to the Affymetrix chromatin immunoprecipitation assay protocol provided by the manufacturer. DNA samples were labeled using the GeneChip WT Double-Stranded DNA Terminal Labeling Kit (Affymetrix) and hybridized with Affymetrix GeneChip mouse promoter 1.0R arrays. The arrays were stained and washed with GeneChip Fluidics Station 450 and scanned with the GeneChip 3000 7G Scanner to obtain a ".CEL" file describing the probe intensities. The instruments were operated using the GeneChip operating software version 1.4.

Bioinformatics

Data flow is summarized in Supplemental Fig. S6. To satisfy gene ID requirements of the bioinformatics analysis, we converted gene IDs under certain circumstances. MAT (Johnson et al. 2006) (bandwidth, 300 bp) was used to analyze the tiling array .CEL files and identify the hypomethylated regions based on tiling probe signals, probe sequences, and copy numbers. xMAN (Li et al. 2008) was used to remap the original tiling probes according to the mouse genome assembly of version mm8 (March 2006 build) from the UCSC genome database (Kuhn et al. 2007). A separate ".bpmap" file, containing a subset of probes for the HpyCH4IV–HpyCH4IV and HpyCH4IV–TaqI fragments, was used to verify the MAT analysis. The data were visualized using the Integrated Genome Browser (http://www.affymetrix.com/support/-developer/tools/download_igb-.affx).

Statistical analysis was performed using the R software package and BioConductor package (Gentleman et al. 2004). The tiling array package in BioConductor was used to examine the reproducibility of the microarray data. MultiExperiment Viewer (MeV in TM4 Microarray Software Suite) (http://www.tm4.org/mev.html) was used for K-means clustering of MATscores (Saeed et al. 2003). Genomic annotations, including Ensembl gene assignments (Birney et al. 2004), were obtained from the Galaxy website (http://g2.bx.psu.edu; Giardine et al. 2005). Transcriptome data were obtained from the GNF SymAtlas website (http://symatlas.gnf.org/SymAtlas/; Su et al. 2002), and annotation and ontology analyses were conducted using g:profiler (http://biit.cs.ut.ee/gprofiler/; Reimand et al. 2007), DAVID 2007 (http://niaid.abcc.ncifcrf.gov/; Huang da et al. 2007), and KEGG pathway database (http://www.genome.jp/kegg/kegg2.html; Kanehisa et al. 2006). EMBOSS (Rice et al. 2000) was applied for DNA sequence analysis, and the BIQ analyzer (Bock et al. 2005) was used to analyze the bisulfite sequencing data. Mouse gene symbols were confirmed by referring to the MGI database (http://www.informatics.jax.org/). Transcription factor motifs 1 kb upstream of TSS were analyzed on the MAPPER database website (http://bio.chip.org/mapper/; Marinescu et al. 2005).

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Potential link between estrogen receptor-α gene hypomethylation and uterine fibroid formation

Hiromi Asada[1], Yoshiaki Yamagata[1], Toshiaki Taketani[1], Aki Matsuoka[1], Hiroshi Tamura[1], Naoko Hattori[2], Jun Ohgane[2], Naka Hattori[3], Kunio Shiota[2] and Norihiro Sugino[1,4]

[1]*Department of Obstetrics and Gynecology, Yamaguchi University Graduate School of Medicine, Minamikogushi 1-1-1, Ube 755-8505, Japan;* [2]*Laboratory of Cellular Biochemistry, Animal Resource Sciences/Veterinary Medical Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan;* [3]*Institute of Life Science, Ajinomoto Co. Inc., 1-1, Suzuki-cho, Kawasaki-ku, Kawasaki 210-8681, Japan*

[4]Correspondence address. Tel: +81-836-22-2286; Fax: +81-836-22-2287; E-mail: sugino@yamaguchi-u.ac.jp

Uterine leiomyomas are the most common uterine tumors in women. Estrogen receptor-α (ER-α) is more highly expressed in uterine leiomyomas than in normal myometrium, suggesting a link between uterine leiomyomas and ER-α expression. DNA methylation is an epigenetic mechanism of gene regulation and plays important roles in normal embryonic development and in disease progression including cancers. Here, we investigated the DNA methylation status of the ER-α promoter region (−1188 to +229 bp) in myometrium and leiomyoma. By sodium bisulfite sequencing, 49 CpG sites in the proximal promoter region of ER-α gene were shown to be unmethylated in both leiomyoma and normal myometrium. At seven CpG sites in the distal promoter region of the ER-α gene, there was a variation in DNA methylation status in myometrium and leiomyoma. Further analysis of the DNA methylation status by bisulfite restriction mapping among 11 paired samples of myometrium and leiomyoma indicated that CpG sites in the distal region of ER-α promoter are hypomethylated in leiomyomas of nine patients. In those patients, ER-α mRNA levels tended to be higher in the leiomyoma than in the myometrium. In conclusion, there was an aberrant DNA methylation status in the promoter region of ER-α gene in uterine leiomyoma, which may be associated with high ER-α mRNA expression.

Keywords: DNA methylation; epigenetics; estrogen receptor-α; ER-α promoter; leiomyoma

Introduction

Uterine leiomyomas are the most common uterine tumors in women of reproductive age. Approximately 20–25% of women of reproductive age are afflicted with this disease (Vollenhoven *et al.*, 1990). They frequently cause serious gynecological problems such as pelvic pain, menorrhagia, dysmenorrhea, reduced fertility and recurrent pregnancy loss (Bajekal and Li, 2000; Stewart, 2001). In addition, uterine leiomyoma is the most common indication for hysterectomy in Japan, as well as in the USA (Farquhar and Steiner, 2002).

Despite the high prevalence rate and tremendous influence on reproductive women, the pathogenesis of uterine leiomyomas still remains to be elucidated. On the basis of the fact that uterine leiomyomas develop only after menarche and markedly shrink under hypoestrogenic conditions such as late menopause, it is presumed that their growth depends on estrogens (Stewart, 2001). Although the increased sensitivity to estradiol is important for the growth of uterine leiomyomas, high circulating estradiol levels are not a necessary requirement. The physiological effects of estrogen are mediated by estrogen receptors (ERs). Among them, ER-α is more highly expressed in uterine leiomyomas than in normal myometrium (Benassayag *et al.*, 1999; Kovács *et al.*, 2001), suggesting a possible link between uterine leiomyomas and ER-α expression level.

Epigenetic mechanisms including DNA methylation and histone modification are known to play key roles in transcriptional regulation. DNA methylation occurs at cytosines within CpG dinucleotides that are clustered frequently in regions of ∼1–2 kb in length, called CpG islands, in or near promoter and first exon regions of genes (Esteller and Herman, 2002). In mammals, 60–70% of CpG sites are methylated in the genomic DNA (Boyes and Bird, 1992). DNA methylation is involved in various developmental processes by silencing, switching and stabilizing genes (Nan *et al.*, 1998; Cho *et al.*, 2001; Imamura *et al.*, 2001; Li, 2002). Although there are differences in the frequency of CpGs in the gene regulatory regions, DNA methylation-dependent gene regulation has been previously reported (Razin and Cedar, 1991; Cho *et al.*, 2001; Imamura *et al.*, 2001). CpG methylation can down-regulate gene expression by preventing the binding of transcription factors or by recruiting repressor molecules (Bird, 1992; Ballestar and Wolffe, 2001).

Accumulating evidence has indicated that increased methylation level of the CpG islands within the ER-α promoter region is highly negatively associated with ER-α expression in a variety of diseases including neoplastic and atherosclerotic lesions (Iwase *et al.*, 1999; Post *et al.*, 1999; Yoshida *et al.*, 2000; Berger and Daxenbichler, 2002). Thus, down-regulation of the ER-α expression is caused by hypermethylation of the CpG islands within the ER-α promoter region. However, the fact that the ER-α expression was higher in uterine leiomyomas than in normal myometrium is different from other ER-α related diseases described earlier. This led us to assume that a different epigenetic abnormality might be involved in uterine leiomyomas. The present study was undertaken to investigate the

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methylation status of CpG sites within the promoter region of the human ER-α gene and to evaluate an association of aberrant DNA methylation status with ER-α gene expression in uterine leiomyomas and normal myometrium.

Materials and Methods

Tissue preparation

Specimens of uterine leiomyomas and corresponding normal myometrium were obtained from 18 women, from 37 to 57 (mean 47.4) years of age, who underwent total hysterectomy. Normal myometrium was obtained from a woman without myoma (49 years, cervical cancer). Informed consent was obtained from all participating patients, and ethical approval was obtained from Yamaguchi University Graduate School of Medicine. Tissues were taken immediately after removal of the uterus, immersed in liquid nitrogen and stored at −80°C until used DNA/RNA extraction. For immunohistochemistry, the specimens were fixed immediately in 10% neutral formalin for ~24 h, embedded in paraffin and cut into 4 μm thick sections.

Immunohistochemistry

The diagnosis of leiomyoma and normal myometrium was established on histological examination with hematoxylin and eosin staining. Immunohistochemistry was performed as described previously (Sugino *et al.*, 2005) using an ER-α monoclonal antibody (ER1D5, mouse, Dako Japan Co. Ltd., Tokyo, Japan). Counterstaining was performed with Meyer's hematoxylin.

Real-time RT–PCR analysis

Total RNAs were isolated from tissues using Isogen reagent (Nippon Gene, Tokyo, Japan) and reverse-transcribed using an ExCript RT reagent kit (TaKaRa, Ohtsu, Japan) according to the manufacturer's protocol, respectively. For PCR amplification, first strand cDNA was synthesized from 1 μg total RNA with reverse transcriptase in 20 μl of reaction mixture. The oligonucleotide primers for ER-α (5′-TGTGCAATGACTATGCTTCA-3′ and 5′-GCTCTT CCTCCTGTTTTTA-3′; 149 bp amplified products) were designed from the human ER-α cDNA sequence (Matsuzaki *et al.*, 2000). Internal control PCR primers for GAPDH (5′-AGGTGAAGGTCGGAGTCA-3′ and 5′-GGTCATTG ATGGCAACAA-3′; 99 bp amplified products) were designed from the GAPDH cDNA sequence (Kaneda *et al.*, 2004). Real-time PCR was performed using LightCycler (Roche Diagnostics, Indianapolis, IN, USA). The reaction mixture contained 10 μl SYBR Premix Ex *Taq* (TaKaRa), 0.2 μM each of primer sets of ER-α or GAPDH and 2 μl cDNA in a total volume of 20 μl. The thermocycling program was 40 cycles of 96°C for 5 s and 60°C for 20 s with an initial cycle of 96°C for 10 s.

Sodium bisulfite genomic sequencing

Genomic DNA was extracted using Genomic DNA kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. The bisulfite reaction, in which unmethylated cytosine is converted to uracil and 5-methylcytsine remains nonreactive, was carried out as previously described (Cho *et al.*, 2001; Imamura *et al.*, 2001) with a slight modification: 2 μg of genomic DNA digested with *Pvu* II was denatured by incubation with NaOH at 42°C for 20 min. After the incubation, sodium metabisulfite and hydroquinone (Wako, Osaka, Japan) were added to the final concentrations of 2.0 M and 0.5 mM, respectively, and the mixture was incubated at 55°C for 16 h. The bisulfite reaction was terminated by incubation with NaOH again at 42°C for 20 min. The DNA fragments covering the transcriptional regulatory region of ER-α gene (−1297 to +279) were amplified by PCR using the following set of primers: Region I (−126 to +279) F: 5′-GTTGTGTTTGGAGTGATGTTTAAGTT-3′, R: 5′-CAATAAAACCATCCCAAATACTTTA-3′; Region II (−670 to −94) F: 5′-GGAAGGGTTTATTTATTTTGGGAGTA-3′, R: 5′-TAACATTAACTT AAACATCACTCC-3′; Region III (−1297 to −731) F: 5′-TTGGGTGTTT GGGATAGTAATTAAA-3′, R: 5′-CTTAATCCCATTAAAAATTCTCAT A-3′. The PCR conditions were 95°C for 10 min, and 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, with a final extension at 72°C for 10 min. The resulting products were subjected to agarose gel electrophoresis and purified using a QIAquick gel extraction kit (Qiagen). The PCR products were cloned into pGEM-T easy vector (Promega, Tokyo, Japan), and 10 or more clones were randomly picked from each of two independent PCRs were sequenced to determine the methylation status. Sequencing was performed using an ABI automated sequencer with BigDye terminators (Applied Biosystems, Foster City, CA, USA).

Bisulfite restriction mapping

The bisulfite-converted DNA was amplified by PCR with a set of primers (−1120 to −645), F: 5′-TATATATATGTGTGTGTGTATGTG-3′ and R: 5′-TACTCCCAAAATAAATAAACCCTTCC-3′. One half of the PCR products were digested with 5 U of *Taq* I (TaKaRa) at 65°C for 3 h. The remaining half was used for undigested control without *Taq* I treatment. *Taq* I recognizes 5′-TCGA-3′ sequences. Because only unmethylated cytosine sites are changed to thymine by sodium bisulfite PCR, PCR fragments from unmethylated genomic DNA are resistant to *Taq* I, whereas those from methylated DNA are digested by the enzyme. The resulting products of the bisulfite restriction mapping were assessed by agarose gel electrophoresis.

Cell culture, demethylation and RT–PCR analysis

Primary human uterine smooth muscle cells and cell culture reagents were purchased from Cambrex, Inc. (Walkersville, MD, USA). Cells were grown to 90% confluence and maintained in SMGM2 medium, which consists of smooth muscle basal medium supplemented with 5% fetal bovine serum, 2 ng/ml recombinant human fibroblast growth factor-β, 5 μg/ml insulin, 0.5 ng/ml recombinant human epidermal growth factor, 50 μg/ml gentamicin sulfate and 50 μg/ml amphotericin B. Medium was changed every other day and all experiments were completed with cells derived from passages 2.

For treatment with a demethylating agent, 5-aza-dC (Sigma, St Louis, MO, USA), that inhibits DNA methylation, cells were seeded at a density of 1×10^6 cells/25 cm^2 tissue culture flask. After 24 h of incubation, cells were cultured with treatment medium containing 1 μM 5-aza-dC for 72 h. The medium was changed daily. After treatment, cells were used for RNA isolation and ER-α mRNA levels were measured by RT–PCR as reported previously (Sugino *et al.*, 1998). Total RNAs were isolated from the cells according to the protocol mentioned above. For PCR amplification, first-strand cDNA was synthesized from 1 μg total RNA with reverse transcriptase in 20 μl of reaction mixture. The oligonucleotide primers for ER-α (5′-TGTGCAATGACT ATGCTTCA-3′ and 5′-GCTCTTCCTCCTGTTTTTA-3′; 149 bp amplified products) were designed from the human ER-α cDNA sequence (Matsuzaki *et al.*, 2000). Internal control PCR primers for ribosomal protein L19 (5′-CTGAAGGTCAAAGGGAATGTG-3′ and 5′-GGACAGAGTCTTGATG ATCTC-3′; 194 bp amplified products) were designed from the L19 cDNA sequence (Sugino *et al.*, 1998). PCR amplification was performed using a programmed temperature control system (PC808, ASTEC, Fukuoka, Japan). The reaction mixture contained 4 μl cDNA, 1 μM each of primer sets of ER-α or L19, GeneAmp 10× PCR buffer, 0.2 mM deoxynucleotide triphosphate, 2.5 mM $MgCl_2$ and 0.05 U AmpliTaq DNA polymerase (Applied Biosystems) in a total volume of 20 μl. The thermocycling program was an initial cycle of 94°C for 5 min, then 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, followed by 10 min of final extension at 72°C. The resulting products were subjected to agarose gel electrophoresis. The level of ER-α expression was determined by quantifying the intensities of the PCR product, compared with the L19 product, using NIH ImageJ software.

Statistical analyses

Wilcoxon signed-ranks test was used for paired samples. A value of $P < 0.05$ was considered significant.

Results

ER-α expression

Immunohistochemical staining for ER-α expression was localized in nuclei of smooth muscle cells, and the staining distribution was homogenous in both leiomyoma and myometrium (Fig. 1A).

Many investigators have reported that ER-α is more highly expressed in leiomyomas than in myometrium (Benassayag *et al.*, 1999; Kovács *et al.*, 2001), suggesting a possible link between

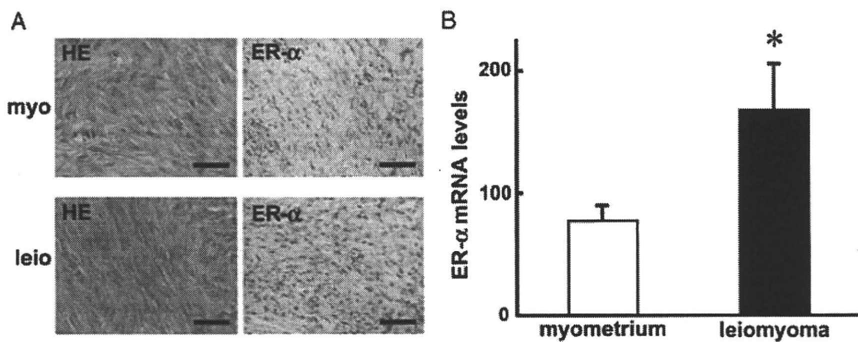

Figure 1: ER-α expression in uterine leiomyoma and normal myometrium.
(**A**) Immunohistochemical staining of ER-α in leiomyoma (leio) and myometrium (myo). Immunohistochemical staining for ER-α was performed on tissue samples obtained from three different patients. HE, hematoxylin–eosin staining. Bar: 50 μm. (**B**) ER-α mRNA expression in leiomyoma and myometrium. Specimens of leiomyomas and corresponding myometrium were obtained from 18 women. Total RNA was isolated from 18 pairs of leiomyomas and myometrium. ER-α mRNA levels were analyzed by SYBR Green I real-time quantitative RT–PCR. Relative ER-α expression normalized to GAPDH was calculated. Values are mean ± SEM. $*P < 0.01$ versus myometrium.

leiomyomas and ER-α expression level. To investigate whether ER-α levels are altered in leiomyoma samples that we collected, the ER-α mRNA was measured in leiomyomas and myometrium by real-time RT–PCR. As shown in Fig. 1B, ER-α mRNA levels in the samples we examined were confirmed as significantly higher in leiomyomas than in myometrium ($P < 0.01$).

Effects of 5-aza-dC on ER-α mRNA expression in human uterine smooth muscle cells

To study the possibility that ER-α mRNA expression is under epigenetic regulation such as DNA methylation, human uterine smooth muscle cells were incubated with 5-aza-dC which inhibits DNA methylation. ER-α mRNA expression in human uterine smooth muscle cells was significantly ($P < 0.05$) increased by 5-aza-dC (Fig. 2).

DNA methylation status of 5′-flanking region of ER-α gene

Since ER-α mRNA expression seemed to be under the regulation of DNA methylation, we examined DNA methylation status of the ER-α promoter region between leiomyoma and myometrium. The 5′-upstream region around the first exon (between about −500 and +200 bp) of the ER-α gene is most importantly involved in the regulation of ER-α expression (Lapidus *et al.*, 1996; Yan *et al.*, 2001; Giacinti *et al.*, 2006). Furthermore, a series of three estrogen response elements (EREs), which lie from −892 to −420 in the ER-α upstream region, is identified as a region with functional importance for ER-α gene transcription (Furguson *et al.*, 1997; Li *et al.*, 2000). Therefore, ~1 kb upstream region together with a part of the first exon of the ER-α gene is considered as important for ER-α gene transcription, and the methylation status of this region was compared between leiomyoma and myometrium.

Figure 3A shows the distribution of CpGs within the transcriptional regulatory region of the human ER-α gene. The ER-α gene has two CpG islands in the promoter region and in the first exon. According to the registered nucleotide sequence of the ER-α (GenBank accession no. AB090237), there were 56 CpG sites in the 1.5 kb genomic sequence including the promoter and exon 1 of the human ER-α gene (Fig. 3A). In the present study, these CpG sites were divided into two regions, the proximal and distal regions. The proximal region included 49 CpG sites around the transcription start site (−556 to +229, designed as Regions I and II), and the distal region included seven CpG sites in the upstream region (−1188 to −790, designed as Region III). First, the methylation status of all the CpG sites between −1188 and +229 (56 CpG sites) were analyzed by sodium bisulfite genomic sequencing for a paired sample of leiomyoma and myometrium (Fig. 3B, Case 1). The proximal region (Regions I and II containing a total of 49 CpG sites) was unmethylated in both tissues (Fig. 3B, Case 1). In the distal promoter region (Region III, seven CpG sites), 47 CpG sites (24.7%) in a total of 190 examined CpG sites were methylated in myometrium, whereas only nine CpG sites (4.9%) in a total of 184 examined CpG sites were methylated

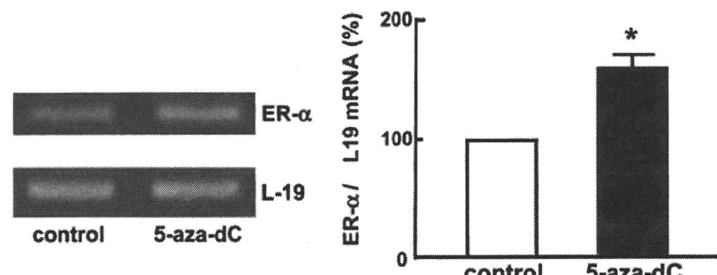

Figure 2: Effects of 5-aza-dC on ER-α mRNA expression in human uterine smooth muscle cells.
Primary human uterine smooth muscle cells were incubated with a demethylating agent, 5-aza-dC (1 μM) that inhibits DNA methylation, for 72 h. After treatment, cells were used for RNA isolation and ER-α mRNA levels were measured by RT–PCR. The intensity of the signals of ER-α was normalized to that of the internal control L19 (the ratio of ER-α to L19). Data were expressed as a percentage of the control value in each incubation. Each bar represents the mean ± SEM of three different experiments. $*P < 0.05$ versus control.

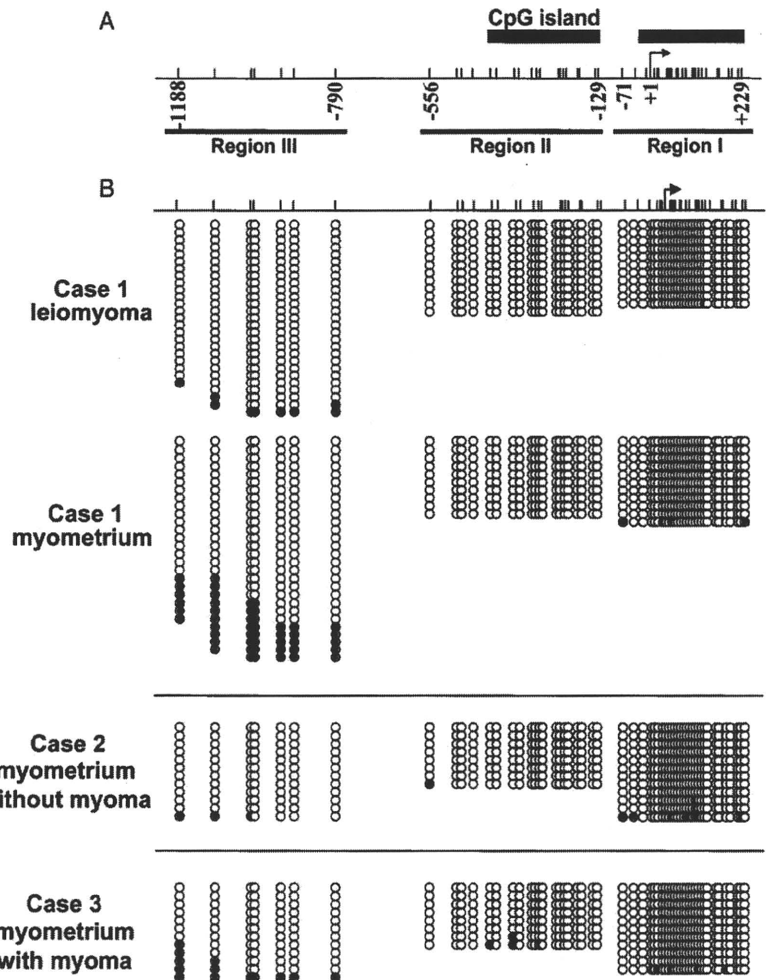

Figure 3: DNA methylation status of the ER-α promoter region in uterine leiomyoma and normal myometrium.
(**A**) Distribution of CpG sites in the promoter region and the first exon of ER-α gene. The position of the transcription start site is designated as +1. The diagram shows a detailed map of ~1.5 kb region around the transcription start site (arrow), in which the 'vertical lines' indicate positions of CpG sites. Thick horizontal lines indicate the region identified as CpG islands. Thin horizontal lines indicate the regions analyzed by bisulfite sequencing (Regions I, II and III). (**B**) DNA methylation status of CpG sites in the promoter region and the first exon of the ER-α gene. Methylation status of all the CpG sites between −1188 and +229 (56 CpG sites) was analyzed by sodium bisulfite genomic sequencing in a paired sample of leiomyoma and myometrium from an individual with myoma (Case 1), normal myometrium from an individual without myoma (Case 2) and myometrium from another individual with myoma (Case 3). Open and filled circles indicate unmethylated and methylated CpG status, respectively.

in leiomyoma (Fig. 3B, Case 1). There was a significant difference in the frequency of DNA methylation between leiomyoma and myometrium (chi-squared test, $P < 0.05$). ER-α mRNA level in the leiomyoma tissue was 6.4 times higher than that in the myometrial tissue in this patient (data not shown).

Second, methylation status was analyzed for normal myometrium from an individual without myoma (Fig. 3B, Case 2). The proximal region (Regions I and II) was unmethylated, and only 3.6% in a total of 84 examined CpG sites were methylated in the distal promoter region (Region III) (Fig. 3B, Case 2). Since there was a difference in DNA methylation status between myometrium from an individual with myoma and without myoma, methylation status was further analyzed in myometrium from another individual with myoma (Fig. 3B, Case 3). The proximal region (Regions I and II) was unmethylated, and in the distal promoter region (Region III), 15.5% in a total of 84 examined CpG sites were methylated (Fig. 3B, Case 3).

Relationship between DNA methylation status of the distal promoter region and mRNA expression in uterine leiomyoma and normal myometrium

Since there was a variation in DNA methylation status in the distal promoter region by sodium bisulfite genomic sequencing, DNA methylation status of this region (six CpG sites; −1096 to −790), a part of the distal promoter region, was analyzed by sodium bisulfite restriction mapping in 11 paired samples of leiomyoma and myometrium. The results from the 11 patients showed two different methylation patterns. Of the 11 cases, nine showed unmethylated status in leiomyomas and a methylated status in myometrium for this region, which is represented as Pattern I in Fig. 4B. The other two cases showed methylated status in both leiomyomas and myometrium, which is represented as Pattern II in Fig. 4B. In the cases who showed Pattern I, ER-α mRNA levels tended to be higher in leiomyoma than those in myometrium (Table I). These

results suggest that CpG sites in the distal region of ER-α promoter are hypomethylated in leiomyomas in most of the patients, and this may be associated with higher mRNA expression in leiomyomas than in myometrium. There seemed to be no relationship between location and size of the leiomyoma and DNA methylation status (Table I).

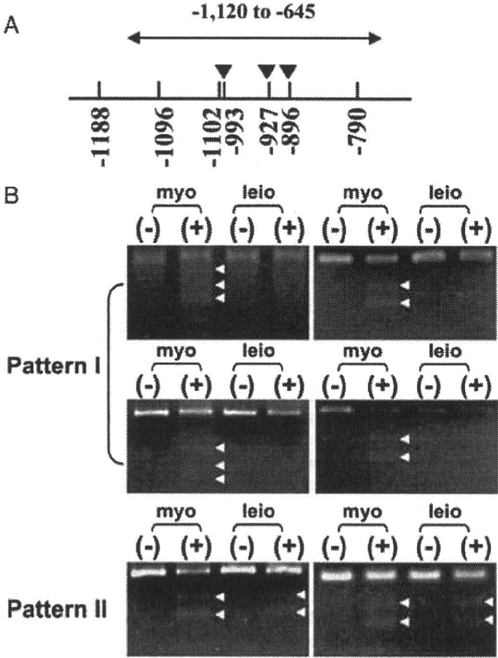

Figure 4: DNA methylation status of the distal promoter region in uterine leiomyoma and normal myometrium.
(A) Diagram of CpG sites ('vertical lines') and *Taq* I recognition sites ('filled triangles') in the distal promoter region. (B) Formation of fragmented DNA after *Taq* I treatment indicates methylated status in this region. Pattern I: unmethylated status in leiomyoma (leio) and methylated status in myometrium (myo) judging by DNA fragmentation in myometrium but not in leiomyoma. Pattern II: methylated status in both leiomyoma and myometrium judging by DNA fragmentation in both myometrium and leiomyoma. (−) undigested control without *Taq* I, (+) *Taq* I treatment. The white arrow heads indicate the fragmented DNA.

Discussion

The present study demonstrated that there were differences in DNA methylation status of the ER-α promoter region between uterine leiomyomas and normal myometrium, suggesting that epigenetic aberration actually occurs in uterine leiomyomas. We found DNA hypomethylation in the distal promoter region of ER-α (−1188 to −790) in a uterine leiomyoma compared with the myometrium by sodium bisulfite sequencing, and further confirmed hypomethylation status of this region in 9 patients out of the 11 patients by bisulfite restriction mapping. Moreover, in these patients who showed unmethylated status of this region in uterine leiomyomas and methylated status in myometrium, ER-α mRNA levels tended to be higher in leiomyoma than in myometrium. Thus, the pathological feature of uterine leiomyomas could be supported by our finding that the aberrant DNA hypomethylation was associated with an increased expression of ER-α that mediates sensitivity to estradiol. It is not surprising that there are cases in which DNA methylation status of the ER-α promoter region is not consistent with ER-α mRNA expression, because DNA methylation may occur heterogeneously and/or gradually and the change in DNA methylation varies among individuals. However, further studies with more samples are needed regarding the relevance of the promoter methylation pattern on ER-α mRNA expression.

A number of reports have addressed the association between aberrant DNA hypermethylation of the ER-α promoter and the ER-α inactivation in a variety of neoplasms such as breast cancer (Lapidus *et al.*, 1996; Yoshida *et al.*, 2000; Yan *et al.*, 2001; Berger and Daxenbichler, 2002; Giacinti *et al.*, 2006), prostate cancer (Li *et al.*, 2000), esophagus adenocarcinoma (Eads *et al.*, 2000), hematopoietic neoplasms (Issa *et al.*, 1996), brain tumors (Li *et al.*, 1998) and colon cancer (Ahuja *et al.*, 1998). In breast cancers, the increased incidence of DNA methylation in the CpG island in the proximal promoter or the first exon of ER-α was highly associated with the loss of ER-α expression (Lapidus *et al.*, 1996; Iwase *et al.*, 1999). In uterine leiomyomas, however, the DNA hypomethylated status was observed in the distal promoter region of ER-α outside the CpG island. In this regard, the epigenetic aberration that we found in uterine leiomyomas is different from those in the previous reports on other tumors or cancers. This is not surprising because it has been reported that DNA methylation of the CpG sites other than CpG islands in the promoter region regulates transcription (Razin and Cedar, 1991; Cho *et al.*, 2001; Imamura *et al.*, 2001). It is also suggested that DNA

Table I. Profile of the samples used for bisulfite restriction mapping.

Patients	Age	Location of leiomyoma	Diameter of the leiomyoma (cm)	ER-α mRNA levels		BRM pattern
				myo	leio	
1	41	Intramural	5	6	42	I
2	49	Intramural	16	31	50	I
3	37	Intramural	8	31	47	I
4	45	Subserosal	7	103	128	I
5	55	Intramural	10	119	120	I
6	53	Intramural	15	29	220	I
7	49	Intramural	7	59	68	I
8	44	Intramural	8	116	133	I
9	50	Intramural	3	100	101	I
10	46	Intramural	15	100	80	II
11	52	Subserosal	9	147	83	II

DNA methylation status of promoter region (six CpG sites; −1096 to −790), a part of the distal promoter region, was analyzed by sodium bisulfite restriction mapping (BRM) in 11 paired samples of leiomyoma (leio) and myometrium (myo). The results from 11 patients showed two different methylation patterns. Nine of the 11 cases showed unmethylated status in leiomyomas and methylated status in myometrium of this region, which is represented as Pattern I in Fig. 4B. The remaining two of the 11 cases showed methylated status in both leiomyomas and myometrium, which is represented as Pattern II in Fig. 4B.

methylation is involved in the regulation of gene expression regardless of richness of CpGs (Shiota, 2004).

The proximal promoter region and the first exon of ER-α are the most important region for ER-α expression (McPherson *et al.*, 1997; Reid *et al.*, 2002). The proximal region is the minimal core promoter of the ER-α gene and determines on–off switching of the ER-α transcription. On the other hand, two EREs, ERE 2 and 3, are present in the distal promoter region and can also regulate transcription of ER-α gene (Treilleux *et al.*, 1997). In addition, the distal promoter region has been reported to contain two ER-α upstream binding factor-1 binding sites, which have a strong transcriptional enhancer activity (Cohn *et al.*, 1999). Thus, the distal promoter region is considered as important for the modulation of ER-α transcriptional level. In fact, the present *in vitro* study revealed that ER-α mRNA expression was increased by 5-aza-dC that inhibits DNA methylation, suggesting that ER-α mRNA expression is under epigenetic regulation. However, further studies including promoter activity assay with methylated reporter constructs are needed to demonstrate that DNA methylation of the distal promoter region actually controls ER-α mRNA expression.

It is of interest to note that DNA methylation of the distal promoter region of ER-α was observed in several sequenced clones of myometrium and that the extent of DNA methylation in this region of the myometrium varies among individuals, suggesting that DNA methylation occurs heterogeneously in the normal tissue, which may be a part of physiological changes in a certain cell type such as smooth muscle cells in myometrium. In fact, there is also a variation in DNA methylation status of the promoter region of ER-β in human endometrial stromal cells among individuals (Xue *et al.*, 2007). Alternatively, DNA methylation seen in the myometrium may be caused by some factors that induce aberrant DNA methylation such as aging, chronic inflammation and possibly viral infection (Ushijima and Okochi-Tanaka, 2005).

This is the first report demonstrating that in uterine leiomyomas there is aberrant DNA hypomethylation in the ER-α promoter, especially outside the CpG island that has been well studied in other clinical cases. Decreased mRNA expression of DNA methyltransferase-3 (DNMT-3) with genome-wide DNA hypomethylation has been reported in uterine leiomyomas compared with myometrium (Li *et al.*, 2003). This suggests that epigenetic alterations are involved in the development of uterine leiomyomas. The aberrant hypomethylation of the ER-α gene could also be caused by the decreased DNMT-3 level.

Recent data have shown that altered expression of a variety of genes contributes to pathogenesis of uterine leiomyomas (Skubitz and Skubitz, 2002; Tsibris *et al.*, 2003; Luo *et al.*, 2005). Taken together with our finding, potential epigenetic alterations such as aberrant DNA hypomethylation are strongly suggested to be involved in pathogenesis of uterine leiomyomas.

In conclusion, there seems to be a potential link between aberrant DNA methylation level and ER-α expression in uterine leiomyomas, and this is the first example that the ER-α promoter region is aberrantly hypomethylated in human disease cases.

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再生医療のためのエピジェネティクスとエピゲノム

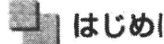
前田千晶* 塩田邦郎*

KEY WORDS

エピジェネティクス，エピゲノム，DNA メチル化，ヒストン修飾，リプログラミング，幹細胞，iPS 細胞，再生医療

SUMMARY

再生医療に用いられる細胞として，組織幹細胞，胚性幹細胞（embryonic stem cell：ES 細胞）につづいて，新たに人工多能性幹細胞（induced pluripotent stem cell：iPS 細胞）が作出された．少数遺伝子導入により多分化能が獲得できることが判明し，"細胞とは？細胞の評価法は？細胞の安定性とフレキシビリティーとは？"などが改めて問われている．エピジェネティクス制御系は，DNA 塩基配列は一定のままで，遺伝子発現を固定・記憶する機構である．"初期化"や"リプログラミング"の分子機構にはエピジェネティクスが深くかかわっている．ゲノム全体のエピジェネティクス情報をエピゲノムという．本稿では，医療への応用が期待される細胞や技術におけるエピジェネティクスとエピゲノム研究の重要性と緊急性を記す．



はじめに

幹細胞は自らを複製，再生する能力（自己複製能）と，さまざまな細胞へと分化する能力（多分化能）を有する細胞である．幹細胞のうち最も多くの細胞に変わることができる細胞が胚性幹細胞（embryonic stem cell：ES 細胞）である．ES 細胞は胚発生初期の着床前の受精卵，胚盤胞の細胞（内部細胞塊）よりつくり出される．一方，組織幹細胞（成体幹細胞，体性幹細胞）は，分化した組織中に存在する未分化な細胞（群）で，通常，すべての種類の細胞には分化できないが，特定系列の複数種類の細胞へ分化が可能な幹細胞を指す．間葉系幹細胞，造血幹細胞，神経幹細胞などが含まれ，成体組織の他，臍帯など多数の組織から発見されている．近年，間葉系幹細胞が，骨組織，脂肪，骨格筋の他，神経細胞，肝細胞，インスリン分泌細胞など，多様な細胞系列に分化転換しうることが報告されてきた．さらに，2006 年に，マウス線維芽細胞から人工多能性幹細胞（induced pluripotent stem cell：iPS 細胞）が作製され[1]，翌年にはヒト iPS 細胞が作出されたことで[2)3)]，再生医療は実現化に向けて大

*MAEDA Chiaki，SHIOTA Kunio/東京大学大学院農学生命科学研究科細胞生化学研究室

きく前進した.

iPS細胞, ES細胞を実際に応用するときに考慮するべきことは, どちらも特定の培養条件下で選択することにより人工的に創出された細胞だという点である. そのため, これらの標準細胞を生体内に求めることはできない. いったん分化した体細胞が多能性を再獲得するメカニズムと, 人工細胞をどのように評価するかに関心が集まっている.

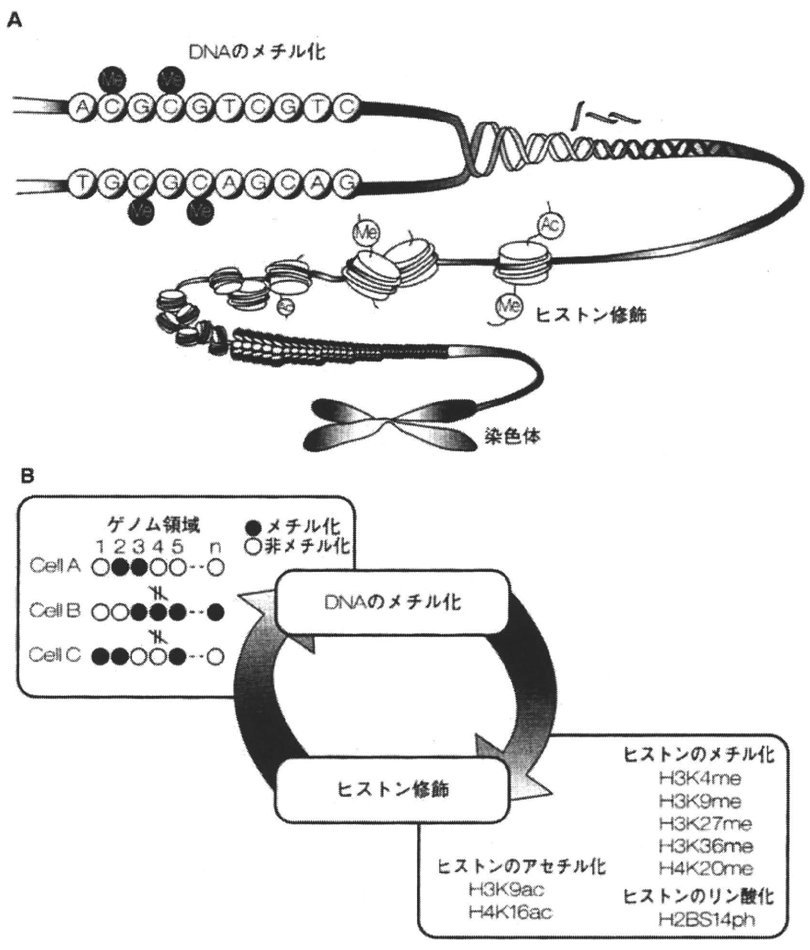
エピジェネティクスとエピゲノムとは

哺乳類の細胞には, 約3×10^9塩基対からなるDNAが含まれており, この配列情報は一部の例外を除いてすべての細胞で共通である. 一方, 細胞はその種類や分化段階に応じて発現する遺伝子を使い分けている. 分化した細胞では, 遺伝子の使い分け機構は細胞分裂をくり返しても維持される. エピジェネティクスとは, 膨大なDNAの塩基配列のカタログから, 必要な情報を選択して利用し, 記憶する機構といえる. エピジェネティクス制御系には, DNAのメチル化, ヒストン修飾(メチル化, アセチル化など), ヒストンバリアントの使い分け, non-coding RNAなどが含まれる(**図❶**). DNAのメチル化は, おもにシトシン, グアニンと連続するCpG配列のシトシンにメチル基が付加される現象を指す. DNAメチル化とヒストン修飾は互いに密接に関連しており, 個体の正常な発生に必須である. ゲノム全域におけるDNAメチル化やヒストン修飾などのエピジェネティック情報の総体をエピゲノムとよぶ.

移植治療のために特定の細胞集団を作出した場合, 得られた細胞が目的の細胞の形質を示し, それ以外の細胞の形質は示さないことをどのように担保するべきであろうか. 細胞の形態や遺伝子発現, および*in vitro*での分化能に加え, エピジェネティクス評価は威力を発揮する. ゲノム上には, 細胞の種類によってDNAメチル化状態の異なる領域, つまり組織・細胞種特異的なメチル化領域(tissue-dependent and differentially methylated region:T-DMR)が多数存在し[4)5)], その細胞のエピゲノムを特徴づけている. 複数のT-DMRのメチル化・非メチル化のパターンの組み合わせを細胞の"DNAメチル化プロフィール"とよぶ. DNAメチル化プロフィールは, 細胞の種類ごとに異なる安定したゲノム上の情報である. このため, DNAメチル化プロフィールを細胞の同定に利用できると考えられる.

エピゲノムはどのように形成されるのか

シトシンへのメチル基転移反応は, DNAメチル基転移酵素(DNA methyltransferase:Dnmt)によって触媒される. ところが, メチル化される領域とされない領域といったDNAメチル化の領域選択性は, Dnmtの酵素活性や発現量のみで決定されるのではない. なぜなら, Dnmtはいずれも DNAに対する配列特異性をもたず, *in vitro*ではすべてのCpG配列に作用するからだ. 領域選択的なDNAメチル化機構には, ヒストン修飾酵素が関係している. クロマチンが弛緩したユークロマチン領域に作用するヒストンメチル基転移酵素であるG9aは, ヒストンH3K9およびH3K27のメチル化を触媒する[6)7)]. G9a欠損ES細胞においてゲノムワイドなDNAメチル化解析をおこなったところ, 解析可能な約1,300座位のうち, 32座位でDNAの脱メチル化が検出された[8)]. これらの領域ではヒストンH3K9あるいはH3K27の脱メチル化も観察され, 確かにG9aの標的領域であると考えられた. G9a自体はDNAメチル基転移活性をもたないことから, このようなG9aの標的領域の一部では, ヒストン修飾状況がDNAメチル化を誘導するような環境をつくっていると考えられる. また, G9aがDnmt1と直接相互作用すること[9)]や, 凝集したクロマチンに結合するヘテロクロマチン蛋白質HP1とDnmt1が協調してはたらくことも報告されてきた[10)]. このように, DNAメチル化とヒストン修飾は相互作用を通して各領域のクロマチン構造を形成している(**図❶**). そこには, HP1をはじめとしたクロマチン結合因子や, メチル化されたDNAに結合するMBDファミリー蛋白質(MeCP2, MBD1-MBD4), RNA分子なども関与する.



図❶ 細胞のエピゲノムとエピジェネティクス機構間の相互依存
A）エピジェネティクス制御系には，DNAのメチル化，ヒストン修飾（メチル化，アセチル化など），ヒストンバリアントの使い分け，non-coding RNAなどの多数の機構が含まれる．ゲノム領域ごとに，付加されているエピジェネティック修飾の組み合わせは異なっており，全体として細胞の種類に特異的なエピゲノムをつくりあげている．
B）DNAのメチル化とヒストン修飾は，相互作用しながらエピゲノム形成にかかわっている．

リプログラミングとはエピゲノムの変化である

iPS細胞作製や核移植クローニングで盛んに用いられてきた，"初期化"あるいは"リプログラミング"とはエピゲノムの変化のことである．多くの場合は定義があいまいなまま，うまく細胞が変化し発生が進行したことをいいかえて，"初期化"あるいは"リプログラミング"がうまくいったといいかえているにすぎない．しかし，細胞分化の基盤としてエピジェネティクス制御系があることは間違いない．細胞の種類によってエピゲノムは異なることから，"初期化"あるいは"リプログラミング"をゲノムレベルで定義すれば，"初期胚や幹細胞と同等のエピゲノムへの書き換え"ということになる．

骨髄由来の間葉系幹細胞（mesenchymal stem cell：MSC）および脂肪組織由来の幹細胞（adipose tissue-derived stem cell：ASC）にDNAメチル化阻害剤である5-アザシチジンを添加すると，筋細胞，心筋細胞への分

化が促進される[11)12)]．一方，MSCに，ヒストン脱アセチル化阻害剤のトリコスタチンA（TSA）を添加し，高グルコース下で培養すると，膵臓β細胞のマーカー遺伝子を発現するようになる[13)]．また，FGF-4を含む複数の因子とTSAとの組み合わせで，ヒトMSCから肝細胞様の細胞もつくられている[14)]．神経幹細胞においては，ヒストン脱アセチル化阻害剤のバルプロ酸（VPA）が，神経細胞への分化を誘導する[15)]．これらの薬剤の作用機序は完全には明らかになっていないが，エピゲノムの変化が組織特異的な遺伝子発現を調節し，ひいては細胞系譜の運命決定にかかわっていると考えられる．

クローン技術を応用してつくられた幹細胞として，核移植胚由来のES細胞（nuclear transfer embryonic stem cell：ntES細胞）がある．ntES細胞は，体細胞から核移植をおこなってクローン胚盤胞を作製し，その内部細胞塊を培養して樹立されたES細胞である[16)]．マウスntES細胞は種々の解析において自然交配の胚盤胞から樹立されたES細胞と区別がつかず[17)18)]，ntES細胞から分化誘導した血球系細胞の移植が治療効果をあげている[19)]．興味深いことに，クローン胚盤胞をあらかじめTSAで処理してからntES細胞株を樹立すると，処理しない場合にくらべて約2〜3倍効率が上昇する[20)]．また，核移植した卵をTSA処理することで，クローン胚盤胞の形成率，およびクローン個体の出生率も上昇する[20)21)]．これらの結果は，エピジェネティクスが個体発生の基礎であるとする考えと矛盾しない．

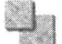
iPS細胞は，体細胞に2〜4つの遺伝子を導入することにより，幹細胞の形質を獲得させた細胞である．形態や分化能はES細胞に類似し，網羅的な遺伝子発現解析においてもES細胞と強い相関を示す．また，その樹立にヒトの胚を利用する必要がなく，患者本人の細胞をもとにつくることができるという利点をもつ．第2世代のマウスiPS細胞（Nanog-iPS）およびヒトiPS細胞では，*Oct3/4*遺伝子をはじめとするいくつかのES細胞マーカー遺伝子が，ES細胞と同様に低メチル化状態であることが確認された．また，一部のヒストン修飾についてもES細胞と近いパターンを示すことが報告されている[22)]．

線維芽細胞からのiPS細胞の樹立率は，マウスでもヒトでも0.001〜0.5%ときわめて低い[2)23)]．多くはES細胞とは似つかない顆粒状のコロニーを形成し，多分化能は獲得しない．iPS細胞樹立の効率化に，DNAメチル化阻害剤やヒストン脱アセチル化阻害剤は有効であるのか，種々の化合物の影響が調べられた[24)]．*Oct3/4*，*Klf4*，*Sox2*，*c-Myc*の4つの遺伝子を導入した線維芽細胞において，5-アザシチジン添加は単独で10倍，またデキサメタゾンとの組み合わせにより，約26倍ものiPS出現効率の上昇をもたらした．また，いずれもヒストン脱アセチル化阻害作用をもつスベロイラニリド・ハイドロザミック酸（SAHA），TSA，およびVPAのなかでは，VPAの効果がとびぬけて高く，未処理にくらべて100倍以上のiPS細胞が得られた．さらに，VPA処理は，癌遺伝子である*c-Myc*を除く3遺伝子導入の線維芽細胞においても，約50倍の効率上昇効果を示した．遺伝子導入していない通常の線維芽細胞へのVPA添加実験から，VPA添加によって，線維芽細胞の網羅的な遺伝子発現のパターンがES細胞のそれに近づくことが示されている[24)]．

ntES細胞，iPS細胞において注意すべきなのは，ゲノム全体をヒストン高アセチル化，あるいはDNA脱メチル化することが，分化多能性の獲得に有利であるとは考えにくい点だ．先に記したように，エピゲノムは細胞ごとに異なり，ES細胞であってもゲノム全域のDNAメチル化状況は決して非メチル化ではない．ES細胞は他の細胞とくらべて異なったDNAメチル化プロフィールを有しているのであって，未分化=DNA低メチル化ではないといえる[4)]．そして，細胞分化やiPS化の過程には，必ず細胞選択過程が含まれる．DNAメチル化やヒストン修飾に影響を与える化合物処理は，ゲノムにエピジェネティクスショックを与えているとすれば理解しやすい．これらの化合物でエピジェネティクス変化が起きた場合，各細胞は生存をかけてエピゲノムをもとに戻すか，あるいは，さらに変化させ，もととは異なった別の状態に落ち着くはずである．どちらにも失敗した細胞は死滅する．薬剤処理をきっかけに引き起こされるエピゲノムの複合的な状況変化が，細胞の選択に偏りを生じさせ，分化多能性細胞の樹立促進を可能にしたと考えるべきであろう．

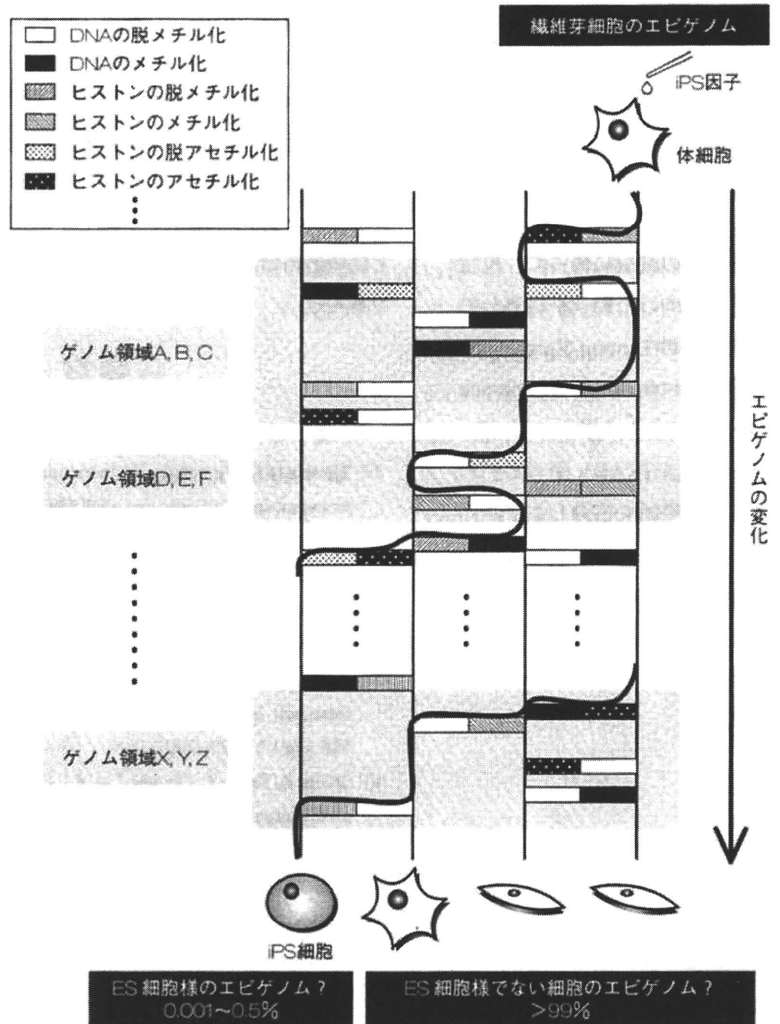
重要なことは，細胞が他の細胞に変化するとき，そこ

にはmRNAや蛋白質の発現変化のみならず，そのもととなるエピゲノムの変化があるということである．体細胞がES細胞様の形質を獲得するためには，ゲノム領域ごとにエピジェネティック修飾が書き換えられ，ES細胞のエピゲノムに近い状態に変化する必要があると考えられる（図❷）．



エピゲノム解析技術

ゲノムワイドなDNAメチル化状況，およびヒストン修飾状況を明らかにするため，網羅的な解析法の開発が進んでいる．これらはおもにマイクロアレイを使うものと使わないものに大別できる．マイクロアレイでは，高密度プローブ実装技術の進歩に伴い，ゲノム配列をそのままタイリングした高解像度のタイリングアレイが使われるようになった．マイクロアレイに供するサンプルの



図❷ リプログラミングとはエピゲノムの変化である
細胞が新たな細胞に変化するとき，そこにはゲノム領域ごとに異なるエピジェネティック修飾の書き換えが起きている．線維芽細胞のエピゲノムがES細胞様のエピゲノムに変化することで，iPS細胞がつくられると考えられる．

調整法としては，DNA メチル化抗体や特定のヒストン修飾の抗体で免疫沈降したゲノム分画を用いる me-DIP 法や ChIP-Chip 法の他，DNA メチル化感受性制限酵素 *Hpa* II と非感受性制限酵素 *Msp* I で切断したゲノムを用いる HELP 法，MIAMI 法などがある．

筆者らのグループは最近，新たなゲノムワイド DNA メチル化解析法として，D-REAM 法を確立した[25]．メチル化感受性制限酵素の認識部位のメチル化状況をタイリングアレイのシグナルに反映させることができる系であり，原理的にはメチル化感受性制限酵素の種類に依存しない解析が可能である．一方，高速シークエンサーの登場により，ハイスループットシークエンスが可能となり，ゲノムワイドな解析において勢いを増している．ゲノムサイズが比較的小さいシロイナズナでは，ゲノムの大部分の DNA メチル化状態が明らかにされた[26]．また，マウスにおいても，分化・未分化細胞の網羅的 DNA メチル化解析などに応用されている[27]．

このようなエピゲノム解読技術の発展に伴って，現在世界ではヒトのエピゲノム解明へ向けた動きが加速している．ヨーロッパを中心に進行中の Human Epigenome Project（HEP）や Epigenome Network of Excellence（NoE）に加え，アメリカの先導による Alliance for the Human Epigenome and Disease（AHEAD）がエピゲノムデータベースの基盤づくりに国際的な協調をよびかけている[28]．このプロジェクトでは，統一された抗体や手法を用い，複数種類の組織・細胞についてエピジェネティック情報を網羅的に記述したエピゲノムマップをつくっていこうとするものである．

おわりに

幹細胞や前駆細胞を用いた再生医療は，現実のものとなりつつある．骨髄由来の幹細胞を用いたクローン病の治療は，臨床試験第Ⅲ相にある他，骨や心臓再生をめざした組織幹細胞治療も臨床試験第Ⅱ相に進んでいる．こうした進展に加え，今後は iPS 細胞の応用研究も新領域を切り拓くことになるだろう．今年7月，筋萎縮性側索硬化症（amyotrophic lateral sclerosis：ALS）の患者の皮膚細胞から樹立された iPS 細胞が，病気の進行で失われる運動神経細胞に分化することができたと報告された[29]．得られた細胞において，形態および神経細胞マーカーの発現は確認された．つぎのステップとしては，エピゲノムの正常性を調べる必要があるだろう．しかし，生体から分離した運動神経細胞と，培養下で樹立したそれ様のもので，ゲノム全域のエピジェネティック状態が寸分違わず一致することは考えにくい．樹立した細胞が神経細胞の表現型を示すには，神経細胞に特徴的に発現する，あるいは発現しない，遺伝子については，生体細胞と同じエピジェネティック状態を示す必要がある．しかし，それ以外の遺伝子領域について，また，反復配列などを含む遺伝子以外のゲノム領域について，どれほどのエピジェネティックな差が許容されるのか．今後の焦点となる重要なトピックである．幹細胞を評価する段階，幹細胞から目的の細胞を樹立する過程，およびできあがった細胞を評価する段階，のいずれにおいてもエピジェネティクスの観点は必要である．メカニズムの解明と臨床応用は，緊密に連携しながら進められていくべきであろう．

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まえだ・ちあき

前田千晶　東京大学大学院農学生命科学研究科細胞生化学研究室

現在の研究テーマは脳のエピジェネティクス.
好きな言葉は「現状維持とは, 急速に世界から遅れていくこと」.

しおた・くにお

塩田邦郎　東京大学大学院農学生命科学研究科細胞生化学研究室

1979年東京大学大学院修了（農学獣博士), 武田薬品中央研究所を経て, 1987年より東京大学助教授, 1998年より教授.
専門はエピジェネティクス.
趣味は写真とソフトボール.
愛読書は山本七平.

幹細胞をエピジェネティクスで評価する

新井良和
八木慎太郎
塩田邦郎

ES細胞（embryonic stem cell）は，哺乳類の体を構成する生来の200種類の細胞とは異なる，人工的に作出された細胞である．ES細胞同様，人工的に作出されたiPS細胞（induced pluripotent stem cell）を評価するにあたり，比較すべきものの拠り所がないまま，再生医療への期待のみが先行しているのが現状である．再生医療時代に相応しい細胞の評価法を確立しなければ，この先の世界で取り残されてしまう．ゲノム全域のエピジェネティクス解析は，このような細胞評価，標準化の有力な手段となるだろう．

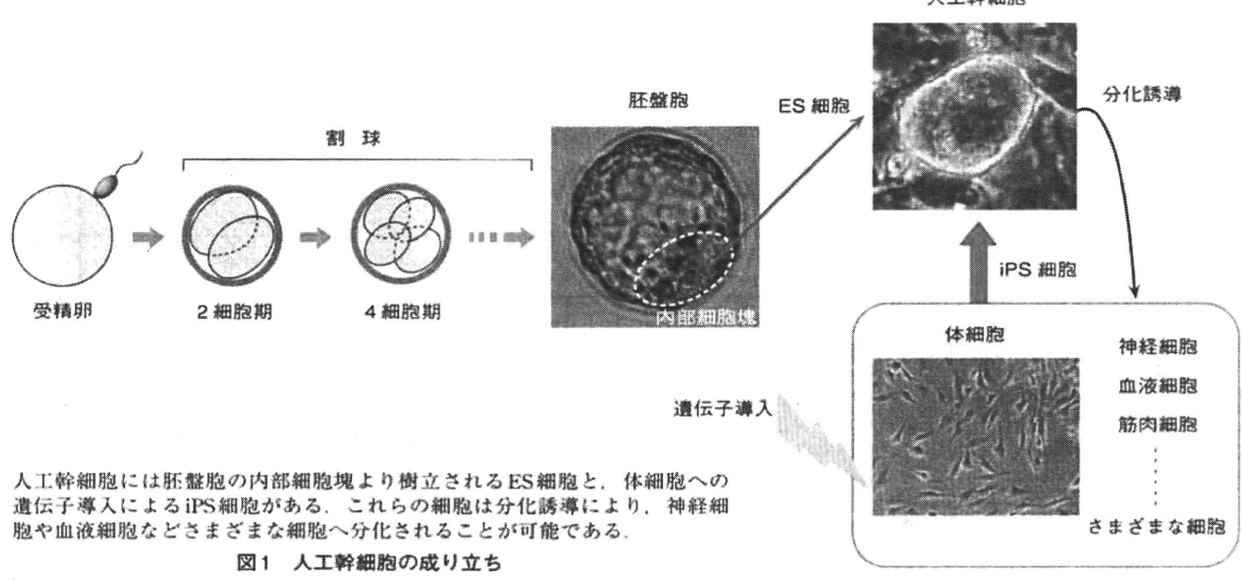
細胞評価にもパラダイムシフトが必要

幹細胞は自らを複製，再生する能力（自己複製能）と，別の違ったさまざまな細胞へと変身（分化）する能力（多分化能）をもつ細胞である．幹細胞のうち最も多くの細胞に変わることができる細胞がES細胞であり，胚発生初期の着床前の受精卵，胚盤胞の細胞（内部細胞塊）よりつくり出される（図1）．

カエルやイモリのように高い組織再生能を示す動物と比べて，哺乳類の場合は，いったん分化した細胞は元に戻ることは難しく，分化した細胞から異なる種類の細胞に変わる能力をもつ細胞を作製することは困難であった．しかし完全に分化した細胞の核を卵に導入することにより，頻度は低いがクローン動物をつくることができること，さらにクローン動物からES細胞も樹立できることから，再分化能を人工的に獲得することは不可能ではないことが証明された．

そして2006年に京都大学の山中伸弥らにより世界で初めて，ES細胞と同様にさまざまな細胞へ分化することができるiPS細胞が樹立された（文献1）．iPS細胞は，患者自身の細胞を基に作製することが可能であるため，ES細胞では避けて通れない倫理的問題や免疫拒絶の問題が回避できる．再生医療の夢が現実に大きく近づいた．

iPS細胞，ES細胞を実際に応用するときに，これから考慮するべきことは，iPS細胞はもとより，ES細胞は，特定の培養条件下で選択することにより人工的に創出された細胞であ



人工幹細胞には胚盤胞の内部細胞塊より樹立されるES細胞と，体細胞への遺伝子導入によるiPS細胞がある．これらの細胞は分化誘導により，神経細胞や血液細胞などさまざまな細胞へ分化されることが可能である．

図1　人工幹細胞の成り立ち

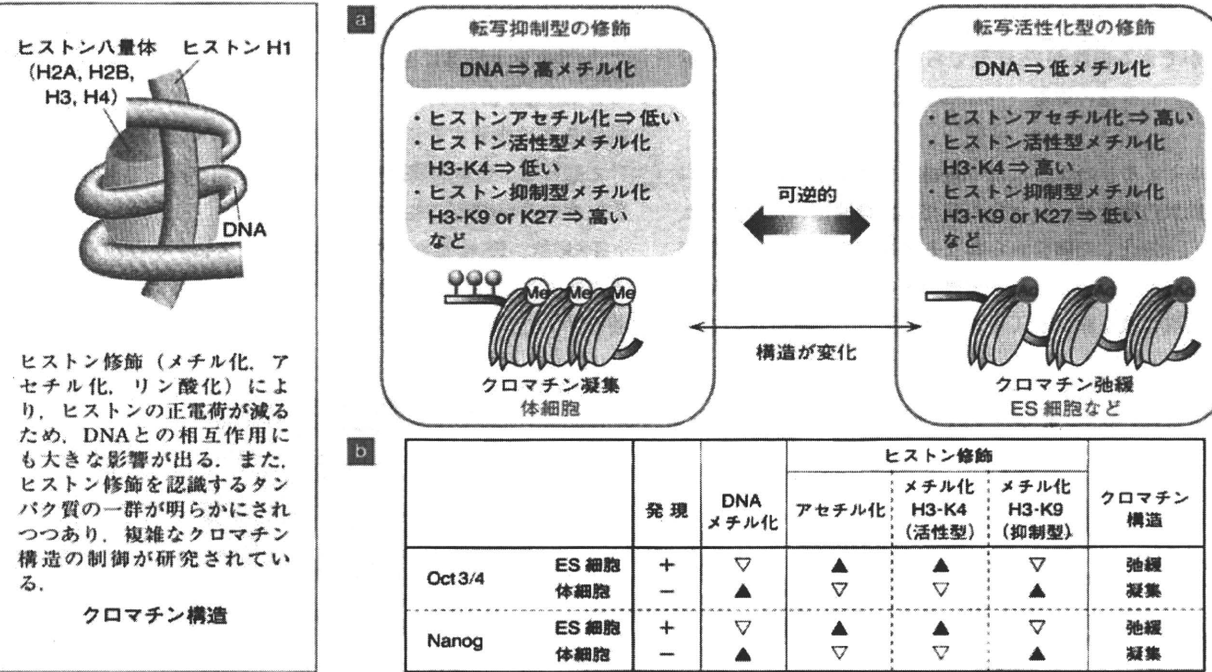
るため，ES細胞の標準細胞を生体内に求めることはできない点である．動物実験に限定して使われていた時代には，細胞の形態と体外培養下での分化能に加え，数種類のマーカー遺伝子の発現および，細胞移植によるテラトーマ（奇形腫）の形成とキメラ形成能などの生体内評価をすれば事足りた．しかし，iPS細胞の創出により，再生医療への応用が現実味を帯びてきた今，動物で検証されてきた方法，たとえばキメラ形成実験などがヒトでは不可能であること，再生医療は個々人を対象とするテーラーメード化された医療になったことを考えると，iPS細胞同様にパラダイムシフトした評価方法が必要である．

幹細胞のマスター遺伝子：*Oct3/4* と *Nanog*

iPS細胞を創出する際の重要な因子である*Oct3/4*や*Nanog*は，ES細胞のマーカー遺伝子として重要であることに加え，ES細胞をES細胞たらしめているマスター遺伝子，つまりES細胞特有の遺伝子の発現量を制御する転写因子であり，これらの因子，さらにこれらによって制御される転写因子がES細胞に特有の遺伝子発現を支えている．たとえば*Oct3/4*遺伝子を欠損した初期胚（胚盤胞）では，ES細胞の基になる内部細胞塊を形成できず，ES細胞を樹立することもできない（文献2）．また，*Oct3/4*の発現量が適当でないとES細胞は多分化能を維持することができない（文献3），など*Oct3/4*の発現は，ES細胞の樹立と維持に必須である（本誌33ページも参照）．そしてこれらのマスター遺伝子の発現を制御している重要な機構はエピジェネティック機構である．

幹細胞のエピジェネティクス

エピジェネティクスとは，「DNAの塩基配列の変化を伴わず細胞切裂後も継承される遺伝子機能の変化を研究する学問領域」を意味する．すなわち受精卵から胎児発生を経て個体が誕生するまで，それぞれの細胞に必要な遺伝子発現をオンにし，不必要な遺伝子をオフにする．一方で，いったん分化決定した細胞では，その発生・分化過程で確立した遺伝子のオン・オフ機構は，細胞分裂後も記憶される必要がある．この遺伝子制御とその記憶機構がエピジェネティック機構であ



		発現	DNAメチル化	ヒストン修飾 アセチル化	ヒストン修飾 メチル化 H3-K4（活性型）	ヒストン修飾 メチル化 H3-K9（抑制型）	クロマチン構造
Oct3/4	ES細胞	+	▽	▲	▲	▽	弛緩
	体細胞	−	▲	▽	▽	▲	凝集
Nanog	ES細胞	+	▽	▲	▲	▽	弛緩
	体細胞	−	▲	▽	▽	▲	凝集

a）遺伝子発現はDNAメチル化やヒストン修飾状況により制御されている．DNAの高いメチル化状況や抑制型ヒストン修飾（ヒストンH3リシン9あるいは27番目（H3-K9 or 27）のメチル化など）によりクロマチンは凝集し，遺伝子の転写は抑制される．一方，DNAの低いメチル化状況や活性型ヒストン修飾（アセチル化やヒストンH3リシン4番目（H3-K4）のメチル化など）によりクロマチンは弛緩し，遺伝子の転写は活性化される．
b）*Oct3/4*や*Nanog*遺伝子が発現するES細胞では，クロマチンが弛緩した活性型の修飾がみられる．一方，発現の認められない体細胞では，クロマチンが凝集した抑制型の修飾がみられる．このようなDNAメチル化とヒストン修飾により，遺伝子は細胞種に固有の発現パターンを示すのである．▲は修飾状況が高いこと，反対に▽は低いことを示す．

図2　*Oct3/4,Nanog*遺伝子のエピジェネティクス制御

り，DNAメチル化とヒストン修飾が中心的な分子機構である．

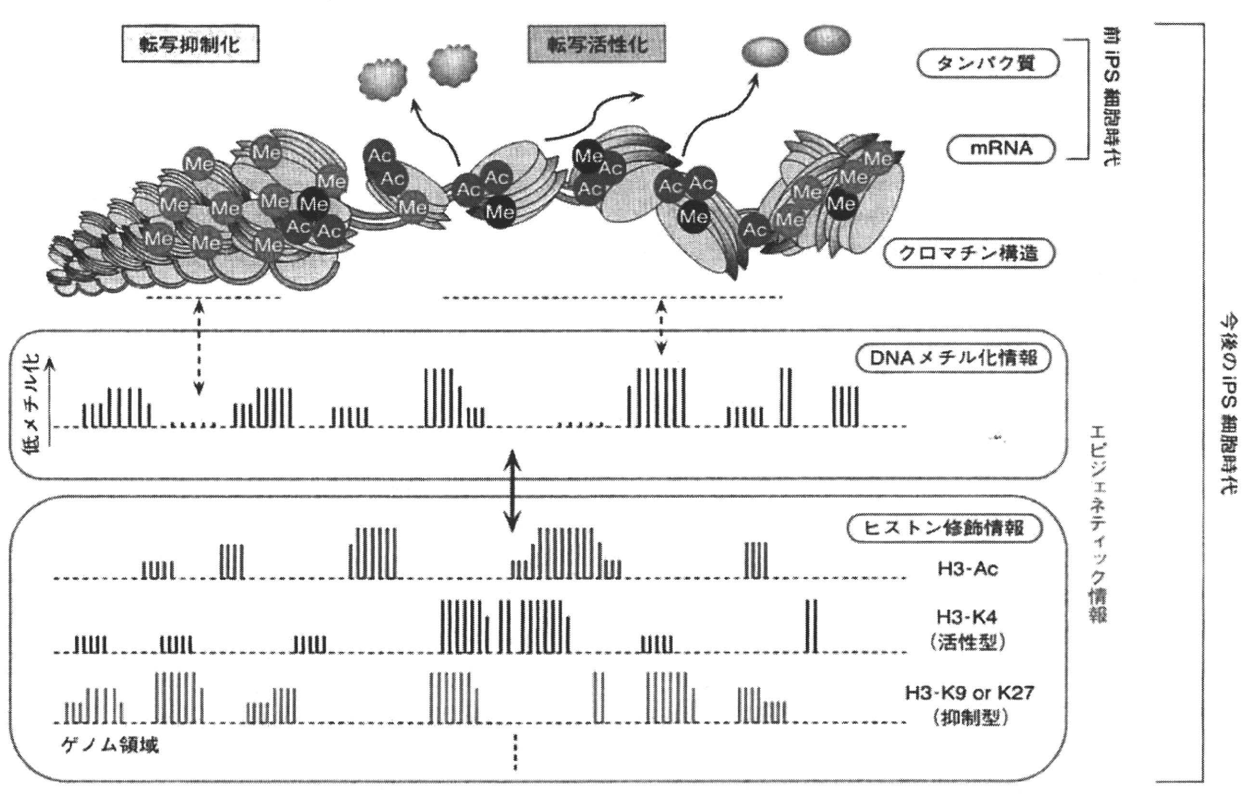
DNAのメチル化は哺乳類ゲノムにみられる唯一の化学修飾であり，おもに，シトシン（C），グアニン（G）の繰返し配列（CpG配列という）のシトシンがメチル化される．一方ヒストンにはさまざまな修飾があり，いくつかの修飾は，短時間に変化する．転写抑制型，転写活性型のヒストン修飾と，DNAのメチル化によりクロマチンの構造が変化し，転写が制御される（図2a）．

ES細胞など*Oct3/4*や*Nanog*が発現している細胞では，これら遺伝子の転写開始点や開始効率を決定するプロモーター領域で，DNAは低メチル化，ヒストンはアセチル化され，転写が活発な状況にある（文献4，5）．一方，*Oct3/4*や*Nanog*の発現が認められない体細胞では，DNAは高メチル化，ヒストンは脱アセチル化され，遺伝子発現が抑制されている．つまり，細胞ごとに異なる*Oct3/4*, *Nanog*の遺伝子発現パターンは，DNAメチル化，ヒストン修飾状況からなる，細胞に固有のエピジェネティックパターンに表されている（図2b）．

エピゲノム：ゲノム広域のエピジェネティクス情報

細胞や組織には，固有の細胞や組織に依存的メチル化領域（tissue dependent differentially methylated region，T-DMRという）があり，ゲノム上には膨大な数のT-DMRが存在する．細胞の種類ごとにT-DMRのメチル化状況は異なり，細胞の種類に特有のメチル化・非メチル化模様，すなわちDNAメチル化プロフィールが形成されている（文献7）．DNAメチル化プロフィールは，細胞のエピジェネティック状況の同一性，類似性，あるいは違いを知る上でよい指標となる．発生上で近縁あるいは同じ細胞系列の細胞（ES細胞と胚性生殖細胞など）の間では似たDNAメチル化プロフィールを示し，逆に発生上で異なった細胞（ES細胞と体細胞など）ではその違いは大きい（文献8）．

ヒトES細胞やマウスiPS細胞での一部のヒストン修飾についてはゲノム全域の解析が行われている（文献9）．ヒストン修飾情報とDNAメチル化情報は互いに密接な関連があり（図3），ヒストンの修飾状況を変化させると，DNAメチ



DNAメチル化情報とヒストン修飾情報は互いに密接な関係にあり，ヒストン修飾状況の変化と共にDNAメチル化も変化する．これらのエピジェネティック情報は，マーカー遺伝子発現を含めたこれらのES細胞，iPS細胞の新たな評価法として期待される．ヒストン修飾には60種以上が発見されており，ヒストン修飾の組合わせによるいわゆるヒストンコード説が成り立っている．

図3　エピジェネティック制御