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Brief Report

Rest Promotes the Early Differentiation of Mouse ESCs but Is Not Required for Their Maintenance

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The functional significance of *Rest* in the maintenance of ESC pluripotency remains controversial. We herein showed that *Rest* is not necessary for the maintenance of mouse ESCs, and instead suggested that the Rest transcriptional repressor connects to the Oct3/4-Sox2-Nanog core regulatory circuitry during early ESC differentiation.

The pluripotency of ESCs is maintained by coordinated expression of a core regulatory circuit of genes that includes Oct3/4, Sox2, and Nanog. Rest (also called Nrsf) is abundantly expressed in ESCs and is a target of the Oct3/4-Sox2-Nanog regulatory network. However, the functional significance of Rest in the maintenance of pluripotency remains controversial. We have generated *Rest* conditional knockout and *Rest*-inducible ESC lines. Conditional ablation of *Rest* showed that it is not required for maintenance of pluripotency, but it is involved in the suppression of self-renewal genes during early differentiation of ESCs. In addition, forced expression of *REST* in ESCs results in rapid differentiation. These results indicate that *Rest* is not necessary for the maintenance of mouse ESCs, and instead suggest that the Rest transcriptional repressor connects to the Oct3/4-Sox2-Nanog core regulatory circuitry during early ESC differentiation.

The transcriptional repressor Rest is a zinc finger protein that binds to a conserved 23 bp motif known as RE1 (repressor element 1, also called NRSE) in a number of genes encoding the fundamental neuronal traits (Chong et al., 1995; Schoenherr and Anderson, 1995). Rest is expressed throughout early development where it represses the expression of neural genes, such as *Syp* and *Syt4* (Schoenherr et al., 1996). Rest is also expressed in ESCs and it has also been shown to be one of target genes of the regulatory circuitry of the pluripotent state in ESCs (Johnson et al., 2008; Sun et al., 2005). However, the functional significance of Rest in the maintenance of pluripotency in ESCs still remains controversial (Buckley et al., 2009; Jørgensen et al., 2009a; Singh et al., 2008). A previous study with a heterozygous *Rest* ESC line combined with an siRNA knockdown indicated that Rest maintains pluripotency through the induction of self-renewal genes, such as *Oct3/4*, *Nanog*, and *Sox2* (Singh et al., 2008). In contrast, Jørgensen et al. generated a *Rest* null ESC line and reported that such *Rest* null ESCs revealed no substantial change in either the Oct3/4 protein levels or alkaline phosphatase activity in comparison to matched wild-type controls (Jørgensen et al., 2009a, 2009b).

In order to elucidate the role of *Rest* in the maintenance of pluripotency, we first generated an ESC line and mice that contained the conditional knockout alleles of *Rest*. The first *Rest* allele in the ESCs (V6.5) was replaced with the KO vector carrying the floxed last exon of *Rest*, which encodes the coRest binding site that is essential for the generation of the silencing complex (Andrés et al., 1999; Grimes et al., 2000), followed by *ires-Gfp* to monitor the transcription of the modified allele ($Rest^{3lox/+}$; Figure 1A). The transient expression of *Cre recombinase* generated a *Rest* floxed ESC line that lacks a drug selection cassette ($Rest^{2lox/+}$). Analyzing the GFP expression allowed us to confirm that *Rest* is expressed in ESCs (Figure 1B).

$Rest^{-/-}$ ESCs were next generated with the floxed *Rest* ESC line together with a plasmid expressing *Cre recombinase* (Figure 1A). After the excision of the floxed *Rest* gene by the transient transfection of *Cre* ($Rest^{+/-\ (1lox)}$), the second *Rest* allele was also replaced with the floxed allele ($Rest^{3lox/-}$). The transient transfection of *Cre* into $Rest^{3lox/-}$ ESCs resulted in the establishment of $Rest^{-/-}$ ESCs that were isogenic to the parental ESCs without any genetic modification except for the *Rest* alleles.

After the recombination of the *Rest* alleles, the lack of a *Rest* transcript in such $Rest^{-/-}$ ESCs was confirmed by a northern blot analysis (Figure 1B; Figure S1A available online). Consistent with the recombination, a FACS analysis revealed a lack of any GFP signal in the $Rest^{-/-}$ ESCs (Figure 1B). In addition, a western blot analysis revealed the lack of any Rest protein in such $Rest^{-/-}$ ESCs (Figure 1B). *Syt4* possesses RE1 and it is expressed while relying solely on dissociation of the Rest repressor complex from the RE1 site for maximal expression (Ballas et al., 2005). The expression of *Syt4* significantly increased in the $Rest^{-/-}$ ESCs, thus indicating that the Rest-targeted gene is derepressed in $Rest^{-/-}$ ESCs (Figure S1B).

Consistent with the findings by Jørgensen et al. (2009a, 2009b), the growth and morphology of the $Rest^{-/-}$ ESCs were indistinguishable from those of wild-type V6.5 ESCs under the self-renewal conditions (under the presence of LIF and MEF). Furthermore, when the expression of the pluripotent genes was compared, the expression of *Nanog*, *Oct3/4*, and *Sox2* in $Rest^{-/-}$ ESCs were not altered

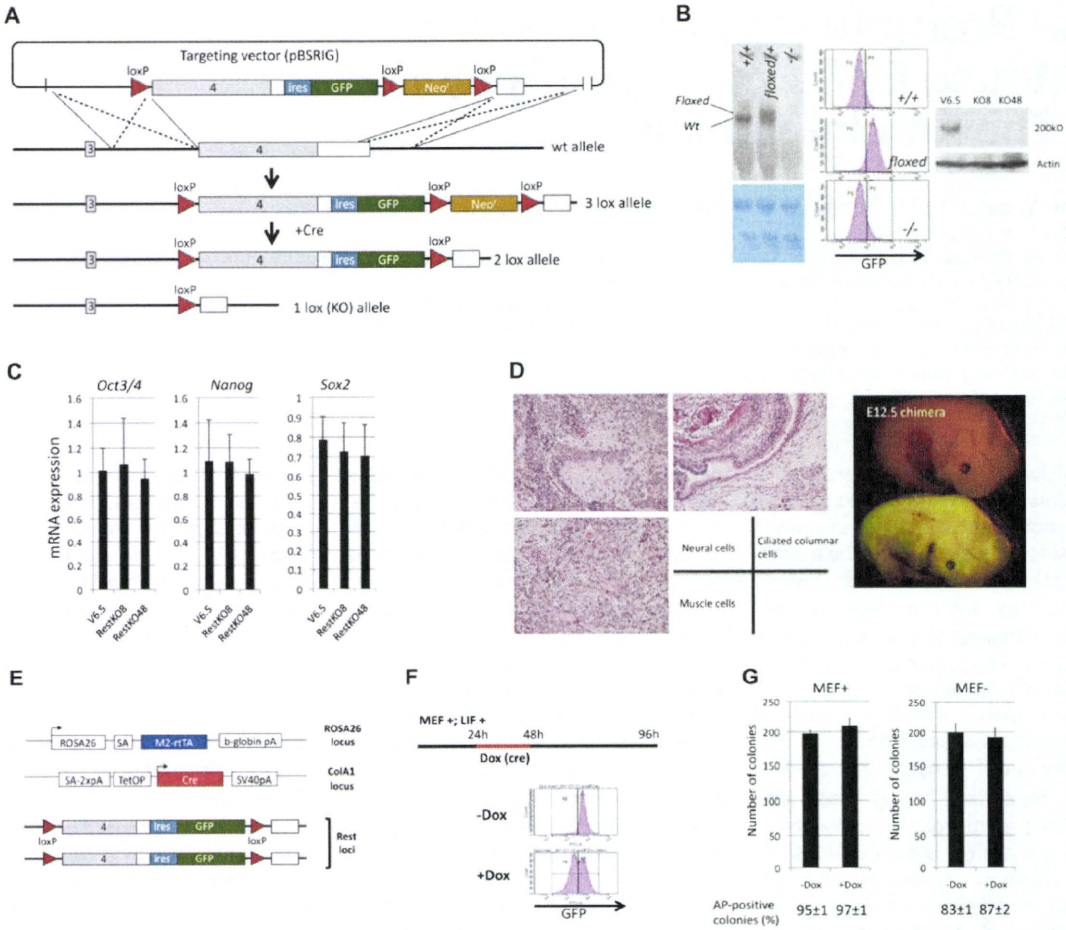

Figure 1. *Rest* Is Not Required for the Maintenance of ESC Pluripotency

(A) A schematic drawing of the *Rest*-conditional knockout vector and targeted *Rest* allele.

(B) A northern blot analysis reveals a lack of *Rest* transcripts. GFP fluorescence is observed to have disappeared in the $Rest^{-/-}$ ESCs. A western blot analysis shows the absence of any Rest protein in two independent knockout ESC lines, RestKO8 and RestKO48.

(C) Transcript levels of pluripotent genes in $Rest^{-/-}$ ESCs. No significant changes in the expression of *Oct3/4*, *Nanog*, and *Sox2* are detectable in the $Rest^{-/-}$ ESCs relative to the control ESCs. Transcript levels were normalized to *β-actin* levels. The data are presented as the average values with SD of six independent samples.

(D) $Rest^{-/-}$ teratomas differentiate into three different germ layers, including neural cells, ciliated columnar cells, and muscle cells. E12.5 chimeric mice were generated by injecting $Rest^{-/-}$ ESCs into blastocysts.

(E) A schematic drawing of the conditional *Rest* knockout ESC line containing doxycycline-inducible *Cre* alleles.

(F) An experimental protocol. Conditional *Rest* knockout ESCs were treated with doxycycline (2 μg/ml) for 24 hr starting at 24 hr and then were harvested at 96 hr after the passage. A FACS analysis revealed the presence of GFP-negative cells, thus indicating the occurrence of *Rest* ablation at 96 hr after passage.

(G) The conditional deletion of the *Rest* gene does not suppress the development of alkaline phosphatase (AP)-positive ESC colonies under the presence or absence of feeder cells. *Rest*-floxed *Cre*-inducible ESCs were exposed to doxycycline and then were fixed after 3 days of exposure. The total number of colonies and the percent positivity for AP are indicated. The data are presented as the mean ± SD of three independent 35 mm wells.

in comparison to those in the control ESCs (Figure 1C). To further examine the pluripotency of $Rest^{-/-}$ ESCs, $Rest^{-/-}$ ESCs were next injected into the subcutaneous tissue of nude mice. $Rest^{-/-}$ ESCs could generate teratomas with evidence of differentiation into three different germ layers (Figure 1D). To fully evaluate the differentiation ability of the $Rest^{-/-}$ ESCs, GFP-labeled $Rest^{-/-}$ ESCs were injected into blastocysts followed by transplantation into the uteri of pseudopregnant mice to generate chimeric embryos (Yamada et al., 2004). Eventually, this generated E12.5 chimeric mice with the widespread contribution of GFP-positive cells into the three germ layers (Figure 1D; Figure S1C).

In order to rule out the possibility that the adaptive responses, which occurred as a result of multiple cell passages, reduced the requirement of Rest-mediated maintenance of ESCs, the initial response of the gene expression was examined after the conditional ablation of the *Rest* genes. For this purpose, an ESC line was derived from transgenic embryo that harbors a doxycycline-inducible *Cre* transgene together with *Rest*-floxed alleles (Figure 1E; *Rest 2lox/2lox*;

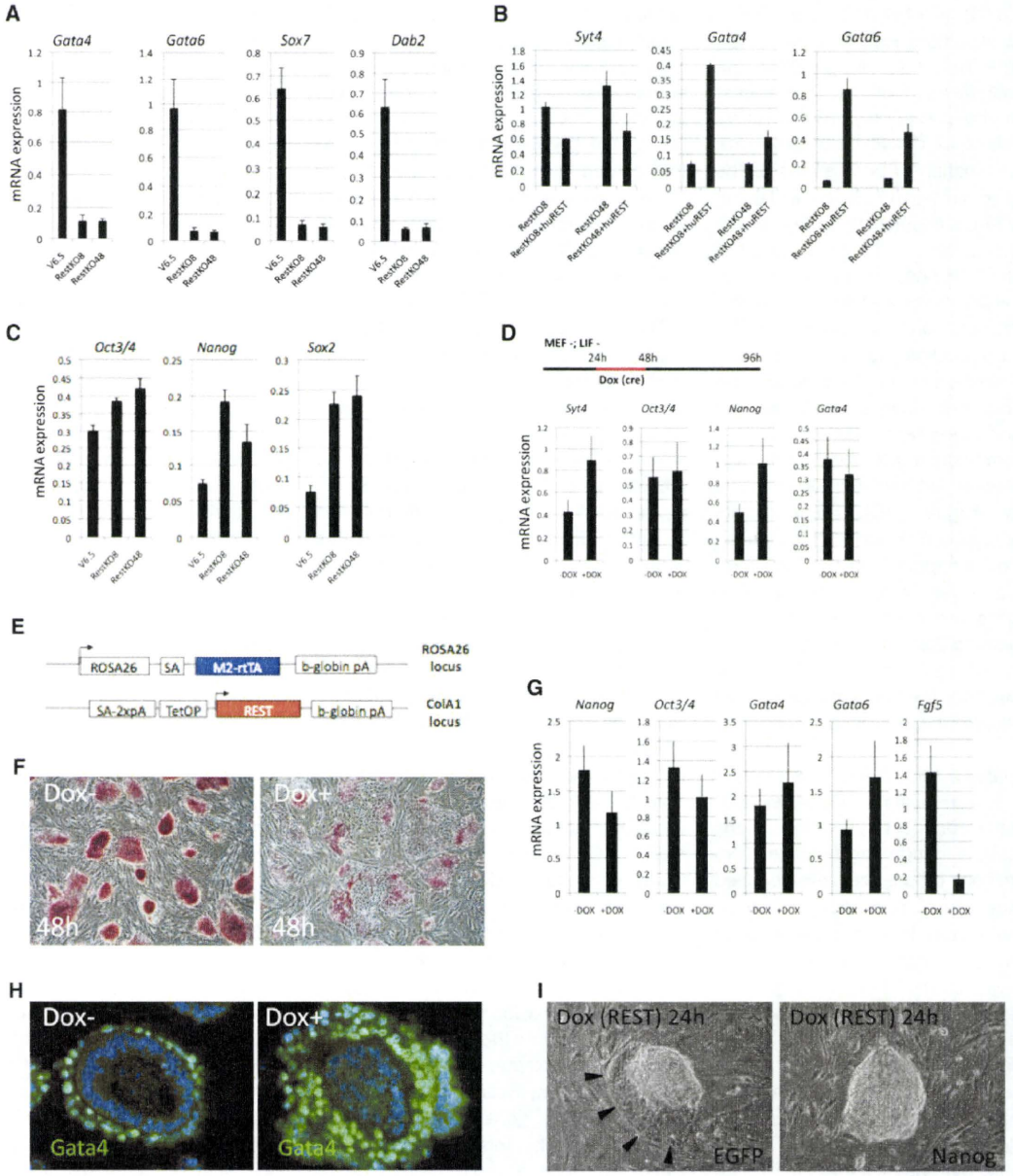

Figure 2. *Rest* Promotes Primitive Endoderm Differentiation in ESCs

(A) Under confluent culture conditions, the expression of *Gata4* and *Gata6* were significantly lower in the *Rest*$^{-/-}$ ESCs in comparison to the control isogenic ESCs (V6.5). The expression of *Sox7* and *Dab2*, which are both markers for the primitive endoderm, are suppressed in *Rest*$^{-/-}$ ESCs. Transcript levels were normalized to *β-actin* levels. The data are presented as the average values with SD of six independent samples.

(B) The exogenous expression of *REST* rescued the suppression of *Gata4* and *Gata6* in *Rest*$^{-/-}$ ESCs. Mean ± SD of three independent samples.

(C) The expression of pluripotent genes in the embryoid body (EB) cells. The expression of *Occt3/4*, *Nanog*, and *Sox2* are upregulated in *Rest*$^{-/-}$ EB cells relative to the control EB cells. The data are presented as the mean ± SD of six independent samples.

(D) Conditional *Rest* knockout ESCs were cultured under differentiation culture conditions and treated with doxycycline (2 μg/ml) for 24 hr starting at 24 hr. The cells were harvested at 96 hr after the passage. The expression of *Syt4*, *Oct3/4*, *Nanog*, and *Gata4* after the conditional deletion of *Rest* under the differentiation culture condition. Note that the expression of *Nanog* and *Syt4*, but not of *Oct3/4*, were upregulated in the doxycycline-treated cells. The data are presented as the mean ± SD of six independent samples.

(E) A schematic drawing of the doxycycline-inducible *REST* ESC line.

(F) 48 hr of the induction of *REST* causes the ESC differentiation into epithelium-like colonies with a decreased AP activity.

(G) The forced expression of *REST* in ESCs leads to decreased expression of *Nanog*, *Oct3/4*, and *Fgf5*, whereas it results in increased expression of *Gata6*. The data are presented as the mean ± SD of six independent samples.

(H) In vitro differentiation of *REST*-inducible ESCs into EBs under the absence or presence of doxycycline. The exogenous *REST* expression results in an increased number of Gata4-positive cells at the periphery of EBs.

Rosa26::rtTA; *Col1a1::tetO-Cre* ESCs; Beard et al., 2006; Hochedlinger et al., 2005). This new ESC line enabled the conditional deletion of the floxed *Rest* genes in the presence of doxycycline. After 3 days of doxycycline exposure, the recombination in both alleles of the *Rest* was confirmed in 70%–80% of these ESCs by FACS (Figure 1F). However, the conditional deletion did not suppress the formation of AP-positive colonies regardless of the presence or absence of feeder cells in comparison to the parental ESCs without doxycycline (Figure 1G). In addition, the expression levels of *Oct3/4* and *Nanog* did not change, whereas the expression level of *Syt4* was derepressed while demonstrating evidence of *Rest* recombination shortly after doxycycline treatment (Figure S1D). These results therefore clearly rule out both the possibility of the adaptation in the long-term culture as well as the notion that feeder cells reduce the requirement of Rest-mediated ESC maintenance. Taken together, our results indicate that *Rest* is not required for the maintenance of ESC pluripotency in these experimental conditions.

Both *Gata4* and *Gata6* were significantly downregulated in the $Rest^{-/-}$ ESCs under confluent culture conditions (Figure 2A), although the findings were not prominent before the cells reached confluence. *Gata4* and *Gata6* are transcriptional factors that promote primitive endoderm differentiation (Fujikura et al., 2002; Niwa, 2007). These findings suggest that the genetic deletion of *Rest* prevents ESCs from differentiating toward the primitive endoderm. The notion of the suppression of primitive endoderm differentiation is confirmed by the decreased expression of both *Sox7* and *Dab2*, markers for the primitive endoderm (Shimoda et al., 2007; Yang et al., 2002), in $Rest^{-/-}$ ESCs (Figure 2A). Consistent with this notion, embryoid bodies (EBs) generated from $Rest^{-/-}$ ESCs revealed a decreased number of Gata4-expressing cells in the periphery of EBs on the histological sections in comparison to the control EBs (13.1 ± 15.0/EB and 30.4 ± 9.02/EB in RestKO8 EBs and V6.5 EBs, respectively, $p < 0.006$ by Student's t test) (Figure S2A). Rescue experiments were performed with a plasmid containing human *REST* cDNA (Grimes et al., 2000) to further investigate the direct association of the genetic deletion of *Rest* and the altered expression of *Gata4* and *Gata6* in confluent $Rest^{-/-}$ ESCs. Importantly, the decreased expression of both *Gata4* and *Gata6* in confluent $Rest^{-/-}$ ESCs were derepressed by the exogenous expression of *REST* (Figure 2B).

The expression of *Nanog*, *Oct3/4*, and *Sox2* were significantly higher in the $Rest^{-/-}$ EB cells than in the control EB cells (Figure 2C). Accordingly, these observations suggest that the delayed repression of self-renewal genes during the early differentiation of ESCs may thus cause the suppression of the early differentiation of $Rest^{-/-}$ ESCs. To further examine the initial response of gene expression upon the early differentiation of $Rest^{-/-}$ ESCs, the differentiation (-LIF, -MEF) of *Cre*-inducible *Rest*-floxed ESCs was induced with/without doxycycline exposure (Figure 2D). At 3 days after doxycycline treatment, the expression of *Nanog*, but not of *Oct3/4*, was observed to be significantly higher in the doxycycline-treated ESCs than that of the nontreated ESCs (Figure 2D). In contrast, a decreased expression of *Gata4* was not detectable at 3 days after doxycycline treatment when the *Syt4* expression had already been derepressed (Figure 2D). These results suggest that a decreased expression of *Gata4* in $Rest^{-/-}$ cells is preceded by an increased expression of *Nanog* and that *Gata4* repression is therefore a secondary effect of *Rest* ablation.

Finally, a doxycycline-inducible *REST* ESC line was generated (Figure 2E; Figure S2B). The forced expression of *REST* led to the rapid morphological changes of ESC colonies into an epithelium-like shape, which was accompanied by decreased AP activity (Figure 2F). In line with such morphological changes, ESCs with exogenous *REST* expressed significantly lower levels of self-renewal genes. The expression of *Gata6* was higher, whereas the expression of an epiblast marker, *Fgf5,* was significantly lower in such ESCs (Figure 2G). Furthermore, an increased number of Gata4-expressing cells in the periphery of EBs was observed in the exogenous *REST*-induced EBs (79.2 ± 19.6/EB and 50.7 ± 17.6/EB in *REST*-induced EBs and control EBs, respectively, $p < 0.004$ by Student's t test) (Figure 2H), thus suggesting that the forced *REST* expression promotes the ESC differentiation into the primitive endoderm. Importantly, the *REST*-induced ESC differentiation was, at least in part, rescued by the *Nanog* overexpression (Figure 2I; Figure S2D).

Although the critical role of the Oct3/4-Sox2-Nanog core transcription circuitry in the maintenance of ESC pluripotency is widely accepted (Boyer et al., 2005, 2006; Chambers et al., 2003; Loh et al., 2006; Mitsui et al., 2003; Niwa et al., 2000), the mechanisms leading to the breakdown of such core circuitry upon the early ESC differentiation are still not well understood (Kunath et al., 2007). The present study demonstrated that *Rest* ablation causes delayed repression of the pluripotent genes, whereas overexpression of *REST* immediately results in the suppression of the pluripotent gene expression. It is noteworthy that the delayed repression of the pluripotent genes by the conditional ablation of *Rest* was predominantly observed in *Nanog*. Given the fact that Rest is a transcriptional repressor and *Nanog* harbors RE1 in its promoter (Johnson et al., 2008), the current results therefore suggest that Rest is involved in the silencing of *Nanog* expression during the early differentiation of ESCs. This notion is also supported by the observation that ectopic *REST* in $Rest^{-/-}$ ESCs predominantly repressed the *Nanog* expression relative to the expression in original $Rest^{-/-}$ ESCs (Figure S2C). These findings suggest that Rest is an external factor connecting to the Oct3/4-Sox2-Nanog regulatory network core circuitry to influence the initial differentiation of ESCs. It is interesting to note that Rest is abundantly expressed in ESCs and it is a target of the Oct3/4-Sox2-Nanog regulatory network core circuitry (Johnson et al., 2008). It is possible that the negative feedback loop through Rest may play

(I) The *Nanog* overexpression dampens the *REST*-mediated ESC differentiation. *REST* was induced in *Nanog*-overexpressing and *EGFP*-overexpressing ESC colonies by the doxycycline exposure. The 24 hr exposure of doxycyline led to the rapid differentiation in *EGFP*-overexpressing ESCs (arrowheads), whereas *Nanog*-overexpressing ESCs retained an undifferentiated morphology. After the 48 hr exposure of doxycyline, 16 out of 25 *EGFP*-overexpressing colonies (68%) started to differentiate, whereas none of *Nanog*-overexpressing colonies (0/21, 0%) revealed the evidence of differentiation (see also Figure S2D).

a role in the stable transcriptional circuitry and in the rapid response upon the early differentiation of ESCs.

The current findings also suggest that Rest promotes the early ESC differentiation. Epiblast and the primitive endoderm are two distinct cell types in the inner cell mass (ICM) of the blastocyst. Genetic evidence indicates that the *Nanog* and *Gata* family transcription factors play a role in the segregation of epiblast and primitive endoderm within ICM (Chambers et al., 2003; Koutsourakis et al., 1999; Mitsui et al., 2003; Soudais et al., 1995). Indeed, Nanog and Gata6 are expressed in the ICM in a mutually exclusive manner (Chazaud et al., 2006), thus indicating the reciprocal control of the gene expression. The current study found that the conditional ablation of *Rest* results in the delayed repression of *Nanog* during the early differentiation of ESCs, whereas *REST* overexpression causes an increased expression of *Gata6*, which is accompanied by the rapid differentiation. In addition, the expression of *Fgf5*, an epiblast marker, was significantly downregulated by the *REST* overexpression. These results suggest that Rest may be involved in the segregation of epiblast and primitive endoderm through modifying the *Nanog* expression.

In summary, the conditional ablation of the *Rest* gene revealed that *Rest* is not absolutely required for the maintenance of ESC pluripotency. These results also indicate that *Rest* plays a role in the suppression of the pluripotent gene expression upon the early differentiation of ESCs.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and two figures and can be found with this article online at doi:10.1016/j.stem.2009.12.003.

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RAPID COMMUNICATION

Hippocampal Epigenetic Modification at the Brain-Derived Neurotrophic Factor Gene Induced by an Enriched Environment

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ABSTRACT: **Environmental enrichment is an experimental paradigm that increases brain-derived neurotrophic factor (BDNF) gene expression accompanied by neurogenesis in the hippocampus of rodents. In the present study, we investigated whether an enriched environment could cause epigenetic modification at the BDNF gene in the hippocampus of mice. Exposure to an enriched environment for 3–4 weeks caused a dramatic increase in the mRNA expression of BDNF, but not platelet-derived growth factor A (PDGF-A), PDGF-B, vascular endothelial growth factor (VEGF), nerve growth factor (NGF), epidermal growth factor (EGF), or glial fibrillary acidic protein (GFAP), in the hippocampus of mice. Under these conditions, exposure to an enriched environment induced a significant increase in histone H3 lysine 4 (H3K4) trimethylation at the BDNF P3 and P6 promoters, in contrast to significant decreases in histone H3 lysine 9 (H3K9) trimethylation at the BDNF P4 promoter and histone H3 lysine 27 (H3K27) trimethylation at the BDNF P3 and P4 promoters without any changes in the expression of their associated histone methylases and demethylases in the hippocampus. The expression levels of several microRNAs in the hippocampus were not changed by an enriched environment. These results suggest that an enriched environment increases BDNF mRNA expression via sustained epigenetic modification in the mouse hippocampus.** © 2010 Wiley-Liss, Inc.

KEY WORDS: **mouse hippocampus; epigenome; BDNF gene; neurogenesis; environmental enrichment**

INTRODUCTION

Over the past few decades, exposure to an enriched environment, which consists of housing groups of animals together in a complex environment with various toys to provide more opportunity for learning and social interaction than standard laboratory living conditions, has been shown to enhance behavioral performance in various learning tasks. Consistent with these behavioral tests, exposure to an enriched environment has been shown to induce biochemical and structural changes in the hippocampal dentate gyrus (DG) and CA1 region, such as an increased number of dendritic branches and spines, enlargement of synapses, and an increased number of glial cells. Moreover, exposure of adult rodents to increased environmental complexity induces hippocampal progenitor proliferation and neurogenesis (Nilsson et al., 1999; van Praag et al., 1999). However, the detailed mechanisms that control neurogenesis in the hippocampus of animals housed in an enriched environment are still unclear.

It has been reported that brain-derived neurotrophic factor (BDNF) promotes neuronal differentiation from endogenous progenitor cells in the ventricular wall of the adult forebrain (Ahmed et al., 1995; Kirschenbaum and Goldman, 1995), and the increased expression of BDNF is required for the environmental induction of hippocampal neurogenesis in rodents (Rossi et al., 2006). The BDNF gene and the regulation of its expression are highly complex, and have been examined in both human and rodent brains (Timmusk et al., 1993; Liu et al., 2006; Aid et al., 2007; Pruunsild et al., 2007). The mouse BDNF gene, which shows a high degree of sequence homology to its human congener, contains multiple 5′ noncoding exons and a single 3′ coding exon for the mature BDNF protein (Aid et al., 2007). These noncoding exons undergo alternative splicing with the common coding exon to produce multiple exon-specific BDNF transcripts. Nine BDNF promoters have been previously identified in the mouse (Aid et al., 2007), and each drives the transcription of BDNF mRNAs containing one of the four 5′ noncoding exons (I, II, III, IV, V, VI, VII, or VIII) spliced to the common 3′ coding exon.

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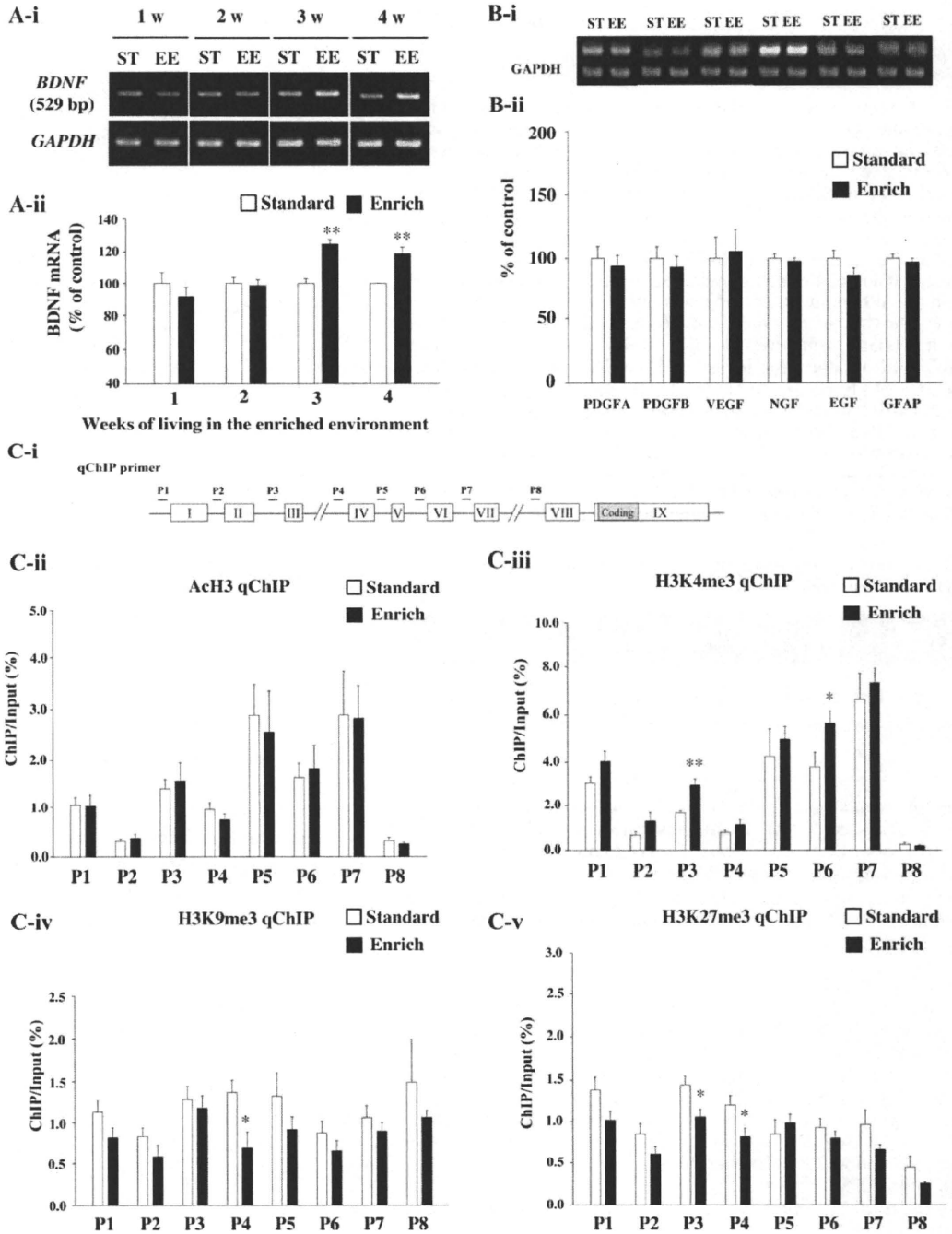
Chromatin remodeling at gene promoter regions is becoming increasingly recognized as a key control point of gene expression. Histone modification represents one prominent form of chromatin remodeling. According to the "histone code theory," different modifications of histones at a particular promoter region, alone or in combination, define a specific epigenetic state that encodes gene activation vs. gene silencing (Jenuwein and Allis, 2001). Intriguing correlations have been found between cellular plasticity, including transformation and such epigenetic modification at a specific gene (Kouzarides, 2007; Borrelli et al., 2008), indicating that possible epigenetic modification at BDNF gene promoters may partly contribute to adult neurogenesis. Therefore, in the present study, we evaluated whether an enriched environment could induce histone modification at several BDNF gene promoters in mice.

Male C57BL/6J mice (Jackson Laboratory), weighing 18–23 g, were used in the present study. Control mice were housed four per standard (16.5 × 26.5 × 13.5 cm^3) plexiglass cage. Mice



Hippocampus

in the enriched environment group were kept eight per large (25.5 × 42.5 × 39 cm^3) wire-mesh, two-storied cage, which contained tunnels and running wheels, for 4 weeks (Supporting Information Fig. 1A).

In the DG of mice housed in the enriched environment for 4 weeks, immunoreactivity (IR) for doublecortin, which is a microtubule-associated protein that is expressed specifically in virtually all migrating neuronal precursors of the CNS and which has been used as a candidate marker for neural migration and differentiation, was increased compared to that in mice housed in the standard cage (Supporting Information Fig. 1B). Furthermore, IR for NeuroD, which is another marker for the differentiation of granule cells in the hippocampus (Miyata et al., 1999), was clearly increased in the DG of mice housed in an enriched environment (Supporting Information Fig. 1C). Additionally, the number of BrdU-positive cells in the DG that were classified as newly dividing cells was markedly increased in mice housed in an enriched environment (Supporting Information Fig. 2A), and theses were clearly colocalized with the neuronal marker NeuN (Supporting Information Fig. 2B). In paralleled with adult neurogenesis, the expression of BDNF mRNA in the hippocampus was significantly elevated after exposure to an enriched environment for both 3 and 4 weeks (Fig. 1A). In contrast, mRNA levels of glial fibrillary acidic protein (GFAP), platelet-derived growth factor A (PDGF-A), PDGF-B, vascular endothelial growth factor (VEGF), nerve growth factor (NGF), and epidermal growth factor (EGF) in the hippocampus were not altered by exposure to an enriched environment for 4 weeks (Fig. 1B).

Under these conditions, a significant increase in histone H3 lysine 4 (H3K4) trimethylation at the BDNF P3 and P6 promoters was observed upon exposure to an enriched environment for 4 weeks. Furthermore, significant decreases in histone H3 lysine 9 (H3K9) trimethylation at the BDNF P4 promoter, and histone H3 lysine 27 (H3K27) trimethylation at the BDNF P3 and P4 promoters were seen in the hippocampus of mice under an enriched environment. In contrast, an enriched environment did not produce the hyperacetylation of H3 in the hippocampus of enriched mice (Fig. 1C).

In terms of changes in mRNA levels of several histone methylases and demethylases in the hippocampus, an enriched environment failed to change the mRNA expression of MLL1, LSD1, Jarid1a, Jarid1b, jmjd2B, jmjd2C, jmjd2D, EZH2, UTX, or jmjd3 (Fig. 2A).

As with histone methylases and demethylases, no significant changes in microRNA9 (miR9), miR124a, miR132, miR133b, or miR145 were observed in the hippocampus of mice housed in an enriched environment for 2 and 4 weeks compared to mice housed in a standard cage (Fig. 2B).

In the present study, we demonstrated hippocampal neurogenesis in mice that were exposed to an enriched environment. This notion is supported by previous reports that exposure of adult rodents to an enriched environment increased neurogenesis in the hippocampus (Kempermann et al., 1997; Nilsson et al., 1999).

During development, growth factors provide important extracellular signals that regulate the proliferation and differentiation of neural stem cells in the CNS (Calof, 1995). Several investigations have examined the role of these factors in the adult brain (Calof, 1995; Kuhn et al., 1997). Furthermore, it has been shown that exposure to an enriched environment increased the expression of BDNF genes (Falkenberg et al., 1992). In support of these findings, the present study showed that the expression of BDNF mRNA in the hippocampus was significantly elevated after exposure to an enriched environment for both 3 and 4 weeks. In contrast, mRNA levels of GFAP, PDGF-A, PDGF-B, VEGF, NGF, and EGF in the hippocampus were not altered under the present conditions. In our *in vitro* study using neural stem cells cultured from the mouse embryonic forebrain, neuronal differentiation was clearly observed following exposure to recombinant BDNF (Supporting Information Fig. 3). These findings raise the possibility that an enriched environment may stimulate expression of the BDNF gene in the hippocampus and, in turn, the enhanced

FIGURE 1. (A) Time course of changes in the expression of BDNF mRNA in the hippocampus. (A-i) Representative RT-PCR for BDNF mRNA in the hippocampus obtained from standard or enriched mice. (A-ii) The intensity of the bands was semiquantified using NIH Image software. The value for BDNF mRNA was normalized by that for the internal standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The value for enriched mice is expressed as a percentage of the increase in standard mice. Each column represents the mean ± S.E.M. of six samples. $^{**}P < 0.01$ vs. the standard group. (B) Upper: Representative RT-PCR for PDGFA, PDGFB, VEGF, NGF, EGF, and GFAP mRNAs in the hippocampus obtained from standard or enriched mice. Lower: The values for mRNAs were normalized by that for GAPDH mRNA. Each column represents the mean ± S.E.M. of six samples. (C-i) Schematic of the BDNF gene: The BDNF gene contains eight noncoding exons I–VIII upstream of the coding exon IX in mouse. Exons I–VIII can each be alternatively spliced next to exon IX, from the 5′ UTR region of different mRNA splice variants, BDNF I-VIII, which can promote the expression of their corresponding transcript variants. For an mRNA analysis of total BDNF, primers were used to amplify exon IX. For ChIP analysis, primers were designed around the putative promoters, P1-P8, which are located upstream of exons I–VIII. (C-ii, iii, iv, v) Stable changes in histone modifications in the hippocampus in mice housed under standard or enriched conditions for 4 weeks. ChIP assays were performed to measure the levels of several histone modifications at the eight BDNF promoters Ex1-Ex8 (P1–8) in the hippocampus using specific antibodies for each modification state. Levels of promoter enrichment were quantified by quantitative PCR. (C-ii) Histone H3 was not altered at BDNF P1–P8. (C-iii) Histone H3K4 trimethylation was increased at BDNF P3 and P6 in mice housed under enriched conditions for 4 weeks. $^{*}P < 0.05$ vs. the standard group, $^{**}P < 0.01$ vs. the standard group. (C-iv) H3K9 trimethylation was decreased at BDNF P4 in mice housed under enriched conditions for 4 weeks. $^{*}P < 0.05$ vs. the standard group. (C-v) Histone H3K27 trimethylation was decreased at BDNF P3 and P4 in mice housed under enriched conditions for 4 weeks. $^{*}P < 0.05$ vs. the standard group.

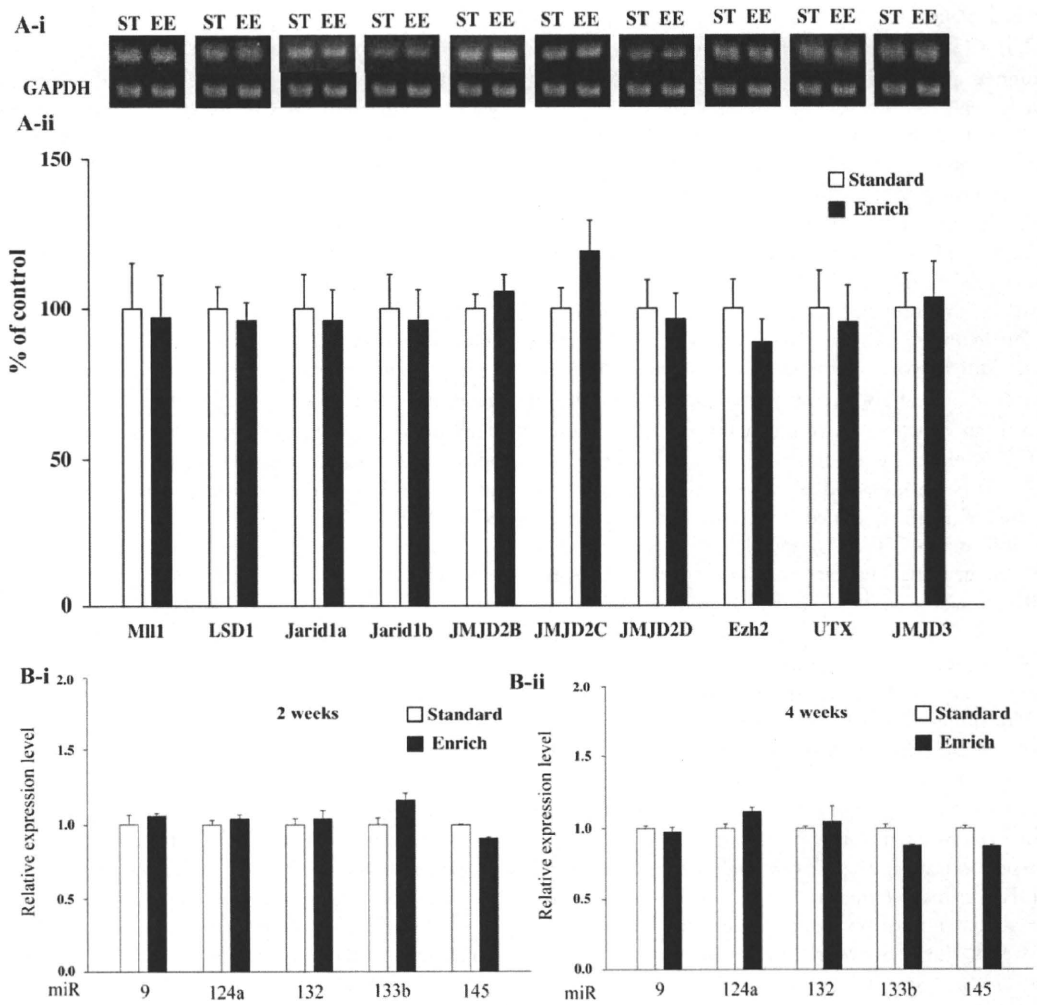


FIGURE 2. (A) Upper: Representative RT-PCR for MLL1 (an H3K4 methyltransferase), LSD1, Jarid1a, and Jarid1b (H3K4 demethylases), JMJD2B, JMJD2C and JMJD2D (H3K9 demethylases), Ezh2 (H3K27 methyltransferase), and UTX and JMJD3 (H3K27 demethylases). mRNAs in the hippocampus obtained from standard or enriched mice. Lower: The value for mRNA was normalized by that for GAPDH mRNA. Each column represents the mean ± SEM of six samples. (B) Expression levels of miRNAs were measured in the hippocampus of mice housed in an enriched environment for 2 weeks (B-i) and 4 weeks (B-ii). The value for miRNA was normalized by that for the internal standard snoRNA202.

BDNF protein may lead to neural differentiation from its precursors in the hippocampal DG.

We next evaluated whether an enriched environment increases BDNF gene expression through chromatin-specific events that promote the expression of distinct transcript variants. In this study we analyzed two active histone modifications (acetylation of histone H3, AcH3, and trimethylation of H3K4) and two repressive histone modifications (trimethylation of H3K9 and trimethylation of H3K27) at different BDNF promoter regions in the hippocampus. As a result, we detected a significant increase in H3K4 trimethylation, an activated histone modification marker, at the BDNF P3 and P6 promoters after exposure to an enriched environment for 4 weeks. Furthermore, significant decreases in H3K9 trimethylation, a repressive histone modification marker, at the BDNF P4 promoter, and H3K27 trimethylation, another repressive histone modification marker, at the BDNF P3 and P4 promoters were seen after exposure to an enriched environment. Under these conditions, we observed that an enriched environment did not produce the hyperacetylation of H3 in enriched mice.

The methylation of H3K9 and H3K27 can be directly modulated by histone methylases and demethylases that target specific lysine residues and methylation states (Jenuwein and Allis, 2001; Kouzarides, 2007). Thus, we investigated whether an enriched environment could alter the mRNA level of several histone methylases and demethylases in the hippocampus. We found that an enriched environment did not change the

mRNA expression of MLL1 (an H3K4 methyltransferase), LSD1, Jarid1a or Jarid1b (H3K4 demethylases), jmjd2B, jmjd2C or jmjd2D (H3K9 demethylases), EZH2 (an H3K27 methyltransferase), or UTX or jmjd3 (H3K27 demethylases).

Recently, microRNAs (miRNAs), a class of small, noncoding RNAs, have been identified as important regulators of many biological processes, including organogenesis and disease development (Kim et al., 2007; Chen et al., 2008; Hutchison et al., 2009). Indeed, it has been shown that epigenetic factors such as DNA methylation, histone modification, and regulatory noncoding RNAs affect the fate of neural stem cells (Chi and Bernstein, 2009). miRNAs have the potential to specifically regulate a large set of target molecules, which may affect the cell's fate in a programmatic way, and the role of miRNAs in stem cell gene networks is being actively explored. Their ability to potentially regulate large numbers of target genes simultaneously suggests that they may be important sculptors of transcriptional networks. In this study, we found that miR9, miR124a, miR132, miR133b, and miR145 are expressed in the hippocampus of adult mice. It has been reported that miR145 regulates Oct4, Sox2, and Klf4 and suppresses the potential of human embryonic stem cells to generate any differentiated cell type (pluripotency) (Xu et al., 2009). miR124, one of these signature miRNAs that is enriched in the brain, regulates adult neurogenesis in the subventricular zone stem cell niche (Cheng et al., 2009). miR132 is localized and synthesized, in part, at synaptic sites in dendrites to regulate synaptic formation and plasticity (Vo et al., 2005). miR9 is expressed specifically in the hippocampus and may be involved in neural stem cell self-renewal and differentiation (Krichevsky et al., 2006; Bak et al., 2008). In the present study, there were no significant changes in miR9, miR124a, miR132, miR133b, or miR145 in the hippocampus of mice housed in an enriched environment for 2 and 4 weeks compared to mice housed in a standard cage. Although further studies are required to investigate the molecular mechanism of hippocampal neurogenesis induced by an enriched environment, we propose that an enriched environment may increase BDNF expression accompanied by histone modification without directly changing the expression of histone H3 methylases and demethylases, and miRs in the hippocampus.

In conclusion, the present study demonstrated that an enriched environment stimulates neuronal differentiation from precursors in the hippocampal DG. Furthermore, the increased expression of BDNF was observed in the hippocampus of mice that had been exposed to an enriched environment. This enrichment induced a significant increase in H3K4 trimethylation at the BDNF P3 and P6 promoters and a significant decrease in H3K9 trimethylation at the BDNF P4 promoter and H3K27 trimethylation at the BDNF P3 and P4 promoters in the hippocampus of mice. These results suggest that an enriched environment may increase BDNF expression with notably sustained chromatin regulation in the mouse hippocampus. This phenomenon could partly explain the hippocampal neurogenesis induced by an enriched environment in mice.

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Introduction: Epigenetics and Cancer

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ABSTRACT

The field of epigenetics has witnessed a recent explosion in our knowledge on the importance of epigenetic events in the control of both normal cellular processes and abnormal events associated with diseases, moving this field to the forefront of biomedical research. Advances in the field of cancer epigenetics and epigenomics have turned academic, medical, and public attention to the potential application of epigenetics in cancer control. A tremendous pace of discovery in this field requires that these recent conceptual breakthroughs and technological state-of-the-art in epigenetics and epigenomics are updated and summarized in one book with cancer focus. This book is primarily intended to academic and professional audience; however, an attempt has been made to make it understandable by and appealing to a wider audience among healthcare workers. The main aim of this book is to produce an authoritative and comprehensive reference source in print and online, covering all critical aspects of epigenetics and epigenomics and their implications in cancer research. This book discusses the state of science and determines the future research needs, covering most recent advances, both conceptual and technological, and their implication for better understanding of molecular mechanisms of cancer development and progression, early detection, risk assessment, and prevention of cancer. In this chapter, we describe the main aim and scope of this book and provide a brief emphasis of each of 22 chapters regrouped into eight major parts.

I. INTRODUCTION

Epigenetics represents a new frontier in cancer research owing to the fact that epigenetic events have emerged as key mechanisms in regulation of critical biological processes and in development of human diseases. Although nongenetic phenomena have long been considered critical for our understanding of the mechanisms and causes of complex diseases such as cancer, it was not until recently that epigenetics has attracted considerable attention of scientific and medical communities. The field of epigenetics has witnessed a recent explosion in our knowledge on the importance of epigenetic events in the control of both normal cellular processes and abnormal events associated with diseases. Historically, the "epigenetics" was used to describe all biological phenomena that do not follow normal genetic rules. The term "epigenetics" was coined by Conrad Waddington in 1942 to describe the discipline in biology that studies "the interactions of genes with their environment that bring the phenotype into being." The field of epigenetics is considered as one of the most rapidly expanding fields of modern biology that has enormous impact on our thinking and understanding of biological phenomena and complex diseases, notably cancer. Since the introduction of the term "epigenetics," a number of biological events that are not coded in DNA sequence itself have been considered as epigenetic phenomena. These include imprinting, position-effect variegation in the fruit fly, paramutations, and X-chromosome inactivation. Recent discoveries in epigenetics and epigenomics revealed that different epigenetic events may share common molecular mechanisms.

In a broader sense, epigenetics can be considered as an interface between genotype and phenotype. Epigenetics encompasses mechanisms that modify the final outcome of the genetic code without altering the underlying DNA sequence. The importance of epigenetic principle is highlighted by the fact that all cells in any given organism share an identical genome with other cell types, yet they exhibit striking morphological and functional properties. Therefore, it is obvious that epigenetic events define the identity and proliferation potential of different cells in the body, the features that are typically deregulated in cancer. Nowadays, the term "epigenetics" may be defined as the study of all changes that are stably transmitted over many rounds of cell divisions, but that do not alter the nucleotide sequence (genetic code). Epigenetic inheritance includes DNA methylation, histone modifications, and RNA-mediated gene silencing, all of which are essential mechanisms that allow the stable propagation of gene activity states from one generation of cells to the next. Consistent with the importance of epigenetic mechanisms, deregulation of epigenetic states is intimately linked to human diseases, most notably cancer (Feinberg and Tycko, 2004; Herceg, 2007; Jones and Baylin, 2007).

Recent discoveries in the field of cancer epigenetics have turned academic, medical, and public attention to the potential application of epigenetic and epigenomics in cancer control. A spectacular pace of discoveries in this field requires that these recent conceptual breakthroughs and technological state-of-the-art in epigenetics and epigenomics are updated and summarized in one book with focus on cancer. Although several books on "epigenetics" are available, these are either becoming increasingly out of date (owing to the fact that the field of epigenetics is rapidly expanding), not focused on cancer epigenetics, or too technical. This book is primarily intended to academic and professional audience; however, an attempt has been made to make it understandable by and appealing to a wider audience among healthcare workers. The main aim of this book is to produce an authoritative and comprehensive reference source in print and online, covering all critical aspects of epigenetics and epigenomics and their implications in cancer research and cancer control. This book discusses the state of science and future research needs, covering most recent advances, both conceptual and technological, and their implication for better understanding of molecular mechanisms of cancer development and progression, early detection, risk assessment, and prevention of cancer. Technological advances in epigenomics for cancer research and molecular epidemiology are also discussed. The content of the book is organized into 22 chapters which can be regrouped into 8 major parts. Particular emphasis is given to: (i) basic epigenetic mechanisms in the regulation of critical cellular processes, (ii) epigenetic events underlying biological phenomena, (iii) cancer epigenome, (iv) epigenetic changes induced by environmental and dietary/lifestyle factors, (v) epigenetic biomarkers, (vi) epigenetic therapy and epigenetic drugs, (vii) application of epigenetics in molecular epidemiology and epigenetic cancer prevention, and (viii) epigenetic databases.

II. BASIC EPIGENETIC MECHANISMS—GHOSTS ABOVE THE GENES

Major epigenetic mechanisms include DNA methylation, covalent posttranslational modifications of histone proteins, and RNA-mediated gene silencing. Different types of epigenetic modifications are intimately linked and often act in self-reinforcing manner in regulation of different cellular processes (Fig. 1.1). Epigenetic mechanisms are essential for embryonic development, cell differentiation, protection against viral genomes, and are likely to be important for the integration of endogenous and environmental signals during the life of an organism (Feinberg *et al.*, 2006; Herceg, 2007; Jaenisch and Bird, 2003). By analogy, deregulation of epigenetic mechanisms has been associated with a variety of human diseases, most notably cancer (Egger *et al.*, 2004; Feinberg and Tycko, 2004; Feinberg *et al.*, 2006; Jones and Baylin, 2002; Ushijima, 2005).

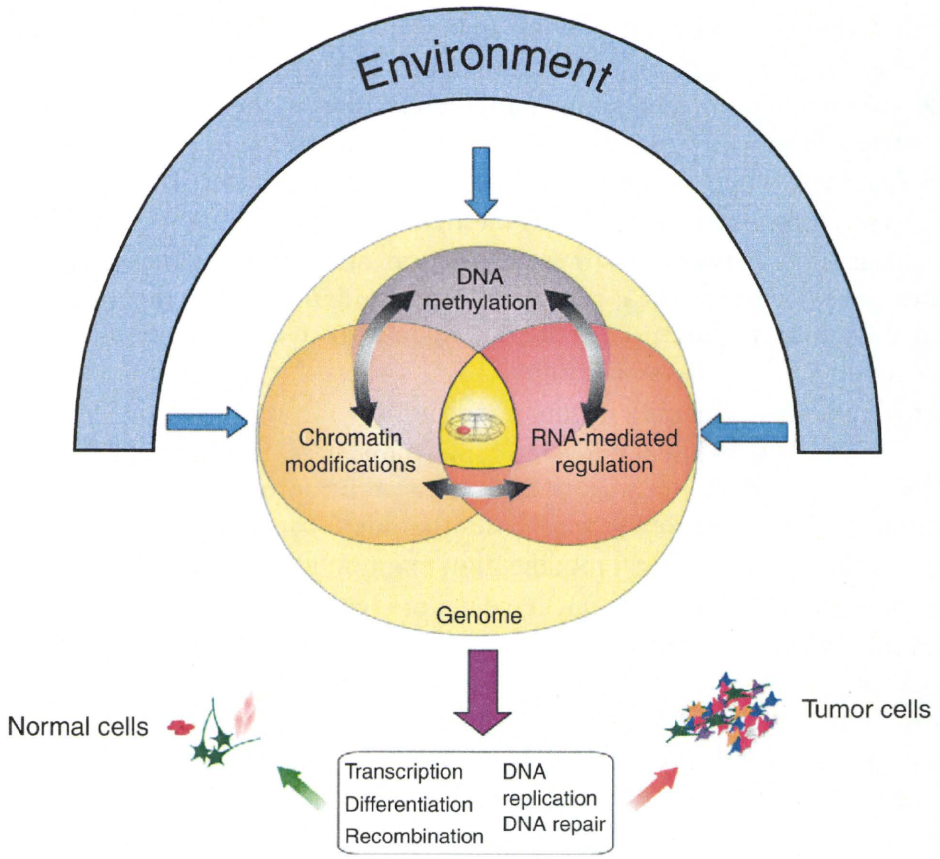

Figure 1.1. An intimate and self-reinforcing cross talk between different types of epigenetic information. Epigenetic mechanisms regulate many cellular processes directly or indirectly and play critical roles in cellular responses to environmental and endogenous stimuli. There is an intimate and self-reinforcing cross talk between different types of epigenetic information. This is proposed to constitute the "epigenetic code" that modulates genetic code in response to endogenous and environmental cues. Epigenetic code is important to maintain gene expression profiles and chromatin structure in a heritable manner over many cell generations, and may dictate cellular outcomes by regulating cellular processes such as gene transcription, proliferation, and DNA repair. Deregulation of epigenetic mechanisms may promote the development of abnormal phenotypes and diseases including cancer. (*Adapted from* Elsevier Ltd. and *Carla Sawan, Thomas Vaissière, Rabih Murr,* and *Zdenko Herceg*: epigenetic drivers and genetic passengers on the road to cancer. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 2008 642(1–2) © 2008 Elsevier B.V.)

DNA methylation and histone acetylation and methylation are the major epigenetic modifications that are most intensively studied in the context of gene transcription and abnormal events that lead to oncogenic process. Once the patterns of these epigenetic marks have been set up, they are propagated autonomously over many cell generations. Wealth of evidence suggests that these marks are dynamically linked in the epigenetic control of gene expression

and that their deregulation play an important role in tumorigenesis (Esteller, 2007; Feinberg *et al.*, 2006; Jones and Baylin, 2002, 2007). Disruption of one of these two epigenetic mechanisms inevitably affects the other. For example, hypermethylation of CpG island in gene promoters triggers deacetylation of local histones, whereas lower levels of histone acetylation (hypoacetylation) seem to predispose to targeted DNA methylation. Therefore, there is an intimate communication between histone acetylation and DNA methylation. Recent studies have begun to reveal the mechanisms underlying these events, although it remains unclear who initiates the talk upon a given environmental or endogenous signal and what is the hierarchical order of epigenetic events during unscheduled gene silencing in tumor cells.

The best-studied epigenetic mechanism is DNA methylation. The methylation of DNA refers to the covalent addition of a methyl group to the 5-carbon (C^5) position of cytosine bases that are located 5′ to a guanosine base. This is a very small chemical modification mark on DNA molecule that does not alter DNA code; however, it may have major regulatory consequences. Aberrant DNA methylation is tightly connected to a wide variety of human malignancies. Two forms of aberrant DNA methylation are found in human cancer: the overall loss of 5-methyl-cytosine (global hypomethylation) and gene promoter-associated (CpG island-specific) hypermethylation (Feinberg and Tycko, 2004; Jones and Baylin, 2007). While the precise consequences of genome-wide hypomethylation are still debated (activation of cellular proto-oncogenes, induction of chromosome instability), hypermethylation of gene promoters is in turn associated with gene inactivation. When hypermethylated, gene promoters become unable to bind the factors that are responsible for gene expression. The gene thus becomes inactivated. A large number of studies indicated that the silencing of tumor suppressor genes and other cancer-related genes may occur through hypermethylation of their promoters. Unscheduled hypermethylation of gene promoters represents an attractive target for early diagnosis, risk assessment, and cancer prevention. For example, the genes that are the target of DNA hypermethylation early in the tumor development, in a high percentage of cases, and in the cancer type-specific manner are of particular interest. In Chapter 2, Manel Esteller and Marta Kulis introduce basic biology of DNA methylation and illustrate the roles of regional hypermethylation and global hypomethylation in carcinogenesis.

Many recent studies have also implicated aberrant patterns of histone modifications and chromatin remodeling in human malignancies. The histone modifications usually occur at the N-terminal "tails" of histones protruding from nucleosomes. These posttranslational modifications include acetylation, methylation, phosphorylation, and ubiquitination. The different histone modifications appear to act in a combinatorial and consistent fashion in regulation of several key cellular processes. This led to the concept known as the "histone code."

The importance of histone modifications is demonstrated by the fact that mechanisms involving these modifications are essential during development and that their deregulation can lead to human diseases. Histone proteins have thus emerged as key carriers of epigenetic information, constituting a fundamental and critical regulatory system that extends beyond the genetic information. Interest in histone modifications has further grown over the last few years with the discovery and characterization of a large number of histone-modifying molecules and protein complexes. Alterations in these chromatin-based processes could lead to mutations in oncogenes, tumor suppressor genes, or DNA repair genes, resulting in genomic instability, oncogenic transformation, and the development of cancer. Importantly, aberrant activity of histone-modifying factors may promote cancer development by misregulating chromatin structure and activity, an example of which is frequently found in human leukemia. Growing evidence suggests that aberrant epigenetic regulation of key cellular processes, most notably gene transcription and DNA repair, may be involved in oncogenesis. Chapter 3 (by Zdenko Herceg and Carla Sawan) introduces basic biology of histone modifications and histone code, and discusses how chromatin modifications may regulate critical cellular process as well as how different forms of epigenetic information in chromatin are disrupted in cancer.

More recently, the role of noncoding RNAs in the control of cellular processes is beginning to emerge. The microRNAs (miRNAs) are small (20–22 nucleotides) RNAs located either within the introns or exons of protein-coding genes (70%) or in intergenic regions (30%), and able to regulate the expression of about 30% of human genes (Bartel, 2004; Calin and Croce, 2006). This regulation is mediated by the direct interaction between miRNAs and messenger RNAs (mRNAs) and it can be accomplished by two mechanisms, translational repression or mRNA degradation (Croce and Calin, 2005; Molnar *et al.*, 2008). It has been proposed that a single miRNA may have several targets as well as a single mRNA may be targeted by different miRNAs. However, the function of most of the miRNAs remains largely unknown. Accumulating evidence indicates that deregulation of miRNAs is linked to several steps of cancer initiation and progression. Interestingly, miRNAs appear to be able to act as either tumor suppressors or oncogenes by affecting distinct genes of gene families involved in critical biological processes such as proliferation and differentiation. Many miRNA genes are located in the genomic loci known as fragile sites and are therefore susceptible to either loss or amplification. Several recent studies indicated that miRNA profiles differ significantly between cancer and normal tissues and also between different tumors. Interestingly, miRNA profiling revealed distinct patterns that may classify cancers according to the developmental lineage and differentiation status, lending miRNAs useful tools in cancer diagnostics and prognosis (Calin and Croce, 2006). Although we are just beginning to understand the complexity of mechanisms regulated by miRNAs, this most

recent type of epigenetic inheritance is likely to provide important information to the overall knowledge in cancer biology. While numerous studies revealed the distinct miRNA profiles in a variety of human malignancies, much less is known about the profiles and function of long noncoding RNAs in cancer (Mercer *et al.*, 2009). In Chapter 4, George Calin and colleagues discuss the basic epigenetic mechanisms involving miRNAs in the regulation of gene expression and their significance in cancer development and progression.

Histone modifications, DNA methylation, and RNA-mediated silencing are epigenetic modifications whose patterns can be regarded as heritable marks that ensure accurate transmission of the chromatin states and gene expression profiles over many cell generations. Accumulating evidence suggests that an epigenetic cross talk, such as interplay between DNA methylation and histone modifications, may be involved in the process of gene transcription and aberrant gene silencing in tumors (Jaenisch and Bird, 2003; Murr *et al.*, 2007; Vaissiere *et al.*, 2008). Although the molecular mechanism of gene activation is relatively well understood, the hierarchical order of events and dependencies in the course of gene silencing during cancer development and progression remains largely unknown. While several studies suggested that DNA methylation patterns guide histone modifications (including histone acetylation and methylation) during gene silencing, other set of studies argues that DNA methylation evolved to take its cues primarily from histone modification states (Jaenisch and Bird, 2003; Thomson *et al.*, 2010). In Chapter 5, Rabih Murr summarizes current knowledge on the interplay between DNA methylation, histone modifications, and miRNAs during gene silencing and its importance in the integration of exogenous and intrinsic stimuli in the control of key cellular processes. Implication of this epigenetic interplay for cancer therapy and prevention is also discussed.

III. EPIGENETIC EVENTS AND BIOLOGICAL PHENOMENA—GENE WIRING TO INSTRUCT INHERITANCE

A. Genomic imprinting and cancer

Genomic imprinting refers to the conditioning of parental genomes mediated by epigenetic mechanism during gametogenesis ensuring that a specific locus is exclusively expressed from either maternal or paternal genome in the offspring. Around 80 genes have so far been found imprinted in humans and mice, although a recent estimation suggested that as many as 600 genes are potentially imprinted. Imprinted genes play critical roles in developmental and cellular processes; therefore, loss of imprinting (LOI) due to epigenetic alterations leads to abnormal biallelic expression, resulting in several human syndromes. Importantly, pathological biallelic expression of several genes caused by LOI is