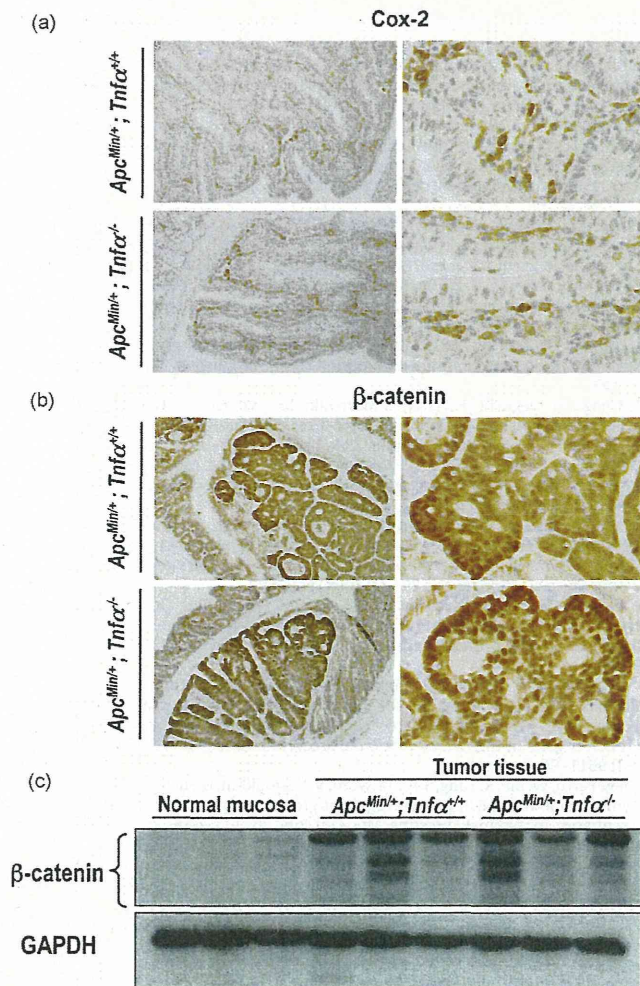

Fig. 4. Cox-2-expressing cells and β-catenin nuclear accumulation in the colon tumor tissues. Colons were immunostained with anti-Cox-2 (a) or anti-β-catenin antibody (b) and representative results from five independent animals are shown here. Original magnification, 100× (left panels), 400× (right panels), respectively. (c) Western blot analysis with anti-β-catenin antibody was performed on cell lysates from both normal colon mucosa and colon tumor tissues. Representative results from three independent experiments are shown here.

4. Discussion

Tnfα is a hormone with a broad spectrum of biological activities, produced mainly by activated macrophages and a variety of other cell types, including activated T cells, mast cells, neutrophils, and astrocytes [31]. Once the protein is efficiently exported from the producing cell, it enters the circulation, where it has a very limited half-life and binds to either high-affinity 55-kDa TNF-receptor-1 (Tnfr1) or low-affinity 75-kDa TNF-receptor-2 (Tnfr2) [32,33]. Popivanova et al. revealed that mice lacking *Tnfr*1 treated with AOM and DSS showed reduced mucosal damage, reduced infiltration of macrophages and neutrophils, and attenuated subsequent tumor formation [11]. These findings indicate Tnfα/Tnfr1 signaling as a crucial mediator of the initiation and promotion of colitis-associated colon carcinogenesis, and suggest that targeting Tnfα may be a useful strategy for prevention and/or treatment of colon cancer in the individuals with IBD.

Table 1
Incidence, multiplicity and tumor volume of large intestinal tumors at week 5.

Genotype	Incidence	Multiplicity[a]	Tumor volume[b]
$Apc^{Min/+}$; $Tnf\alpha^{+/+}$	26/26, 100%	16.15 ± 5.84	3.90 ± 2.25
$Apc^{Min/+}$; $Tnf\alpha^{-/-}$	25/25, 100%	14.27 ± 7.71	3.81 ± 2.49

Statistical significance of differences was evaluated by Student's *t*-test.
[a] Number of tumors/mouse, the mean ± SD.
[b] Tumor volume was calculated as length × width × width × 0.526, the mean ± SD.

In contrast [11], the current study revealed that, despite a deficiency in the *Tnfα* expression, both chemically induced colonic inflammation and the tumor formation in *Tnfα* deficient mice were not attenuated in comparison to those of *Tnfα* +/+ control mice. In addition, the expression of pro-inflammatory factors in the colon mucosa exposed to DSS was not altered in comparison to wild-type control mice. Moreover, no significant differences were observed in either the infiltration of Cox-2-positive inflammatory cells or the nuclear β-catenin accumulation of tumor cells between *Apc* Min/+:*Tnfα* +/+ and *Apc* Min/+:*Tnfα* −/− mice, both of which have been shown to be significantly suppressed in *Tnfr*1 deficient mice [11].

Although Tnfα is the ligand for the Tnfr1, it is noteworthy that Tnfr1 also binds with tumor necrosis factor-β (Tnfβ) or lymphotoxin (LT) [34]. Tnfβ shares about 30% structural homology with Tnfα [35,36], and Tnfα and Tnfβ are functionally indistinguishable with respect to receptor binding and activation of Nf-kB in HL60 cells [37]. Although DSS-induced *Tnfβ* expression was almost equal in both wild-type and *Tnfα* deficient mice, it might be possible that Tnfβ is involved in the induction of colonic inflammation and inflammation-related colon tumorigenesis induced by DSS. This finding is consistent with the previous findings that activation of Nf-kB is associated with DSS-induced inflammation-related colon tumorigenesis [13,38] and that the maximum activation of Nf-kB with Tnfα and/or Tnfβ requires only a small fraction of the total number of Tnf-receptors to be occupied [39].

Previous studies demonstrated that several inflammation-related factors including *Il1*β, Stat3 and iNOS are involved in DSS-induced inflammation-related colon tumorigenesis [38,40,41]. In addition, the possible interaction between iNOS, TnFα and *Il1*β is suggested to play a role in the tumor promotion of inflammation-related colon carcinogenesis [42]. Given such intricate inflammatory responses, it is also possible that the Tnfα-independent signal may promote the inflammation-related mouse colon tumorigenesis in the present study.

There is increasing evidence that the anti-Tnfα monoclonal antibody, infliximab, is an effective therapy for IBD, including Crohn's disease and ulcerative colitis [43–49]. Infliximab is a chimeric monoclonal antibody that binds not only the soluble subunit of Tnfα but also the membrane-bound precursor of Tnfα [50,51]. Infliximab inhibits a broad range of biological activities of Tnfα by blocking the interaction of Tnfα with its receptors. Given the fact that the genetic deletion for *Tnfr*1 significantly suppressed inflammation-related colon tumorigenesis, it was expected that the blockage for Tnfα with the use of infliximab could be a useful strategy for both the chemoprevention and therapy for tumorigenesis. Indeed, previous studies demonstrated that antibodies against Tnfα strongly suppress the development of inflammation-related CRC [11,15]. In contrast, the current study revealed that the genetic ablation of Tnfα alone did not either reduce colon inflammation or attenuate tumor formation. Our results may therefore suggest that an indirect action of anti-Tnfα antibodies exerts a tumor suppressive effect in inflammation-related CRC. A previous study demonstrated that anti-Tnfα monoclonal antibody binds to the transmembrane form of Tnfα, thus resulting in the efficient killing of the Tnfα-expressing cells by both antibody-dependent cellular toxicity and complement-dependent cytotoxicity effector mechanisms [51,52]. The mode of action of anti-Tnfα monoclonal antibody for the treatment of IBD and IBD-related tumorigenesis might be attributed principally to the lysis of the inflammatory

cells rather than blocking the interaction of Tnfα with its receptors.

In conclusion, the current study revealed that the genetic ablation of *Tnfα* results in no detectable effect on either the suppression of DSS-induced colonic inflammation or the attenuation of inflammation-related mouse colon tumorigenesis. These observations suggest that intricate inflammatory responses are involved in the inflammation-related mouse colon tumorigenesis.

Conflicts of interest

No conflicts of interest.

Acknowledgments

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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

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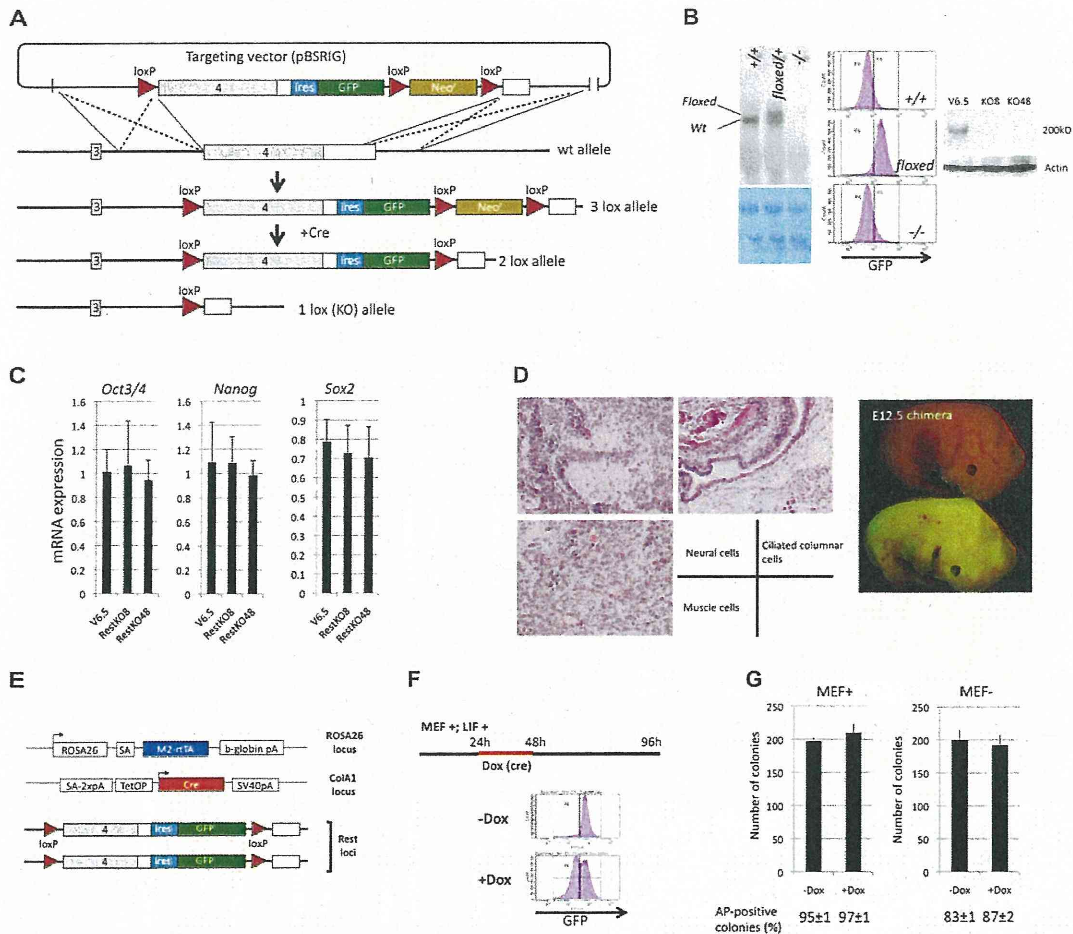

Figure 1. *Rest* Is Not Required for the Maintenance of ESC Pluripotency

(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*;

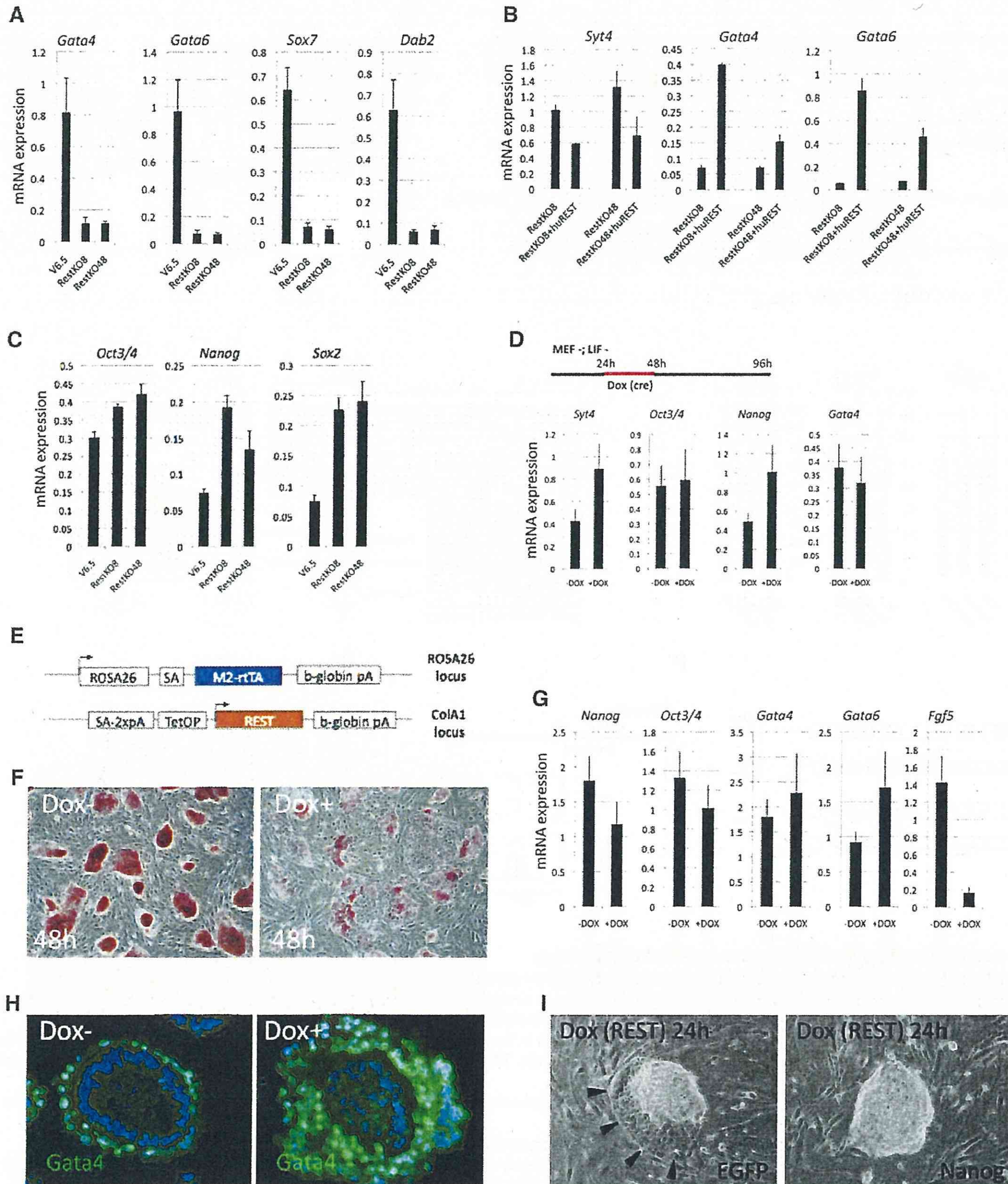

Figure 2. *Rest* Promotes Primitive Endoderm Differentiation in ESCs

(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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