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# ***Rest* promotes the early differentiation of mouse ESCs but is not required for their maintenance**

Highlights

*Rest* is not required for mouse ESC maintenance.

*Rest* promotes the early differentiation of mouse ESCs.

Rest connects to the Oct3/4-Sox2-Nanog core regulatory circuitry in ESCs.

50-word summary

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.

# *Rest* promotes the early differentiation of mouse ESCs but is not required for their maintenance

Yasuhiro Yamada[1, 2, 3, 5, 6], Hitomi Aoki[4, 5], Takahiro Kunisada[4] and Akira Hara[1]

[1]Department of Tumor Pathology and [4]Department of Tissue and Organ Development, Regeneration, and Advanced Medical Science, Gifu University Graduate School of Medicine, Gifu, 501-1194, Japan.

[2] Center for iPS Cell Research and Application (CiRA), Institute for Integrated Cell-Material Sciences, Kyoto University, Kyoto 606-8507, Japan.

[3] PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho Kawaguchi, Saitama, Japan.

[5]These authors are equally contributed to this work.

[6]Correspondence should be addressed at; y-yamada@cira.kyoto-u.ac.jp

Running Title;

*Rest* promotes the early differentiation of mouse ESCs

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 knock-out and *Rest*-inducible ES cell 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; Jorgensen et al., 2009a; Singh et al., 2008). A previous study using a heterozygous *Rest* ES cell line combined with an siRNA knock-down 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, Jorgensen et al. generated a *Rest* null ES cell 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 (Jorgensen et al., 2009a; Jorgensen et al., 2009b).

In order to elucidate the role of *Rest* in the maintenance of pluripotency, we first generated an ES cell line and mice which 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 (Andres 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 ES cell line which 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 using the floxed *Rest* ES cell 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 (**Figures 1B, S1A**). 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 Jorgensen et al. (Jorgensen et al., 2009a; Jorgensen et al., 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 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 pseudo-pregnant 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 (**Figures 1D, 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 ES cell line was derived from transgenic embryo that harbors a doxycycline-inducible *Cre* transgene together with *Rest*-floxed alleles (**Figure 1E**, *Rest 2lox*/*2lox*; *Rosa26*::*rtTA*; *Col1a1*::*tetO-Cre* ES cells) (Beard et al., 2006; Hochedlinger et al., 2005). This new ES cell 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 from 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 using 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 non-treated 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* ES cell line was generated (**Figures 2E, S2B**). The forced expression of *REST* led to the rapid morphological changes of ES-cell colonies into an epithelium-like shape, which was accompanied by decreased ALP 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 (**Figures 2I, 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; Boyer et al., 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, while 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 the 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 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.

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Figure legends

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 ES cell 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 ES cell line containing doxycycline inducible *Cre* alleles.

(F) An experimental protocol. Conditional *Rest* knockout ESCs were treated with doxycycline (2 μg/ml) for 24 hrs starting at 24 h and then were harvested at 96 h after the passage. A FACS analysis revealed the presence of GFP-negative cells, thus indicating the occurrence of *Rest* ablation at 96h 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 35mm-wells.

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 hrs starting at 24 h. The cells were harvested at 96 h 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* ES cell line.

(F) Forty-eight hrs of the induction of *REST* causes the ESC differentiation into epithelium-like colonies with a decreased ALP 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.

(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 twenty-four hour exposure of doxycyline led to the rapid differentiation in *EGFP*-overexpressing ESCs (arrowheads), whereas *Nanog*-overexpressing ESCs retained an undifferentiated morphology. After the forty-eight hour exposure of doxycyline, sixteen 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).

**Figure1**

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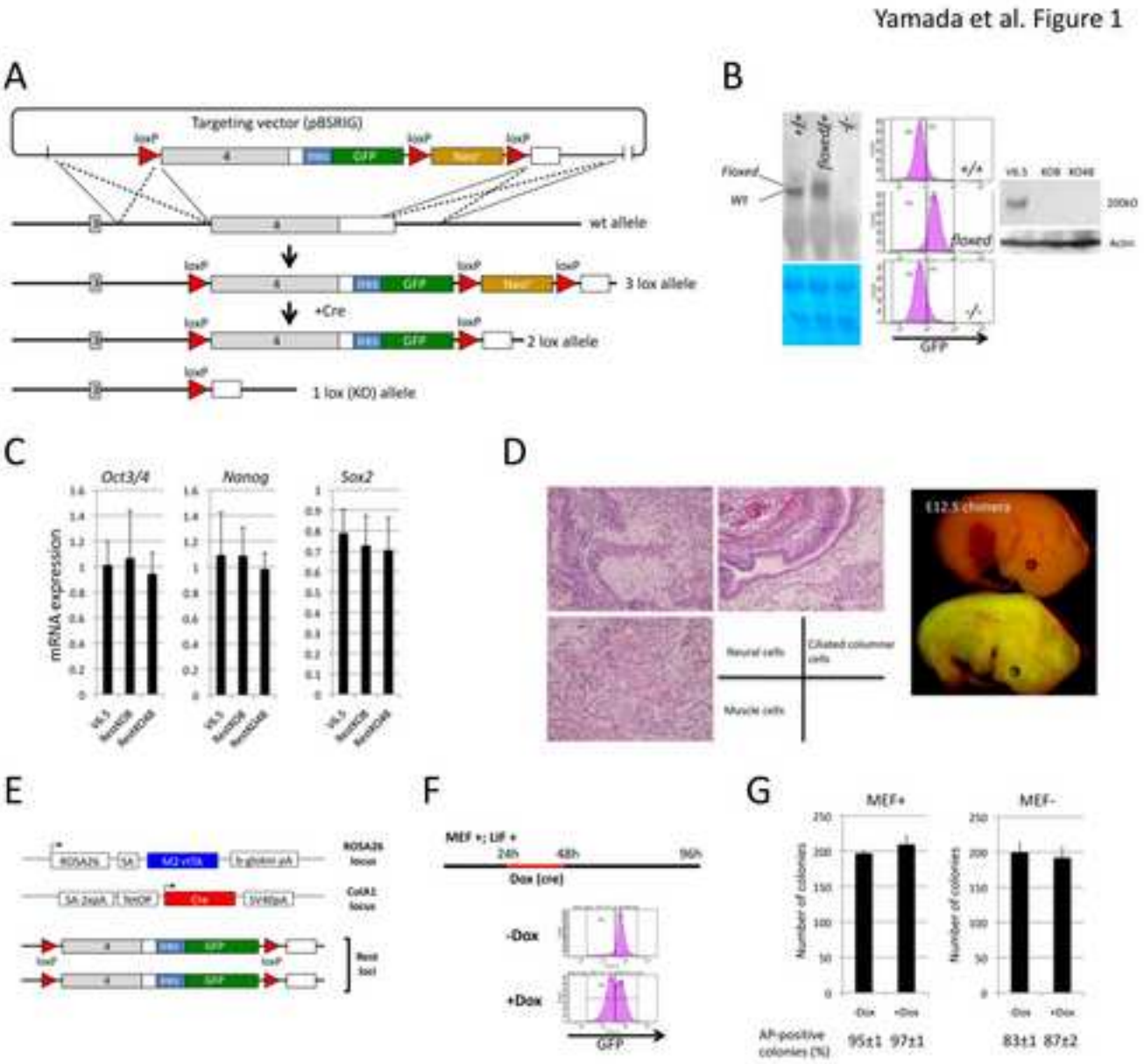

Figure2

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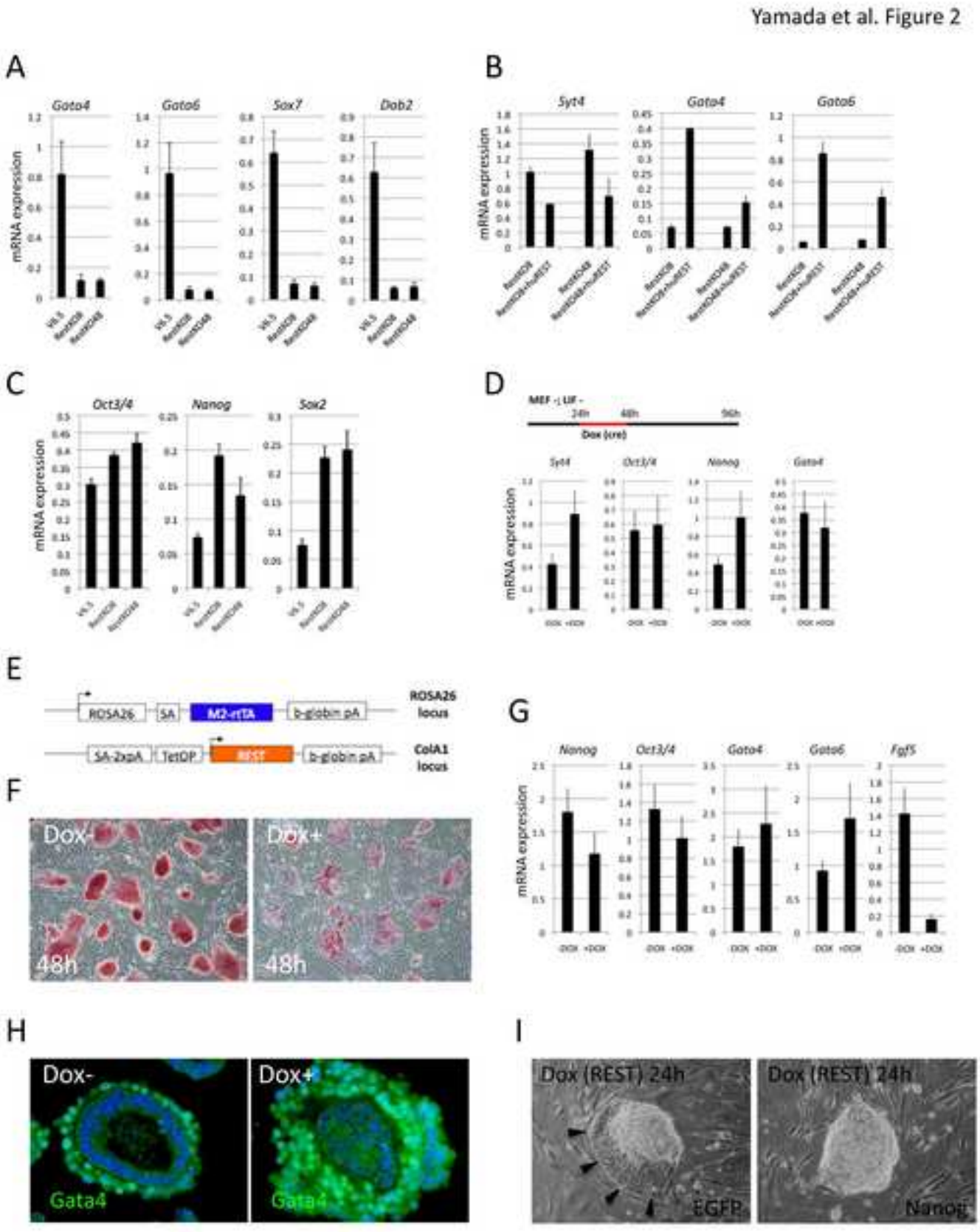

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## Inventory of Supplemental Information

1. Supplemental Experimental Procedures

*Construction of the Rest-conditional knockout vector*.

For the generation of a *Rest*-conditional knockout vector, DNA fragments for the 5' and 3' arms and exon 4 of the *Rest* gene were amplified by PCR from the BAC clones that contained a genomic locus of the *Rest* gene (BACPAC Resources Center, CHORI). These fragments together with the fragment of *IRES-EGFP-floxed pgk Neo* were subsequently cloned into the pBluescript II SK vector (Stratagene), thus generating a plasmid pBSRIG, which was used to generate the conditional knockout construct (**Figure 1A**). In the pBSRIG, a loxP site was inserted into the intron just 5' of the exon 4, which encodes the coRest biding site. In addition, a drug selection marker in the form of a *neomycin phosphotransferase* gene under the control of the *phosphoglycerate kinase 1* gene promoter (*pgk-neo* cassette flanked by loxP sites) was inserted into the 3'UTR of the *Rest* gene.

*Generation of ES cells and mice*.

Plasmid pBSRIG was linearized and electroporated into F1 (129SvJae x C57BL/6; V6.5

line) mouse ESCs and selected for drug resistance against neomycin. Selection was initiated 24 hr postpulse with G418 (300 μg/mL). The correctly targeted clones (3lox ESCs) were confirmed by a standard Southern blot analysis. In order to remove the *pgk-neo* cassette, 3lox ESCs from a targeted clone were transiently transfected with an expression vector encoding *Cre-recombinase* and the puromycin resistance gene (pCre-PAC). After electroporation, the cells were selected with puromycin for 48 hr to enrich for cells that had transiently expressed *Cre-recombinase* and puromycin resistance gene. The removal of the *pgk-neo* cassette was confirmed by Southern blotting, and by death in a G418-containing medium (2lox ESCs). *Rest* -/- ESCs (1lox ESCs) were obtained by repeated targeting using pBSRIG and pCre-PAC. ESCs from one properly targeted subclone in which the neomycin resistance cassette had been removed (2lox ESCs) were used for injection into blastocysts. The resulting chimeras were crossed with C57BL/6 mice to generate the founder colony. Human *REST* cDNA was amplified from a plasmid containing the human *REST* gene (Grimes et al., 2000) and it was introduced into the KH2 ES cell line as described previously (Beard et al., 2006; Hochedlinger et al., 2005) to generate the doxycycline inducible *REST* ES cell

line. *EGFP*- and *Nanog*-overexpressing *REST*-inducible ESCs were generated by electroporation with plasmids containing the *CAG promoter-EGFP-ires-Zeocin* gene (a gift from Hitoshi Niwa) (Niwa et al., 1991) and the *CAG promoter-Nanog-ires-Zeocin* gene, respectively, followed by Zeocin selection at a concentration of 40 μg/ml. After 4 days selection, ES-cell colonies were treated with doxycycline (2 μg/ml) to induce the *REST* gene.

*ES cell culture conditions*.

Mouse ESCs (V6.5) were cultured under self-renewal conditions on feeder cells in standard ES media (Knockout DMEM (Gibco) supplemented with 15% FBS (Hyclone), 1X nonessential amino acids, 2mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (all from Gibco), 0.1mM β-mercaptoethanol (Sigma), and with 1000 U/mL LIF (ESGRO, Millipore)) on 0.2% gelatin-coated tissue culture dishes.

*Derivation of the conditional Rest knockout ES cell line containing doxycycline inducible Cre alleles*.

The conditional *Rest* knockout ES cell line containing doxycycline inducible *Cre* alleles was derived from mouse E3.5 embryos that contain conditional *Rest* knockout (2lox) alleles together with both the *Rosa 26*-M2rtTA allele and *ColA1*-tetOP-Cre allele by the standard methods. AP staining was performed as described previously (Tsuji et al., 2008).

*Teratomas and chimeric mice*.

To obtain *Rest* -/- teratomas, $2x10^6$ *Rest* -/- ESCs (1lox ESCs) were injected into the subcutaneous tissue of nude mice. Teratomas were harvested 3 weeks postinjection, then fixed overnight in 10% buffered formalin, paraffin embedded, and finally processed with hematoxylin and eosin staining. *Rest* -/- ESCs were transfected with the constitutive *EGFP* expressing retrovirus to visualize the *in vivo* contribution (Yamada et al., 2004). EGFP-labeled *Rest* -/- ESCs were then injected into BDF1 blastocysts, followed by transfer into the uteri of pseudopregnant ICR mice. The embryos were dissected at E12.5 and GFP signals were examined under a fluorescent stereo microscope (SZX16, Olympus) and the digital images were captured with an

Olympus DP70. The embryos were then fixed overnight in 10% buffered formalin, paraffin embedded, and processed with GFP immunostaining with anti-GFP antibody (Invitrogen).

*In vitro differentiation.*

For EB formation, ESCs ($1.0 \times 10^5$) were seeded in non-adherent 24-well plates and then were cultivated in an ES medium without LIF. EBs were then collected and processed for both RNA extraction and histological analyses. Gata4 immunostaining was performed with anti-Gata4 antibody (Santa Cruz Biotechnology, inc.) on paraffin-embedded sections. The number of Gata4-expressing cells in the periphery of EB was determined using the Gata4-immunostained histological section (EBs 120-150 μm in diameter on histological sections, n=10).

*Northern blot and quantitative RT-PCR.*

Total RNA was prepared using either TRIzol reagent (Invitrogen) or RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. For a Northern blot

analysis, 5 μg of total RNA was analyzed by non-radioactive filter hybridization (Roche) with either the *Rest* Exon2 probe or the *Rest* Exon4 probe. The first strand cDNA was synthesized from 1 μg of total RNA using SuperScript First-Strand Synthesis System (Invitrogen) using oligo dT primers according to the manufacturer's instructions. Real-time PCR was performed with SYBR Premix EX Taq (Takara) using a LightCycler 2.0 System (Roche) for each gene of interest. The primer sequences used in qRT-PCR analyses were obtained from the PrimerBank (http://pga.mgh.harvard.edu/primerbank/). Amplification curves and gene expression were normalized to the housekeeping gene *β-actin*, used as an internal standard. The average values with standard deviations of six or three independent samples are shown.

2. Supplemental References

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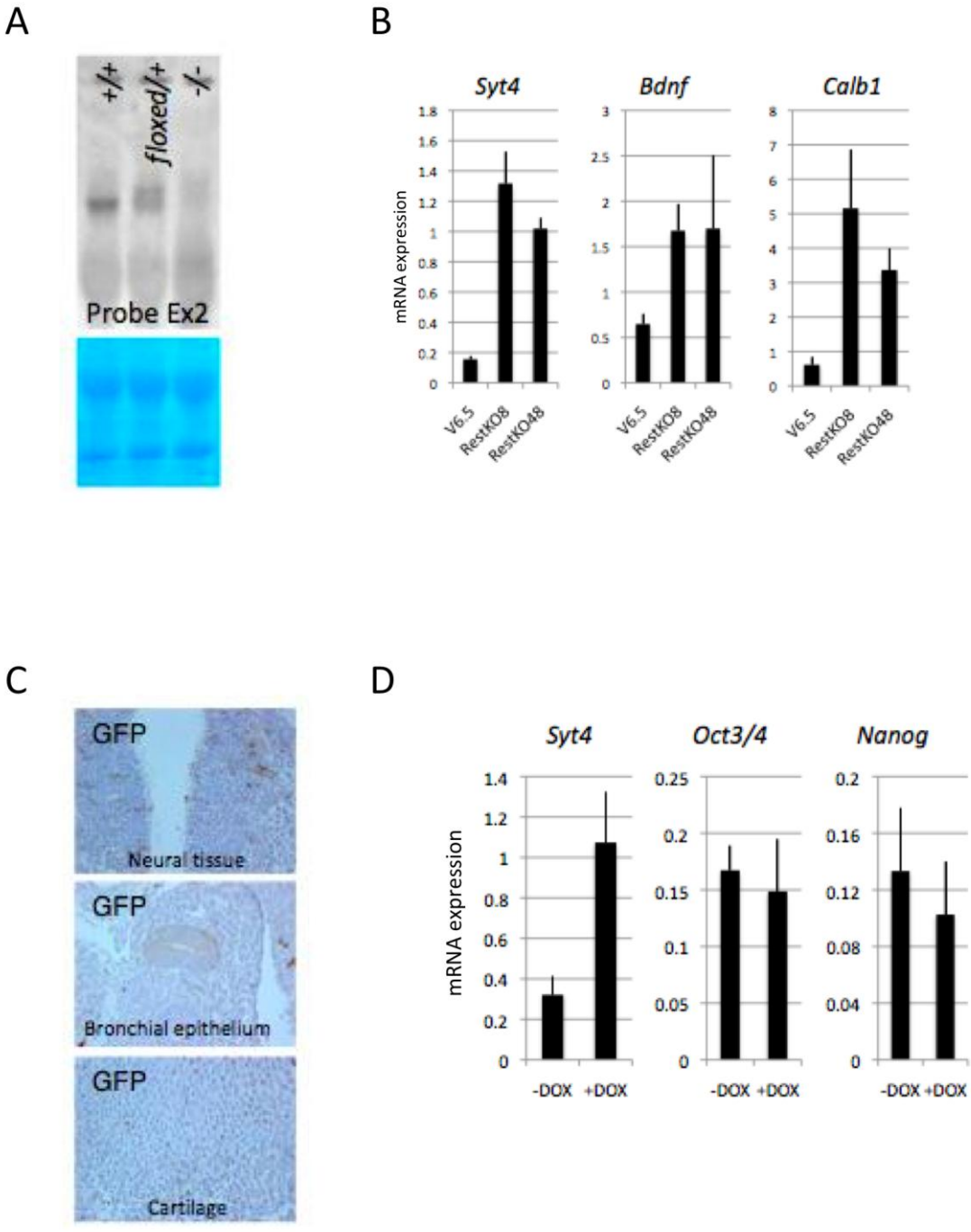
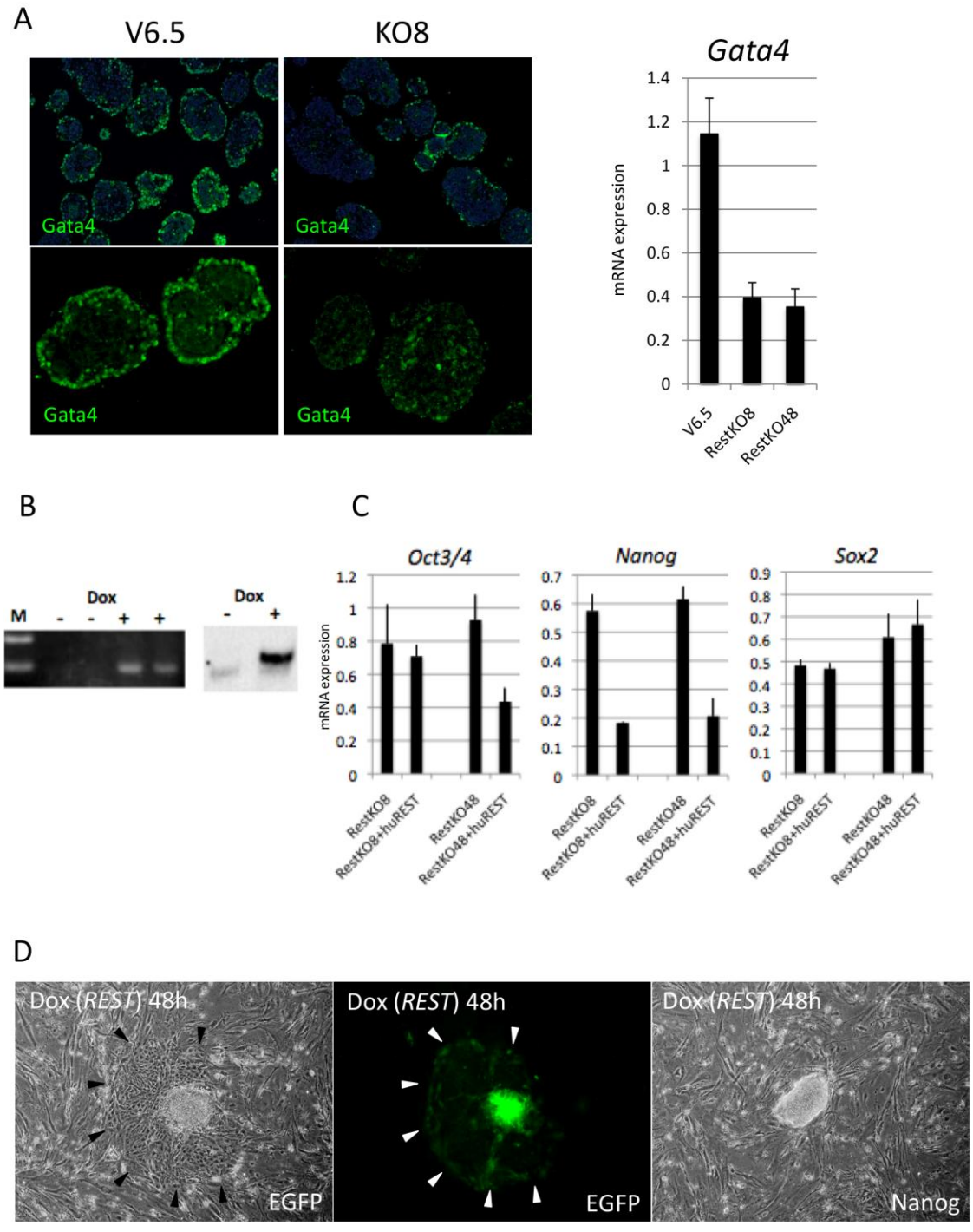
3. Figure S1, related Figure 1.



4. Figure S2, related to Figure 2.



5. Supplemental Figure Legends

Figure S1

(A) A Northern blot analysis using the probe for the remaining exon2 reveals no altered transcript from the remaining *Rest* promoter.

(B) Loss of Rest transcripts leads to the upregulation of the Rest-targeted neuronal genes, *Syt4*, *Bdnf* and *Calb1*. Transcript levels were normalized to *β-actin* levels. The data are presented as the average values with SD of six independent samples.

(C) E12.5 chimeric mice were generated by injecting *Rest* -/- ESCs into blastocysts. GFP immunostaining reveals that GFP-positive *Rest* -/- cells differentiated into neural tissue, bronchial epithelium and cartilage *in vivo*.

(D) The expression levels of *Syt4*, *Oct3/4* and *Nanog* after the conditional *Rest* deletion. The expression of *Oct3/4* and *Nanog* did not change after the *Rest* ablation, whereas the expression of *Syt4* had been derepressed. The data are presented as the mean±SD of six independent samples.

Figure S2

(A) *In vitro* differentiation revealed the EBs generated from *Rest* KO ESCs to contain a decreased number of Gata4-expressing cells 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). Consistently, the expression of *Gata4* was significantly lower in the *Rest* -/- EBs in comparison to the control isogenic EBs (V6.5).

(B) *REST* expression was detectable only in doxycycline-treated cells by RT-PCR. A Western blot analysis reveals ectopic REST expression in doxycycline-treated ES cells.

(C) The ectopic expression of *REST* suppresses the *Nanog* expression in *Rest* -/- ES cells. The data are presented as the mean±SD of three independent samples.

(D) The *Nanog* overexpression blocks the *REST*-mediated ESC differentiation. *REST* was induced in *Nanog*-overexpressing and *EGFP*-overexpressing ESCs by doxycycline exposure. After the forty-eight hour exposure of doxycyline, sixteen out of 25 *EGFP*-overexpressing colonies (68%) started to differentiate into an epithelium-like shape (arrowheads), whereas none of *Nanog*-overexpressing

colonies (0/21, 0%) revealed the evidence of differentiation.