RE1-silencing Transcription Factor (REST) Is Required for Nuclear Reprogramming by Inhibiting Transforming Growth Factor β Signaling Pathway*

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Differentiated cells can be reprogrammed by transcription factors, and these factors that are responsible for successful reprogramming need to be further identified. Here, we show that the neuronal repressor RE1-silencing transcription factor (REST) is rich in porcine oocytes and requires for nuclear reprogramming need to be further identified. Here, we show transcription factors, and these factors that are responsible for successful reprogramming.

Embryonic cells differentiate into all three germ layers of the body as development progresses. Once differentiated, the reversion of the differentiated state to pluripotency is strictly limited in normal development. However, experimentally the differentiated state can be returned to the pluripotent state by transcription factors (1, 2). Despite numerous attempts, the factors responsible for successful nuclear reprogramming still need to elucidate. Transcription factors maintaining the pluripotent state of embryonic stem cells (ESC) are called pluripotent factors, and they have an important role in nuclear reprogramming, such as Oct4, Sox2, and Nanog (3, 4). Thus, we can identify and characterize reprogramming factors by screening the pluripotent factors.

The repressor element 1 (RE1)-silencing transcription factor (REST), as a zinc finger protein, binds 21-bp RE1 sites and functions as a key negative regulator of neurogenesis, so it is also called neuron-restrictive silencer element (5). Recently, REST has been reported to induce gene expression by recruiting TET3 to the DNA for directed 5hmC generation and Nuclear SET domain-containing protein 3-mediated H3K36 trimethylation in neurons (6). Furthermore, REST has different roles in different cellular contexts, such as oncogenic and tumor-suppressor functions and hematopoietic and cardiac differentiation (7, 8). In 2008, REST was proved to maintain self-renewal and pluripotency of mouse ESCs through suppression of microRNAs and believed to be a major pluripotent factor (9, 10). However, it has not been elaborated in nuclear reprogramming. Here, we provide evidence that REST plays a unique role in NT-mediated reprogramming as a suppressor of the TGFβ signaling pathway in pig.

Results

Expression Pattern of REST—We first investigated the expression of REST in porcine oocytes, nuclear transfer (NT), and parthenogenetic activation (PA) embryos by real-time PCR and Western blotting analysis. Porcine fetal fibroblasts (PFFs) were used as donor cells to construct NT embryos, and REST was observed in the cells by Western blotting. Large amounts of REST mRNA and protein were stored in oocytes. After activation, REST mRNA was significantly decreased in NT and PA embryos (p < 0.001) and maintained at a low level from the four-cell to blastocyst stages (Fig. 1A), and REST protein was also degraded in one- and two-cell NT and PA embryos (Fig. 1B). We performed immunofluorescence analysis to locate REST protein in oocytes and embryos. REST was dispersed in the MII oocyte cytoplasm (Fig. 2, A and A′, n = 17), and was incorporated into transferred donor nuclei in NT embryos when the nuclei were condensed at 2 h post-NT (Fig. 2, B and B′, n = 15) and decondensed at 6 h post-NT (Fig. 2, C and C′, locked nucleic acid; Q-PCR, quantitative PCR; iPSC, induced pluripotent stem; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.
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n = 10). In donor cells, REST was also incorporated into the nuclei (Fig. 2, D and D'). To confirm whether maternal REST could be incorporated into the transferred donor nuclei, hREST-GFP mRNA was injected into oocytes at least 2 h before NT. In control, GFP mRNA was injected and the GFP signals were dispersed in the embryos at 2 (Fig. 2, E and E', n = 10) and 6 h (Fig. 2, F and F', n = 20) and 6 h (Fig. 2, G and G', n = 12) post-NT. But in the hREST-GFP mRNA-injected embryos GFP signals were obviously observed in the transferred donor nuclei at 2 (Fig. 2, D and D', n = 20) and 6 h (Fig. 2, H and H', n = 16) post-NT. In in vitro fertilization (IVF) and PA embryos, maternal REST was not incorporated into the nuclei when they were condensed (Fig. 2, I and I', n = 12; K and K', n = 14) and incorporated with the nuclei when they were decondensed (Fig. 2, J and J', n = 14; L and L', n = 17). These results demonstrate that maternal REST is incorporated into transferred donor nuclei, suggesting it may function in the process of NT-mediated nuclear reprogramming.

Inhibition of REST in NT Embryos—To test the role of REST in nuclear reprogramming, its function was inhibited by injection of anti-REST antibody into MI oocytes at least 2 h before NT, PA, IVF, and intracytoplasmic sperm injection (ICSI). The successful injection of the antibody used here was verified by immunofluorescence analysis (Fig. 3, A and A', n = 15; B and B', n = 15). In addition, no or a weak signal was detected by immunostaining the anti-REST antibody-injected oocytes at 2 h post-injection (Fig. 3, C and C', n = 16), indicating that the injected antibody had been degraded and would not affect donor cell-derived and zygotic REST; and, effectively matching the anti-REST antibody to the porcine REST among whole oocyte proteins was verified by Western blotting (Fig. 3D). Then the in vitro developmental competency of porcine NT embryos, regarded as a stringent test of reprogramming efficiency, was examined. The rates of cleavage and cell numbers of blastocyst showed no significant difference among NT embryos with no injection (Con-NT), anti-REST antibody injection (anti-REST-NT), and IgG (IgG-NT) injection, but the proportion of anti-REST-NT embryos that developed to blastocysts was significantly lower than that of IgG-NT and Con-NT embryos (7.27 versus 20.8 and 21.53%, respectively; p < 0.05; Table 1). More anti-REST-NT embryos were arrested at the two- or four-cell stage in comparison with IgG-NT and Con-NT embryos (54.39 versus 30.33 and 28.09%, respectively; p < 0.05; Table 1). To further confirm the results, REST-specific locked nucleic acid (REST-LNA) was injected into oocytes at 33 h of in vitro maturation (IVM). Q-PCR and Western blotting analysis showed REST mRNA and protein were effectively reduced in oocytes at 42 h of IVM by REST-LNA injection (p < 0.001; Fig. 3, E and F). Consistent with anti-REST antibody, the proportion of NT embryos developed to the blastocyst stage was significantly decreased in the REST-LNA injection group (3.06 versus
17.46 and 15.37%; \( p < 0.05 \); Table 2). We also overexpressed REST by injection of hREST-GFP mRNA into oocytes, and it had no significant effect on development of NT embryos. In contrast to that in NT embryos, injections of anti-REST antibody and hREST-GFP mRNA did not affect embryonic development of IVF, ICSI, and PA embryos (Table 1). Therefore, we suggest that REST is required for successful nuclear reprogramming.

**Inhibition of REST Up-regulates TGF\( \beta \) Signaling Pathway in NT Embryos**—REST binds RE1 sites to repress gene expression throughout the body (11). To determine how maternal REST regulates NT-mediated nuclear reprogramming, we searched REST-targeted genes in pig. A consensus RE1 based on the sequences of 32 known RE1 elements, NT(T/C)AG(A/C)(A/G)CCNN(A/G)G(A/C)(G/S)AG, was used to screen porcine in the UCSC genome sequence database (susScr3) by using a PERL script (11). The number of putative RE1s identified in the porcine genome was 1,662, and there are 324 genes that have RE1s within 10 kb from their transcriptional start site (supplemental Table S1). Pathway analysis showed that REST putative targeted genes were widely involved in the TGF\( \beta \) signaling pathway (\( p < 0.001 \), FDR = 0.1642; Fig. 4A). We therefore decided to examine expression of several key genes of the TGF\( \beta \) pathway in NT embryos. By RT-PCR analysis, the expressions of TGF\( \beta R1 \), ACVR2A, ACVR2B, Smad2, and Smad3 were not detected in oocytes and were high in donor cell PFFs. In one-cell NT embryos, the expressions of these genes were at relatively low levels but dramatically up-regulated after REST deficiency (Fig. 4B). Consistent with RT-PCR results, Q-PCR showed that REST inhibition significantly enhanced the expressions in NT embryos from the one- to four-cell stages (Fig. 4C; \( p < 0.001 \)). Moreover, we observed an increase of Smad3 and phosphorylated Smad3 (Smad3-p) expressions in two-cell REST-deficient NT embryos by Western blotting and immunofluorescence analysis (Fig. 4, D and E). The results show that inhibition of REST up-regulates the TGF\( \beta \) signaling pathway in NT embryos.

**Up-regulation of TGF\( \beta \) Pathway by Inhibition of REST Is Involved in Reprogramming Failure**—To determine whether up-regulation of the TGF\( \beta \) pathway in REST-deficient NT embryos leads to the failure of NT-mediated nuclear reprogramming, a specific TGF\( \beta \) pathway inhibitor, SB431542, was used. We found 0.1 \( \mu \)M SB431542 treatment for 12 h post-activation had no negative effect on development of PA embryos (Table 3), so NT embryos were treated as the method. SB431542 treatment could dramatically decrease Smad3 and...
Smad3p in normal and REST-deficient NT embryos detected by Western blotting and immunofluorescence analysis (Fig. 4, D and E), indicating the TGFβ pathway was efficiently inhibited by SB431542. Then, we asked whether TGFβ pathway inhibition in REST-deficient NT embryos can rescue the failure of nuclear reprogramming. As expected, SB431542 treatment successfully rescued the embryonic development to blastocysts (anti-REST + DMSO-N T versus Con + DMSO-N T and anti-REST + SB-N T, 6.48 versus 18.14 and 22.94%, respectively; p < 0.05; Table 1). Furthermore, the development of NT embryos was significantly enhanced by SB431542 treatment (Con + DMSO-N T versus Con + SB-N T, 18.14 versus 28.93%, respectively; p < 0.05; Table 1). These results indicate that the failure of NT-mediated reprogramming in REST-deficient NT embryos can, at least to some extent, be attributed to TGFβ pathway up-regulation and TGFβ pathway may block nuclear reprogramming. In induced pluripotent stem (iPS) cells technology, inhibition of TGFβ pathway promotes reprogramming through inducing Nanog (12). Here, we also found that the expressions of Nanog in one- and two-cell NT embryos were remarkably enhanced after TGFβ pathway inhibition checked by Q-PCR and Western blotting analysis (p < 0.001; Fig. 5, A and B). Taken together, our results indicate the REST repressing TGFβ pathway regulates NT-mediated reprogramming (Fig. 5C).

### Discussion

Differentiated cell nuclei can be reprogrammed to a pluripotent state by NT into oocytes, iPS technology, and cell fusion with ESCs (2, 3, 13, 14). NT mediated-reprogramming has been proven to be the most efficient way (15), and complex transcription factors are significant in the process. Therefore, identification and characterization of these factors will provide us important information on nuclear reprogramming. In the study, we found that REST was rich in oocytes and required for NT-mediated reprogramming through inhibiting TGFβ pathway in pig.

REST is a zinc finger protein, and represses neuronal gene transcription in nonneuronal cells (16–18). Mice that lack REST exhibit malformations in the developing nervous system and die by embryonic day 11.5 of embryogenesis. However, these mice appear normal until embryonic day 9.5 (16). This is

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**TABLE 1**

| Groups | Repeats | Embryos | Cleavage | Blastocyst | No. of blastocyst cells | Embryos arrested at two four-cell stage |
|--------|---------|---------|----------|------------|------------------------|---------------------------------------|
| NT     | Con     | 3       | 122      | 86 (71.9 ± 6.67) | 25 (21.53 ± 3.12)* | 38.33 ± 8.33 | 33 (28.07 ± 3.43)* |
|        | IgG     | 3       | 131      | 100 (78.33 ± 7.84) | 26 (20.8 ± 2.27)* | 39.43 ± 10.56 | 40 (30.33 ± 8.33)* |
|        | Anti-REST | 3     | 137      | 104 (75.52 ± 11.11) | 9 (7.27 ± 3.50)* | 32.89 ± 8.50 | 72 (54.39 ± 4.11)* |
|        | Con + DMSO | 3     | 169      | 136 (81.28 ± 4.73) | 30 (18.14 ± 5.72)* | 35.25 ± 8.38 | 54 (32.48 ± 5.87)* |
|        | Anti-REST + DMSO | 3 | 166      | 130 (79.31 ± 5.25) | 45 (28.93 ± 4.33)* | 41.26 ± 7.64 | 40 (25.74 ± 5.37)* |
|        | Anti-REST + SB | 3 | 164      | 126 (78.26 ± 6.82) | 9 (6.48 ± 7.78)* | 30.52 ± 10.65 | 91 (56.84 ± 5.21)* |
|        | Anti-REST + SB | 3 | 169      | 132 (79.10 ± 5.08) | 37 (22.94 ± 5.09)* | 37.41 ± 9.62 | 42 (25.25 ± 5.42)* |
|        | hREST-GFP mRNA | 3 | 96       | 74 (78.13 ± 10.95) | 23 (23.09 ± 4.13)* | 35.14 ± 7.50 | 26 (27.52 ± 3.88)* |
|        | GFP mRNA | 3       | 87       | 64 (75.00 ± 8.86) | 16 (19.92 ± 5.80)* | 32.25 ± 10.32 | 24 (29.32 ± 5.53)* |
|        | Con     | 3       | 120      | 82 (70.08 ± 6.67) | 17 (15.7 ± 4.08) | 43.00 ± 7.53 | 54 (45.26 ± 7.92)* |

**Discussion**

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consistent with our observations that REST deficiency has no obvious effect on early embryonic development of IVF and PA embryos. REST was found to incorporate into the condensed and decondensed transferred donor nuclei in one-cell NT embryos; and REST inhibition by anti-REST antibody and REST-LNA injections remarkably decreased the in vitro developmental competency of NT embryos. So, we believe that REST is required for NT-mediated reprogramming. REST can bind RE1 sites to repress gene expression (5). In porcine genome, 1,662 RE1s and 324 corresponding genes were identified. These numbers are comparable with human and mouse (11). Pathway analysis showed that TGFβ/H9252 signaling pathway could be suppressed by REST. Confirming that, up-regulation of the TGFβ/H9252 pathway was observed in REST-deficient NT embryos, revealing TGFβ pathway is suppressed by REST in NT embryos.

**FIGURE 4.** Maternal REST suppresses the TGFβ signaling pathway in porcine NT embryos. A, putative REST-targeted genes in the TGFβ signaling pathway. Putative REST targeted genes were marked by red. B, expression of REST targeted genes in TGFβ pathway checked by RT-PCR. 1, MII oocytes; 2, donor cells; 3, one-cell NT embryos; 4, anti-REST one-cell NT embryos. C, expression of REST targeted genes in the TGFβ pathway checked by Q-PCR. CON, NT embryos; anti-REST, anti-REST NT embryo. Asterisk (*) indicates \( p < 0.001 \); D, Western blotting analysis of Smad3 and Smad3-P in NT embryos; E, immunofluorescence analysis of Smad3-P in porcine two-cell NT embryos, a and a’, NT embryo (n = 14); b and b’, anti-REST NT embryo (n = 15); c and c’, NT embryo treated by SB431542 (n = 14); d and d’, anti-REST NT embryo treated by SB431542 (n = 17); e and e’, IVF embryos (n = 8); f and f’, PA embryos (n = 12). Green, Smad3-P; blue, DNA. Scale bar, 50 μm.

**TABLE 3**
Effect of SB431542 with different concentrations on in vitro development of porcine PA embryos

| Groups | Repeats | Embryos | Cleavage % | Blastocyst % |
|--------|---------|---------|------------|--------------|
| Con. 3 | 121     | 98 (79.91 ± 6.36)a | 32 (27.64 ± 7.09)a |
| 1 μM 3 | 119     | 69 (56.38 ± 3.19)b | 16 (14.53 ± 7.81)b |
| 0.5 μM 3 | 124     | 84 (66.94 ± 5.48)c | 22 (18.42 ± 4.53)c |
| 0.1 μM 3 | 131     | 96 (74.48 ± 5.42)c | 34 (26.41 ± 4.19)c |

Note: values with different superscripts within columns denote significant differences (\( p < 0.05 \)).
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TGFβ pathway has been implicated in the development and maintenance of various organs (19, 20), and is necessary for the maintenance of self-renewal and pluripotency of both human and mouse ESCs (21). During embryonic development, the pathway is believed to play critical roles in the specification of cell identities in embryonic and extra embryonic lineages of the post-implantation embryo (19, 22–24). Before implantation, embryonic phenotypes for loss-of-function mutation of the pathway are not detected (20). Transcriptome sequencing and analyzing pig embryos in vivo and in vitro also show that the TGFβ pathway is not active well before maternal zygotic transition at the four- to eight-cell stages (25). Those data suggest the function of the TGFβ pathway is suppressed during early embryonic development. In the study, activation of the TGFβ pathway at a certain level was detected in NT embryos, but not in IVF and PA embryos, and the activation in NT embryos could be attributed to donor cell PFS in which the TGFβ pathway activated. Here, we proposed that REST was required to silence the TGFβ pathway in NT embryos, and in IVF and PA embryos, the pathway was unactivated and, in the regard, REST was not needed. So, the function of REST is only necessary for NT embryos, not for IVF and PA embryos. In addition, inhibition of REST up-regulated the TGFβ signaling pathway in NT embryos. So, we believe inhibition of the TGFβ pathway by REST may be involved in successful nuclear reprogramming.

To test the point, SB431542 was used to treat NT embryos. SB431542 treatment could successfully rescue the development failure of REST-deficient NT embryos and improve developmental potential of normal NT embryos. The results reveal that the TGFβ pathway may have a negative effect on NT-mediated reprogramming. It has been demonstrated that TGFβ pathway inhibition can replace Sox2 and promote the completion of iPS reprogramming through induction of the reprogramming factor Nanog (12). Coincidentally, high level Nanog expression was observed in the TGFβ pathway-inhibited NT embryos. Previous reports have been shown that inhibition of the TGFβ pathway by SB431542 increases Bmp signaling (26) and Bmp signaling induces Nanog expression (27). The cross-talk between TGFβ and Bmp signaling may result in Nanog induction. We conclude that inhibition of the TGFβ pathway improves NT-mediated reprogramming perhaps by up-regulation of Nanog.

So far, many studies have focused on identification of reprogramming factors (28–35). In the study, we demonstrate that REST acts as a repressor of the TGFβ pathway and is critical for NT-mediated nuclear reprogramming, and inhibition of the TGFβ pathway by SB431542 treatment promotes the reprogramming efficiency in pig. In addition to better understanding the detailed mechanism of how TGFβ pathway inhibition contributes to increased reprogramming efficiency, whether or not our observation can be generally applied to other animal species warrants future investigation. The simplicity of SB431542 treatment during NT makes the testing of our approach worthwhile. If so, SB431542 treatment has the potential to enhance cloning efficiency in a broad range of mammalian species, including humans. Our method could hold great promise for human therapeutic cloning (36).

**Experimental Procedures**

*hREST-GFP* mRNA in *in Vitro Transcription and Plasmid Construction*—pEGFP-C1 and hREST-GFP (RG211570, OriGene) plasmids were linearized before *in vitro* transcription. RNA synthesis and poly(A) tailing were carried out with a MEGA script T7 Kit (Ambion, Carlsbad, CA) according to the manufacturer’s instructions.

**Oocyte and Embryo Manipulations**—Before NT, IVF, ICSI, and PA, 10 picoliters of 1 mg/ml of anti-REST antibody (ab21635, Abcam), 100 ng/μl of GFP and hREST-GFP mRNAs solution were injected into matured oocytes. After injection, oocytes were kept for at least 2 h before manipulations, which allows the antibody to bind endogenous REST. Moreover, 10 picoliters of 10 μM REST-LNA (Locked Nucleic Acid, Exiqon) was injected into porcine oocytes with the first polar body collected at 33 h of IVM (35), and the oocytes matured at 42 h were used for NT, ICSI, and IVF. The procedure for porcine NT, PA, and IVF has been described previously (35). After fusion, 0.1 μM SB431542 was used to treat NT embryos for 12 h. Cumulus cell-free oocytes were directly activated by the same parameters as for the somatic cell nuclear transfer procedure to produce PA embryos.

ICSI was performed by using an inverted microscope (Olympus IX71, Olympus Optical Co. Ltd.) with a piezoeuctuated micromanipulator (PMAS-CT150; Prime Tech Ltd, Tsuichiura, Japan). A 100-ml drop of HEPES-M199 containing 0.5% (v/v) FBS and a 20-ml drop of 4% (w/v) polyvinylpyrrolidone (M, 360,000; Sigma) were placed in a 35-mm dish and covered with
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### Table 4

The primer list

| Gene   | Primer sequence (5’-3’)                          | Length (bp) | Accession number |
|--------|--------------------------------------------------|-------------|------------------|
| 18S rRNA | F: TCCAAAGAGAATCTGCGGGGAA  
|         | R: CCTCCAGTGGTCTCTGTTTTGA | 149         | NR002170         |
| REST   | F: AGGCGCGAGTGCTAAGGACAG  
|         | R: GTTGCTGTGGAGTTTGTGG   | 192         | GU7991112.1     |
| ACVR2A | F: ACGGCTCAAACTGTGTTGACCTCA  
|         | R: CTGGGAGTAGCTCCTTGTAATGT | 187         | NM_001204765.1  |
| ACVR2B | F: CACCGAGGAGTCATCTACAAAGCC  
|         | R: CAGTTGCTTGAGGAGATCTCCTG | 165         | NM_001005350.1  |
| ACVR1B | F: AGGCGCGAGTGCTAAGGACAG  
|         | R: GTTGCTGTGGAGTTTGTGG   | 112         | NM_001195322.1  |
| TGFB1  | F: AGAAGCAGATCTGACACGACATACCAG  
|         | R: AGCTATTTCCAGAATACTACCCCTT | 172         | NM_001038391.1  |
| Smad2  | F: GCTGCTCTTCCGCGCAGTCGCG  
|         | R: AGTTCTGCCTCCCTGCTGTTTG | 123         | NM_001256148.1  |
| Smad3  | F: CACGCCACCCAGATGACCACAG  
|         | R: CTCTAGATGAGGAGACCCAGAC  | 145         | NM_214137.1     |
| Nanog  | F: CCTCCAGTGGATCTGCTTTAC  
|         | R: CATCTTGTGGTGGGCTGAG    | 118         | AY596464        |

### Immunostaining

- Oocytes and embryos without zona pellucida were transferred to cold 40 mM sodium phosphate, pH 7.6, containing 50 mM NaCl, 50 μM sodium orthovanadate, 20 μM MG132, 2 μM matrix metalloprotease inhibitor III (444264, Calbiochem), and 1% protease inhibitor mixture III (539134, Calbiochem).
- Homogenization was carried out with a Tekmar homogenizer by three 15-s bursts with a minute cooling between. Homogenates were centrifuged for 1 h at 100,000 x g. The supernatant solutions are referred to as “soluble” fractions. The pellets were suspended in 0.2 ml of complete buffer containing 1% ASB-14 and were mixed every 15 min for 2 h with Radnoti glass pestles (Unitek, Monrovia, CA). After centrifugation for 1 h at 100,000 x g, the supernatants, referred to as “membrane extracts” were removed, and the pellets were discarded. About 50 embryos of each soluble and membrane extract for each gene were separated by lithium dodecyl sulfate-polyacrylamide gel electrophoresis on 4–12% BisTris NuPAGE gels (the gels have been run under the same experimental conditions) and transferred to PVDF membranes (Invitrogen); nonspecific binding was blocked by overnight incubation in 1% casein in PBS at room temperature. Antibodies against REST (ab21635, Abcam), Smad3 (ab40854, Abcam), Smad3-p (ab118825, Abcam), and Nanog (500-P236, Peprotech) were used, and β-actin (A1978, Sigma) served as a loading control. After a 2-h incubation at room temperature with secondary antibodies, protein bands were detected by enhanced chemiluminescence with the RPN2108 kit (Amersham Biosciences) and BioMax Light film (Eastman Kodak Co.).

### Western Blotting

- The procedure for Western blotting analysis has been described previously (11). Oocytes or embryos were fixed in freshly prepared 4% paraformaldehyde in PBS, permeabilized in 1% Triton X-100 in PBS, and left in blocking solution (1% BSA in PBS) for 1 h. For immunolabeling, the embryos were incubated overnight with anti-REST (ab21635, Abcam), anti-Nanog (500-P236, Peprotech), or anti-Smad3-p (ab118825, Abcam) antibodies; washed three times, and incubated for 1 h with secondary antibody FITC-labeled donkey anti-mouse IgG (A21202, Invitrogen) diluted 1:1000 with blocking solution. Immunofluorescence of injected oocytes and one-cell NT embryos without anti-REST antibody (only secondary anti-
body) was used to analyze REST antibody injection and degradation. Samples were washed and counterstained with 5 μg/ml of Hoechst 33342. Fluorescence was detected and imaged using a Nikon fluorescence microscope.

Statistical Analysis—Statistical analysis was performed using SPSS 13.0 for Microsoft™ Windows. Data are shown as the mean ± S.D. One-way analysis of variance was used to assess any differences between groups. The Duncan method was used for pairwise comparisons followed by a Bonferroni correction. p < 0.05 (two-tailed) was considered statistically significant.

Author Contributions—Z. H. L. and Q. R. K. designed and conceived the experiments; B. T. X., Q. R. K., and H. Z. conducted the oocyte and embryo manipulations; Q. R. K. and T. Q. H. conducted molecular experiments; J. Y. L. contributed to bioinformation analysis; R. Y. W. conducted the cell manipulations. Z. H. L. and Q. R. K. wrote and all authors reviewed the manuscript.

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