Acta Biochim Biophys Sin 2012, 44: 385–393 | © The Author 2012. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gms016.
Advance Access Publication 16 March 2012

Original Article

NRSF/REST is required for gastrulation and neurogenesis during zebrafish development

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Repressor element 1-silencing transcription factor (REST) was recognized as a transcription suppressor regulating nervous system differentiation. However, the role of REST during early development has not been clarified. We cloned the zebrafish homolog of human REST. Real-time polymerase chain reaction results showed that zebrafish REST mRNA was both maternal and zygotic with the higher expression level from blastula to the late segmentation period. Whole-mount *in situ* hybridization showed that REST was strongly expressed in the blastoderm since dome stage and dynamically expressed mainly in developing brain, especially in the border of the brain subdivisions in early segmentation period. Knockdown of REST using translation blocking morpholino (MO-tra) technique resulted in gastrulation delay or even blockage, and subsequently led to embryo lethality by early segmentation period with deficient neurogenesis. However, splicing blocking morpholino for REST did not show obviously abnormal phenotype until 48 hpf (hours post-fertilization), indicating that maternal REST was an important regulator for gastrulation. Further study revealed that the abnormal development in MO-tra morphants was at least partly due to the dysfunction of protein transportation from the yolk to the blastoderm. Our results showed that REST (especially maternal supplied REST) was required for gastrulation and neurogenesis during zebrafish early embryogenesis.

Keywords NRSF/REST; *in situ* hybridization; morpholino; maternal; gastrulation; neurogenesis

Received: November 20, 2011 Accepted: January 11, 2012

Introduction

Protein complexes that contain chromatin-modifying enzymes play an important role in regulating tissue-specific gene expression. It has been shown that a single transcription factor, the repressor element 1-silencing transcription factor (REST), also called neuron-restrictive silencer factor, could act as a hub for the recruitment of multiple chromatin-modifying enzymes, affecting neural gene regulation [1]. REST was originally proposed as a silencer of neuronal gene expression in non-neural tissues [2]. However, the recent study showed that the roles of REST were much complex.

The expression and activity of REST varied in embryonic stem cells (ESCs), neural stem cells (NSCs), non-neural cells, and lower in neuron cells [3,4]. Besides, its main function differed due to the cell type. REST is expressed highly in ESC and involved in the self-renewal and pluripotency [5–8]. Downregulation of the REST expression will facilitate cell-fate decision during ESC and NSC differentiation [4,9]. In some non-neural cells, REST is involved in the establishment and maintenance of tissue-specific character [10–12]. Moreover, REST acts as both an oncogene and an anti-tumor gene depending on the cellular context [13–15]. Study on REST knockout mice by Chen *et al*. [16] showed that homozygous embryos developed unaffectedly till embryonic Day (E) 9.25, but abnormally soon after that, and died by E11.5. However, whether the maternal REST contributes in early embryonic development was not known. Since the homozygous knockout mice are embryonic lethal, it is not a suitable model to address the question.

Here, we examined the effects of the REST gene on early development in zebrafish. We isolated and characterized the zebrafish REST gene. In addition, we depicted the temporal and spatial expression of zebrafish REST, modulated its function with morpholino knockdown, and investigated the probable mechanism with special attention focused on the role of this gene during gastrulation and neurogenesis of embryonic zebrafish.

Materials and Methods

Zebrafish and embryos maintenance

Zebrafish (AB strain) were raised and maintained at ~28.5°C under a 14 h light/10 h dark photoperiod as described by Westfield [17]. Embryos were staged by morphological features as described by Kimmel *et al*. [18]. To facilitate visualization of RNA during *in situ* hybridization, 0.003% phenylthiourea (PTU; Sigma, St Louis, USA) was used to treat the cultured embryos to block pigment formation. The related embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline.

This work was approved by Institutional Animal Care and Use Committees (IACUC) in Shanghai Research Center for Model Organisms (Shanghai, China) with the approval ID 2010-0010.

Cloning of zebrafish REST gene

REST cDNA was obtained by polymerase chain reaction (PCR) with primer pair cREST (**Table 1**). The PCR product was digested by *Eco*RI and *Xho*I and then inserted into vector pCS2+, designated as pCS2-REST. For overexpression or rescue experiment, the pCS2-REST was linearized by *Apa*I and used as a template for REST mRNA transcription. The capped REST mRNA was transcribed *in vitro* using SP6 RNA polymerase, and injected into one-cell stage embryos at a dose up to 4 ng/embryo using IM-300 (Narishige, Tokyo, Japan).

Real-time quantitative PCR

Semi-quantitative real-time PCR was carried out to test the REST temporal expression pattern. The E13 primer pair (PCR product laid across exon1 and exon3 of REST gene) was used to detect mature REST mRNA, and immature REST mRNA was detected by I1E2 primer pair, with the PCR product laid across intron1 and exon2. β-Actin was used as internal control (**Table 1**). PCR amplification was performed using cDNA as the template and the conditions were 95°C for 2 min, 40 cycles of 95°C 20 s, 58°C 20 s, and 72°C 30 s, followed by a melt process from 60 to 99°C. The negative control contains no sample template.

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed as described previously [17]. PCR was carried out with the E13 primer pair. The PCR product was cloned into pMD20-T vector (TaKaRa, Dalian, China), which contained SP6 promoters. Two oriented inserted vectors were obtained by sequencing identification. Both plasmid DNAs were linearized with *Eco*RI and transcribed *in vitro* by SP6 RNA polymerases for either the antisense or sense REST RNA probe. The probes were labeled with digoxigenin (DIG; Roche, Basel, Switzerland) and used as positive or negative probe, respectively.

Table 1 Sequences of primers for PCR

Primers	Sequence (5′→3′)
cREST	tgtaGAATTCatgtctcagccggtgtttc
	tactCTCGAGtcatttgccccctgtgccg
E13	AGCAACTCTAAAGGCGTGA
	GCAGTTATCGCATTTGAA
I1E2	TGTCAGTGCAGGTCAGGT
	GTGTCTTGTCAGATGGGTT
β-Actin	CTGCAGACCTTGTGACTGTGCGGGG
	GGTAGCCTGTAAGACTTTCTTAATC

Table 2 Sequences of morpholinos

MO	Sequence (5′→3′)
MO-tra	CAGTGGGAAACACCGGCTGAGACAT
MO-spl	TGTTTGAGACGCACCAGTGTGAGTC
MO-splcon	TcTTTGAcACGCAgCAGTcTGAcTC
MO-con	CCTCTTACCTCAGTTACAATTTATA

Morpholino oligo and RNA injection

REST gene-specific antisense morpholino oligonucleotides were purchased from Gene Tools LLC (Philomath, USA). Four morpholinos (MOs) including MO-tra, MO-spl, MO-splcon, and MO-con (standard negative control oligo) were designed (**Table 2**).

MO-tra was a REST translation blocking MO, and MO-con was used as its control. MO-spl was a REST splicing blocking MO. MO-splcon was 5 nucleotides mismatched of MO-spl and used as a control. Microinjection was carried out into embryos at the one-cell stage.

For rescue experiment, the embryos were first injected with the capped REST mRNA and then with the MO at a dose described in the text.

All images were captured using a Nikon SMZ1500 (Tokyo, Japan) except for **Fig. 4** using an IP-20 (Olympus, Tokyo, Japan) under an IX-12 (Olympus).

Primers for marker genes

After the control or MO-tra was microinjected into one-cell stage embryos, each 50 embryos that survived were collected and lysed using Trizol (Invitrogen, Carlsbad, USA). The mRNA was exacted and reverse transcripted as a template for real-time PCR. Primers for zebrafish embryonic developmental stage marker genes were shown below: Pou5f (also called Oct-3/4, a marker gene for ESCs [19]),

Table 3 Sequences of primers for real-time PCR

Primers	Sequence (5′→3′)
Pou5f	AGAGGTGGTTGAACGAGGCCGA
	ACCAAACCAGGGTGCAAGCCG
Sox2	GGACCAACGGAGGCTACGGC
	CACTGTCCGGCCCGGGAATG
Snail1	ATGCCTCGGTCTTTCCTG
	TCCGTCCTTCATCTTCTTCC
Goosecoid	CCGCTGGGATGTTTAGTA
	CATAACCTGTATGAATACACGGACA
NTL	ACTCACCCAACTTCGGCGCG
	CCATGGGGCCGTTACTGGGC
Egr2b	TCCACCTCCACCTGTCCCA
	GGCGGTAATTTGAAAGAGTCCA
Nestin	GGCAGCCAACAACTATCA
	TTTCTCCAACTCCAGGGT
NeuroD1	ATGCCTCCAACTGAACCC
	AGACCCGCTGCCTGATAG
NCAM	TAGTCATAACACTGCCTTGC
	TGCCCTGAGATTCCTTTT

Sox2 (a marker of the earliest neuroectoderm [20]), Snail1(a marker for late blastula [17]), Goosecoid (a marker to locate shield formation and the earliest cells in the axial hypoblast [17]), NTL (also called no tail, a marker for mesoderm [17]), Egr2b (also called krox20, a marker for the neural plate [17]), Nestin (a marker for NPC [21]), NeuroD1 (a proneural marker [22]), and NCAM (a mature neural marker [22]) (**Table 3**).

Enhanced green fluorescent protein injection

Enhanced green fluorescent protein (EGFP; 32.6 kDa) made by our lab was injected into the yolk of MO-tra or MO-con embryos at eight-cell and 3 hpf (hours post-fertilization) stage at a dose of 1 mg/ml, respectively.

Statistical analysis

Data are represented as the mean ± SEM. One-way analysis of variance followed by *t*-test was used to compare two groups. The criterion for significance was $P < 0.05$. All statistical analyses were performed using Origin 7.5 (OriginLab, Northampton, USA).

Results

Cloning of zebrafish REST cDNA

Full-length REST cDNA of zebrafish was cloned, sequenced, and submitted to GenBank (GenBank accession no. HQ646369). The 2565-bp cDNA encoded a predictive protein of 854 amino acids (aa). It had a lysine-rich domain (200 aa between 452 and 651 aa), and 9 C2H2-type zinc-fingers, which is highly conserved in vertebrates [23], such as human (ENSP00000311816), mouse (ENSMUSG00000029249), rat (ENSRNOP00000002837), and *Xenopus* (ENSXETG00000005323).

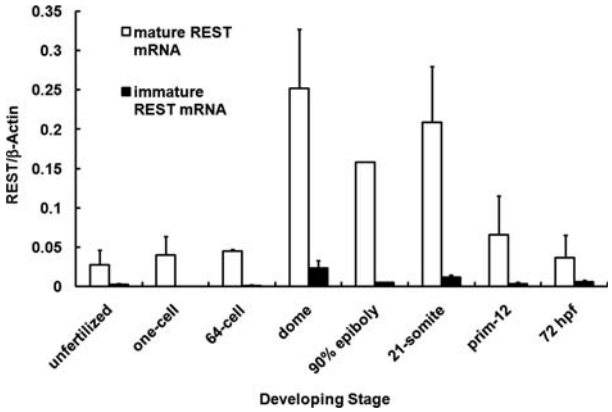

Figure 1 Amount of REST transcripts in different stage embryos examined by real-time PCR Both maternally and zygotic supplied REST mRNA was detected by real-time PCR. Error bars represented SD based on two independent experiments.

Temporal expression of zebrafish REST transcript

The expression time course of the zebrafish REST transcript was determined by real-time PCR. Total RNAs were extracted from eight different stages of embryos (unfertilized egg, one cell, 64-cell, dome stage, 90% epiboly, 21-somite, prim12, and 72 hpf) and subjected to real-time PCR analysis, and the primers of E13 and I1E2 were used to amplify mature REST mRNAs and immature REST mRNAs, respectively. β-Actin was used as a control (**Fig. 1**). Results showed that mature REST mRNA was expressed at all the above stages, indicating that mRNAs were both maternal and zygotic supplied. As both the mature and immature REST mRNA levels rapidly increased at dome stage, the activation of the REST zygotic expression was consistent with the onset of mid-blastula transition (MBT).

In situ hybridization analysis of REST gene during zebrafish embryo development

To gain insight into the role of the zebrafish REST gene, we further analyzed its spatiotemporal expression pattern during embryo development using whole-mount *in situ* hybridization (**Fig. 2**). Results showed that the REST transcripts were ubiquitously expressed at high oblong stage [**Fig. 2(A)**]. At 30% epiboly stage, REST transcripts were distributed mainly in inner undifferentiated embryonic cells [**Fig. 2(B)**], suggesting that maternal REST mRNA may be involved in the pluripotency of embryonic cells. By the 70% epiboly stage, REST mainly appeared in shied

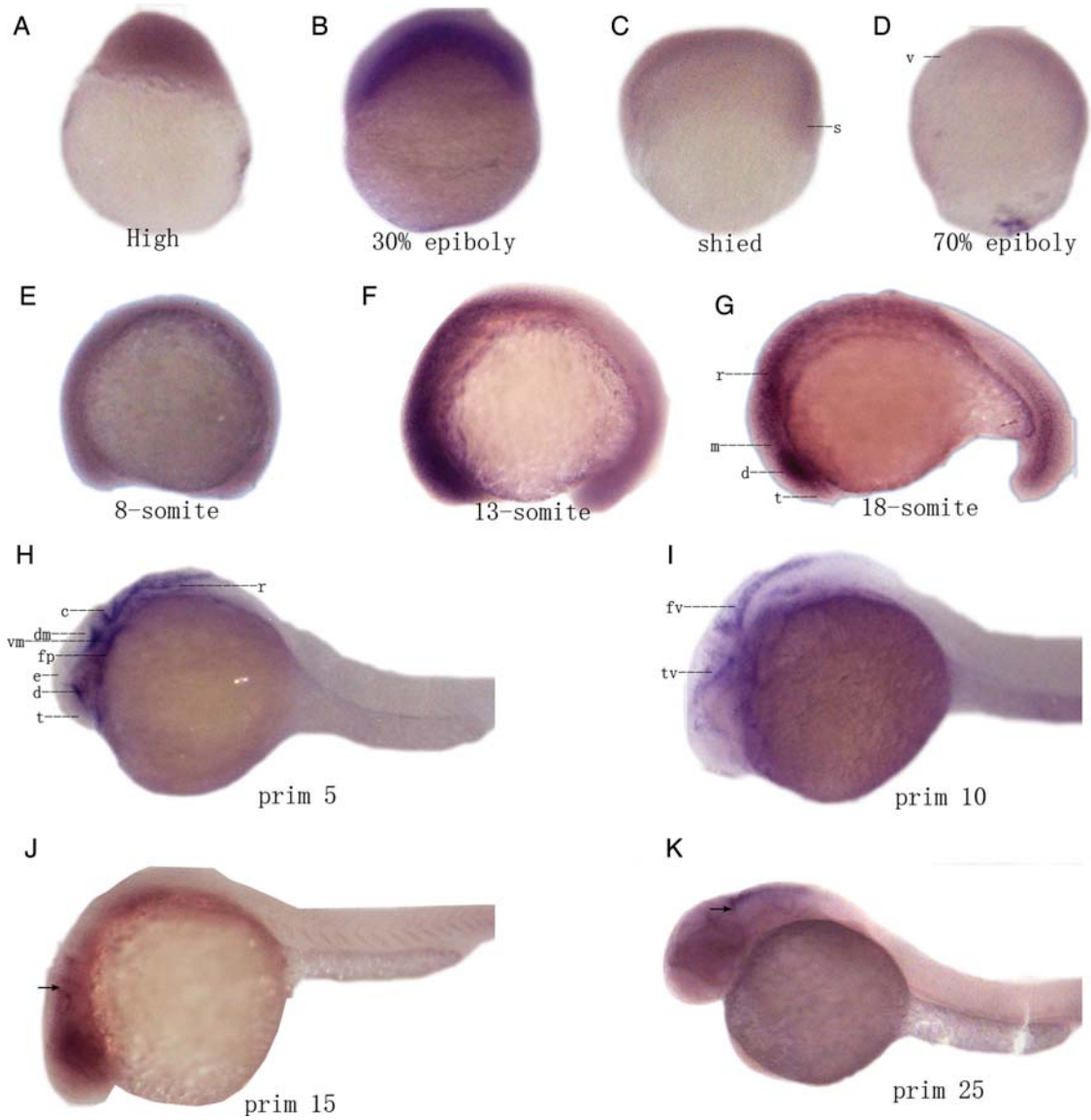

Figure 2 Expression patterns of zebrafish REST gene during embryogenesis (A–D): Lateral view, the dechorionated embryos with animal pole to the top. (E–M): left-side view, with anterior to the left. (A) High oblong. (B) 30% epiboly. (C) Shied. (D) 70% epiboly. (E) 8-somite. (F) 13-somite. (G) 18-somite. (H) Prim5. (I) Prim10. (J) Prim15. (K) Prim25. Arrow indicated MHB. s, shied; v, evacuation zone; t, telencephalon; d, diencephalons; e, epiphysis; dm, the dorsal midbrain (tectum); vm, the ventral midbrain (tegmentum); fp, floor plate; c, cerebellum; r, rhombomeres; fv, the fourth ventricle; and tv, the third ventricle.

[**Fig. 2(C,D)**]. With the development going, REST transcripts were distributed universally at 8-somite stage [**Fig. 2(E)**], then limited in the head and tail bud at 13-somite stage [**Fig. 2(F)**]. At 18-somite stage, REST mRNA emerged in different brain regions [**Fig. 2(G)**] and were located in the borders between the telencephalon and the diencephalon, the epiphysis and the rudiment of the hypothalamus, the tectum and the tegmentum, the cerebellum and the hindbrain rhombomeres, and the hindbrain rhombomeres and fourth ventricle [**Fig. 2(H)**]. By prim10, REST expression in the floor plate diffused and the mRNA in neuromeres borders became obscure [**Fig. 2(I)**]. By prim15, REST transcripts were concentrated mostly in the ventral brain. In the dorsal brain, the REST transcripts were weakly expressed in the midbrain–hindbrain boundary (MHB) [**Fig. 2(J)**]. REST expression was maintained at prim25 stage [**Fig. 2(K)**].

Maternal supplied REST is necessary for gastrulation

To further study the function of REST during embryogenesis, two MOs were designed: MO-tra targeting the translation starting point and MO-spl targeting the splicing between intron 1 and exon 1 region of REST, respectively. MO-tra was used for knocking down total REST mRNAs including both maternal and zygotic ones and MO-spl targeted to zygotic transcripts only.

Abnormal phenotypes in MO-tra-treated embryos were mainly shown in growth retardation and malformation

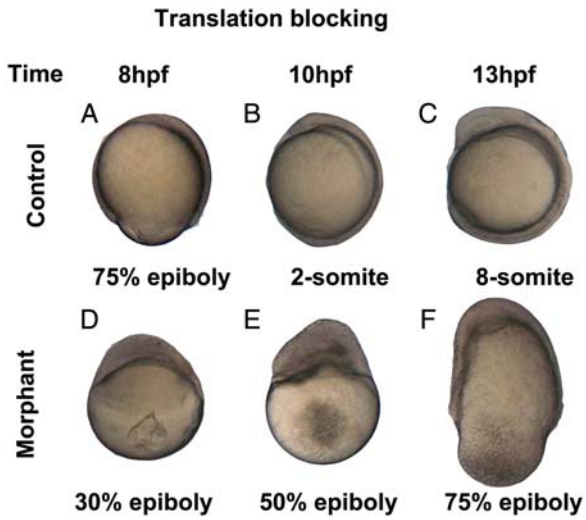

Figure 3 Phenotypes of REST knockdown zebrafish embryos by translation blocking morpholino (A–C) MO-con-injected groups. (D–F) MO-tra-injected groups. Because of growth retardant, embryos were staged by morphological features. (A) 75% epiboly. (B) 2-somite. (C) 8-somite. (D) 30% epiboly. Obvious growth retardant could be figured compared with MO-con of 8 hpf. (E) 50% epiboly. Brachet's cleft (arrow) normally showed in the normal embryos at 75% epiboly stage, but the MO-tra-injected embryo showed epiboly not more than 50%. Also malformation of YSL and High refractive index mass in the yolk could be seen. (F) 75% epiboly. Convergence never occurred; malformation of yolk and high refractive index mass in the yolk could be seen.

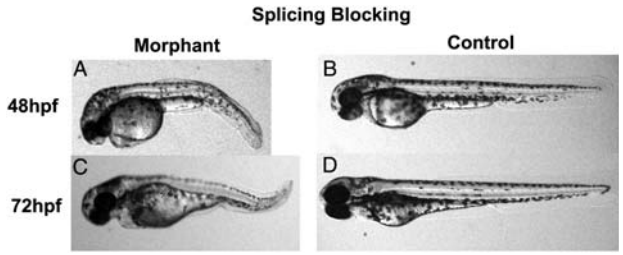

Figure 4 Phenotypes of REST knockdown zebrafish embryos by splicing blocking morpholino (A) MO-spl group embryos of 48 hpf. (B) MO-splcon group embryos of 48 hpf. (C) MO-spl group embryos of 72 hpf. (D) MO-splcon group embryos of 72 hpf.

(**Fig. 3**). At 8 hpf, most embryos (>95%) in the MO-con group had entered 75% epiboly stage, while embryos in the MO-tra group (>95%) were still at the dome stage [**Fig. 3(A,D)**]. Maturity of embryos in the MO-tra group at 10 hpf was equivalent to 4–5 hpf of the control group with obvious malformation (normal/mild/severe: 12/134/103) including irregular yolk syncytial layer (YSL) and asymmetrical yolk in severe cases [**Fig. 3(E)**]. At 13 hpf, malformations appeared more severe in more MO-tra-treated embryos (normal/mild/severe: 12/47/133). During gastrulation, the obvious blockage of the epiboly of deep cell multilayer manifested poor ingression, involution, convergence, and extension, which led to all kinds of abnormal gastrulae (malformed/normal: 58/64). No shied was formed in most embryos even if the epiboly exceeded 50%, causing dorsal–ventral axis and anterior–posterior axis missing. Some with less severe malformation (survives/totals: 5/249) could survive 1 dpf (day post-fertilization), but neither brains nor somites appear normal. For example, no brain subdivide could be distinguished at all.

However, no obvious morphological abnormality was observed when treated with MO-spl until 2 dpf. At 48 hpf, obviously abnormal phenotypes (78 in 196) such as shortened head, bent somites, the heart chamber edema, and the reflexive movement deficiency were evident in the MO-spl group (**Fig. 4**).

Real-time PCR with E13 primers showed the amount of REST transcripts in these morphants decreased 10 fold than the control group at long-pec stage, while the I1E2 PCR products were increased significantly than the control (hundreds of fold).

Taking all embryos of MO-spl into consideration, real-time PCR with E13 primers and I1E2 primers showed that MO-spl was effective to block REST mRNA mature in all stages since there was an increase in immature REST mRNA [**Fig. 5(B)**] while only little change of mature RNA at the four stages after MBT in MO-spl-treated animals [**Fig. 5(A)**]. So it was suggested that MO-spl was efficient for blocking the splicing of zygotic messenger RNA transcript of REST, and it mainly affected the organogenesis during latter segmentation period.

Rescue with injection of REST mRNA significantly attenuated both the MO-tra and the MO-spl caused abnormality by reducing severe morphants and increasing mild ones (in the rescue group, normal/mild/severe: 96/47/43).

REST is required for neurogenesis

To clarify the role of REST during early development, we examined the expression of the marker genes for different stages of early embryogenesis by real-time PCR (**Fig. 6**). Results showed that as to the morphants at the sphere and shied stage, transcripts of Oct-3/4/Pou5f1, Snail1, Goosecoid, NTL, Egr2b, and Nestin were increased, while there were no detectable changes in NeuroD1 and NCAM. However, eight out of nine marker genes were significantly upregulated at the morphants that survived to 21-somite stage, while the mature neural marker NCAM could not be detectable in the morphants. It was reversed in the control group. Considering the growth retardation caused by REST knockdown, we inferred at 6 hpf, differentiation from ESC to NSC and gastrulation were arrested, and at 21 hpf, differentiation from NSC to neuron were arrested in neurogenesis, and none of the morphants could really reach the phase when the mature neuron developed. During the process, the following affairs were involved: ESCs differentiation (Pou5f), the earliest neuroectoderm formation

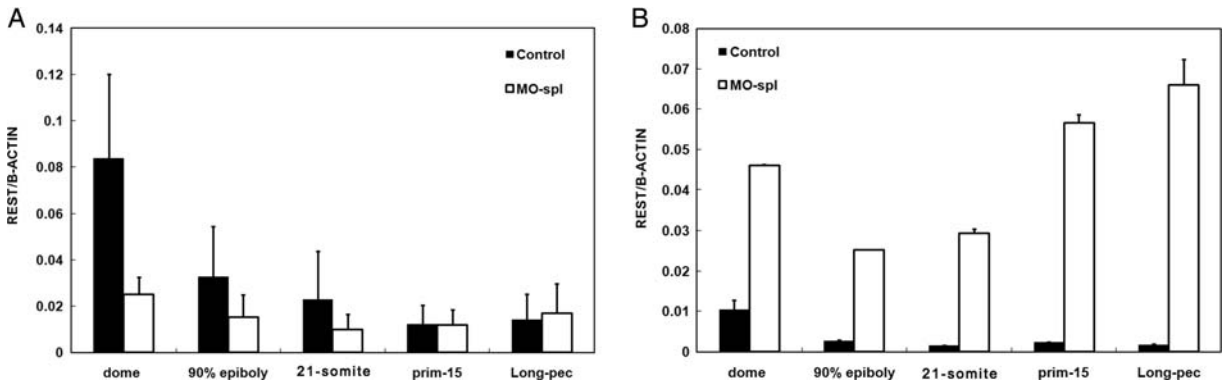

Figure 5 Amount of REST transcripts in MO-spl morphants examined by real-time PCR Error bars represented SD based on two independent experiments. (A) Mature RNA was examined in different stages in both the control group and the MO-spl group. (B) Immature RNA was examined in different stages in both the control group and the MO-spl group.

(Sox2a), epiboly, ingression, involution, convergence and extension (NTL, Snail1, and Goosecoid), the neural plate formation (Egr2b), NPC differentiation (Nestin, and NeuroD1), etc. It was suggested that REST was necessary for embryogenesis especially for gastrulation and neurogenesis.

REST is important for protein transportation from yolk

To study the mechanisms of REST function during gastrulation, we underwent protein transport experiment during early development. EGFP proteins were injected into the yolk of MO-treated embryos at eight-cell and 3 hpf stage, respectively. For the eight-cell injected group, we could observe that the EGFP in MO-tra embryos were transported from the yolk to the blastoderm as that in MO-con [**Fig. 7(A)**]. However, for the 3 hpf-injected group, EGFP in MO-tra group could not go across the YSL and subsequently accumulated to the yolk side of YSL in sphere stage, and formed a highlighted semi-circle beneath the embryo at 21 hpf [**Fig. 7(B)**]. Results suggested that REST might function in protein transport during early development; absence of REST led to failure of protein (maybe others involved, such as RNA) transport and thus led to gastrulation delay, even arrest.

Discussion

In this study, we showed that the maternal REST played an important role in the early developmental stages in zebrafish and the embryonic death of MO-tra morphants might be caused in part by blockage of protein transportation from the yolk to the blastoderm.

Real-time PCR showed that the REST mRNA was abundant during gastrulation and early segmentation period but decreased at later development stages. This is in agreement with previous studies in mice that the amount of REST decreased from ESC to NSC, and then to fully differentiated neuronal cells [24]. The whole-mount *in situ* hybridization indicated that REST was widely distributed in undifferentiated cell mass. With the forming of the brain subdivision, REST was highly expressed at the borders of the subdivision. The REST expression declined at the borders after the subdivision was completed, but remained high in the midbrain, suggesting that REST may be involved in neurogenesis. The early expression profile of REST in zebrafish embryos is also consistent with previous reports in rat brain [25].

Results of REST knockdown by MO-tra suggested that REST transcripts from maternal source were of crucial importance to embryogenesis in late blastula and gastrula period. The real-time PCR analysis of developmental stage marker genes also fitted the morphological result and suggested that the gastrulation and neurogenesis including ingression, involution, convergence, and extension were blocked after inhibition of maternal REST mRNA. This result may be understandable since many REST target genes are involved in formation of cellular matrix, cytoskeleton, and maintaining of cellular communication [26,27]. On the other hand, MO-spl that specifically inhibited the maturation of zygotic REST transcripts perturbed development during the hatching period. Thus, maternal REST transcripts are responsible for early-stage embryonic development while embryonic REST transcripts continue to play a crucial role during late stages. This result cannot be obtained from previous study on REST knockout mice that showed no morphological differences being observed between homozygous mutant embryos and wild types before E9.25 because of their existence of maternal REST mRNA.

In EGFP transportation experiment, protein transportation from the yolk was blocked after REST knockdown and was accumulated beneath the YSL, suggesting that REST was important to the transportation of proteins from the yolk during early embryonic development. It was well known that maternal materials including proteins and

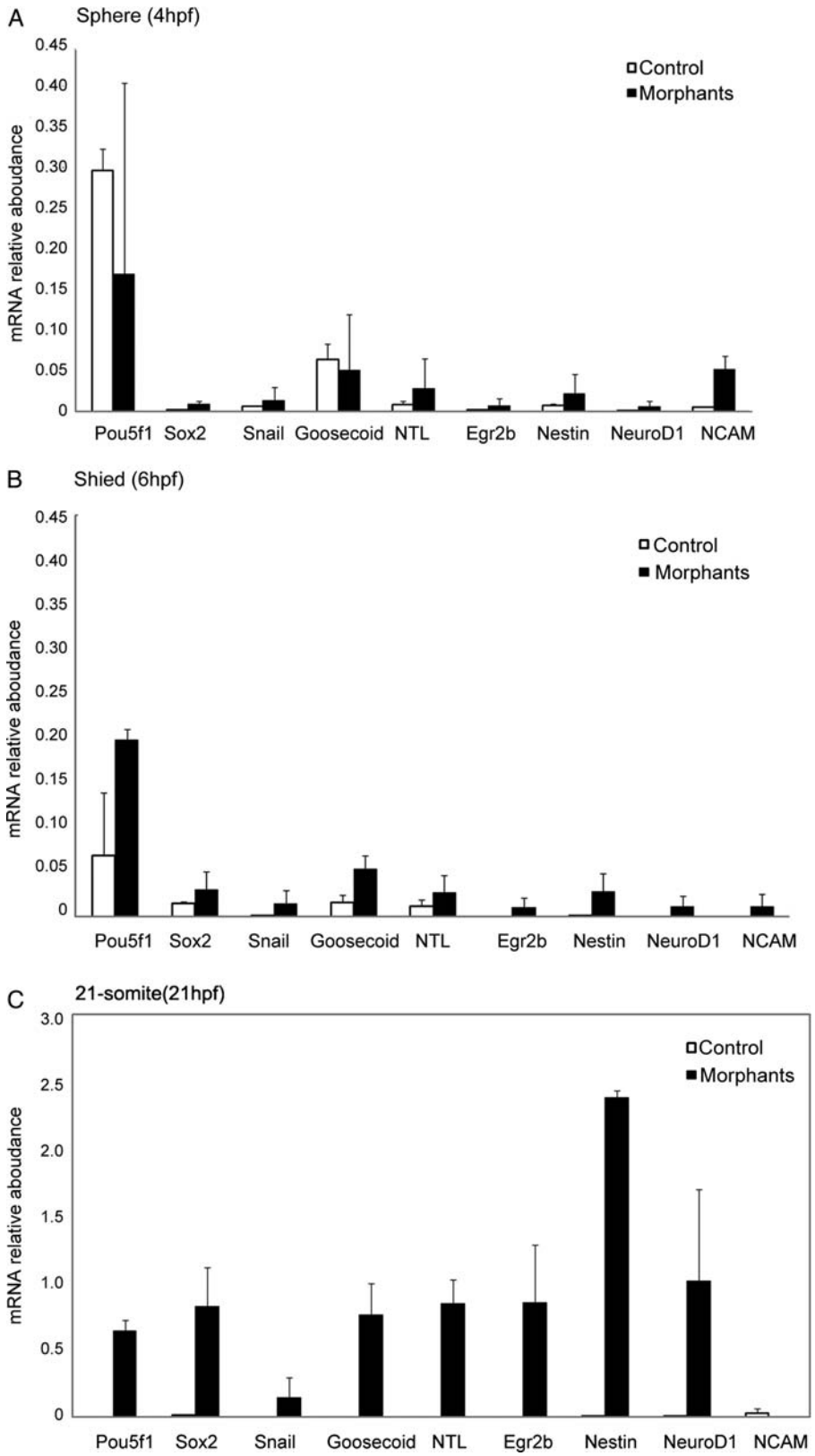

Figure 6 ESC, NSC, and neuron marker genes were examined by real-time PCR in both control and morphants groups (A) Sphere stage. (B) Shied stage. (C) 21-somite stage. It showed that transcripts of the following markers: Pou5f1 (ESC maker), Sox2 (the earliest neuroectoderm), Snail1 (late blastula marker), Goosecoid (shield marker), NTL (mesoderm marker), Egr2b (the neural plate marker), Nestin (NPC marker), and NeuroD1 (proneural marker), were significantly decreased from sphere to 21-somite stage in the control while increased in the morphants. However, the expression of NCAM (mature neuron marker) was the direct opposite, and even undetectable at 21-somite stage in the morphants.

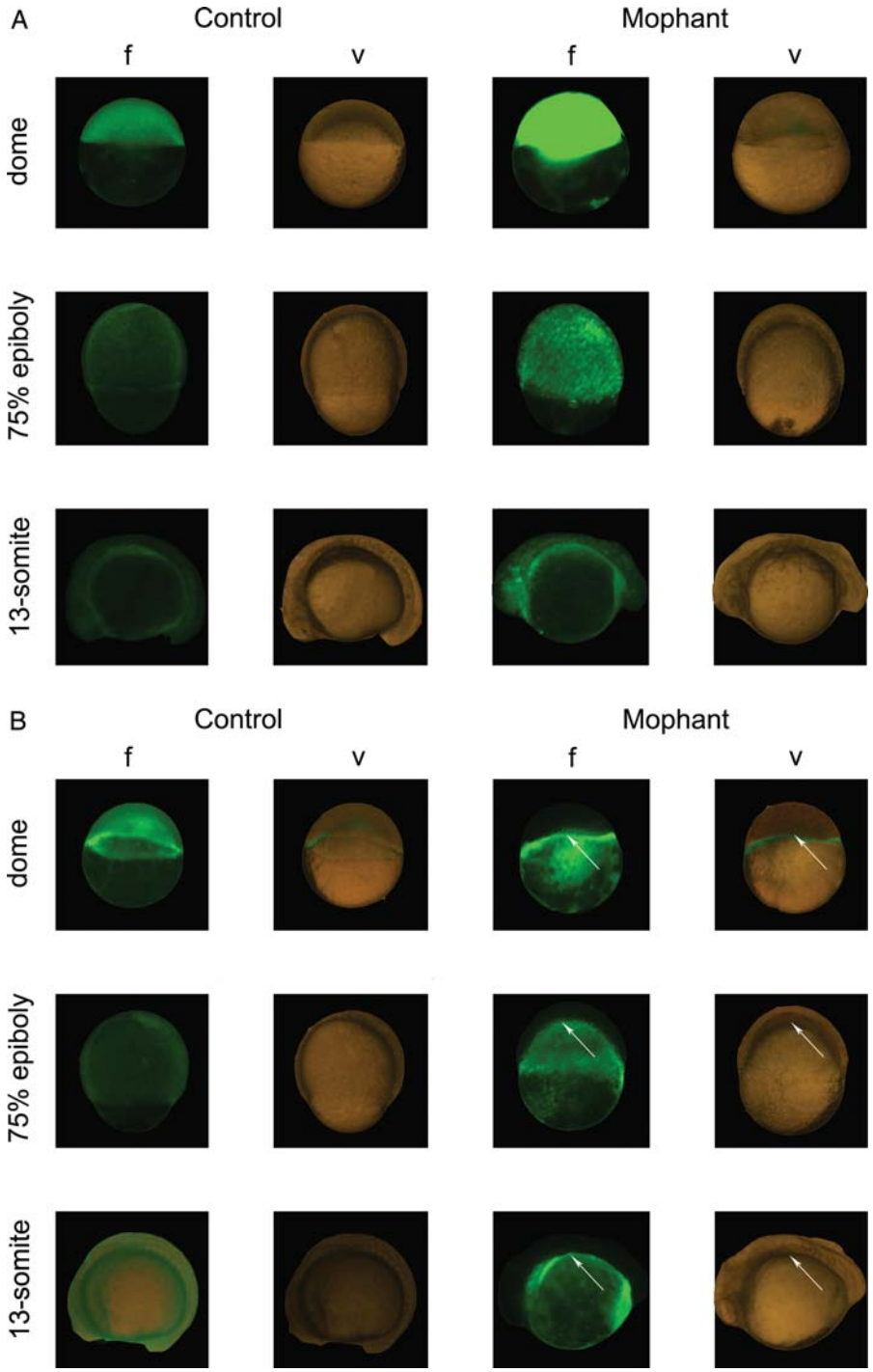

Figure 7 EGFP transportation test (A) EGFP proteins were injected into the yolk of MO-treated embryos at eight-cell stage. (B) EGFP proteins were injected into the yolk of MO-treated embryo at 3 hpf stage. MO-con used as control. Photographs were taken at dome stage (4.7 hpf), 75% epiboly (8h pf), and 13-somite (15.5 hpf) under fluorescent light (f) and visible light (v), respectively. The arrow indicates YSL.

RNAs were stored in the yolk. In metazoan, zygotic genome kept inactive and no RNA was transcripted for some time after fertilization. It is generally believed that most material with poor liposolubility or strong charge and polarity could hardly pass through membranes, and their transportation mainly depends on specific transport proteins or channel proteins located in YSL [17,28]. As a consequence of transportation blockage, blastula and gastrula became abnormal and result in malformation of embryos because of nutrients deficiency. Our results of REST knockdown experiment showed the importance of REST mRNA, especially the maternal ones, in early development of zebrafish embryos. To our knowledge, it is the first time to show that the REST plays a role in regulation of protein transportation from yolk in early embryonic developmental stages.

The present study is the first systematic investigation in expression and functions of REST for early embryonic development, which could not be carried out in mammalians due to embryonic lethality and technical difficulties. The high level of maternal REST mRNAs discovered by this study and the surprising finding that REST is involved in protein transportation regulation between the yolk and the embryo strongly suggest the importance of REST expression during early embryonic development besides its known roles in neurodifferentiation.

Acknowledgements

We thank Prof. Du (National Zebrafish Resource of China) for the zebrafish gifted. We also thank Prof. William Jia for his assistance in preparing the manuscript.

Funding

This work was supported by the grants from the National Basic Research Program of China (2010CB945500, 2011CB965103, 2011CB943804, and 2012CB944603); Science and Technology Commission of Shanghai Municipality (10140901400, 10410703800) and E-Institutes of Shanghai Municipal Education Commission (E03003).

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