Short Report

Stem Cell INVESTIGATION

The *Vox* mRNA and protein expression in zebrafish Pou5f3 MZ*spg* mutant embryos

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Abstract: The transcription factor of pluripotency Pou5f3 is considered to enhance the *Vox* expression. This conclusion was based on the study of mRNA expression, but the expression of the Vent-family proteins was not analyzed. We compare spatiotemporal distribution of the *Vox* and *Vent* mRNAs and the proteins in embryos of wild type zebrafish (WT) and MZ*spg* (spiel ohne grenzen) mutants devoid of both maternal and embryonic Pou5f3 functions. We revealed the *Vox* mRNA and its protein in both the WT and mutant embryos during the cleavage period. They were probably prestored maternally. The quantity of the prestored protein, unlike the mRNA, in the mutants was visibly less than that in the WT embryos. The Pou5f3, therefore, had no influence on the *Vox* mRNA maternal synthesis, but it affected the maternal Vox protein synthesis. During the blastula and gastrula periods the MZspg mutants, but not the WT, failed to synthesize the new *Vox* mRNA, while the prestored maternal mRNA was gradually degrading. At these stages the WT and mutant embryos displayed minor visual quantitative difference in staining of Vox protein. The *Vent* mRNA was not maternally prestored and its zygote synthesis slightly depended on the Pou5f3. The Vent protein in mutants and WT was synthesized on the new zygote mRNAs. By the gastrula period, the Vent staining of the WT and mutant embryos were almost comparable. The data obtained suggest the existence of mechanisms sustaining a required Vox and Vent proteins level, but these mechanisms are not directly dependent on the Pou5f3.

Keywords: Embryogenesis; MZspg; Pou5f3; Vent; Vox; zebrafish

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Introduction

Pluripotency is an ability of a cell to differentiate into any embryonic tissue. In vertebrata, the property of pluripotency is inherent for every cell of early embryos (at least up to gastrulation). All the pluripotent types of cells, including the embryonic stem cells (ESC), express the transcription factors: Pou5f3 (outdated Pou5f1/Oct4), Sox2 and Nanog, which play a principal role in maintaining the pluripotency. They are called "transcription factors of pluripotency". These key factors control the expression of thousands of genes in ESC (1-4). In zebrafish embryos, 12–15% of genes become active in quick and coordinated manner (5) at the time of zygotic genome activation (ZGA) beginning after 10 cell divisions. The role of genome global activators in zebrafish is performed by several transcription factors—homologues of the transcription factors of pluripotency—Pou5f3, SoxB1 and Nanog (6,7). Under their influence there are activated tissue-specific genes in different parts of embryo (4,8,9). In particular, it was shown that in the MZ*spg* (spiel ohne grenzen) mutants devoid of both maternal and embryonic Pou5f3 functions, the expression of 595 genes was almost halved (8). Pou5f3 in the embryo acts as an activator of gene expression determining ventral fates by direct activation of the *Vox* promoter, and this is a phylogenetically conserved mechanism. In wild type embryos, the Vox expression was enhanced by the Pou5f3, but it was partially suppressed in the mutants (10). However,

the gene expression was estimated only on the level of the mRNA synthesis. Here we investigated the expression of *Vox* and *Vent* mRNA and encoded proteins in the WT and mutant embryos.

Methods

Embryos, stages

Zebrafish (*Danio reria*) developmental stages were identified according to tables (11). We used the embryos during the Cleavage (8-64 cells), Blastula (sphere) and Gastrula (shield) periods.

Production of antibodies

Rabbit polyclonal antibodies against zebrafish Vox and Vent were produced by Almabion Company (Russia) using peptides pvldvqepekktrphvpc and skfsvewlsqsfhdqekc, respectively. Both antibodies were purified by affinity chromatography and conjugated with horseradish peroxidase. The same conjugate with rabbit polyclonal antibodies against human immunoglobulin (Imtek, Russia) was used in control experiments.

Whole-mount immunostaining of embryos and Western blotting

Embryos were fixed according to Klymkowsky lab manual (12) in MEMFA solution followed by 20% DMSO—80% methanol (Dent's fixative) and then whole-mount immunostaining was carried out according to standard procedure with slight modifications (13). Western-blot analyses were performed with single appropriate HRP-conjugated antibodies using SuperSignal Western Blot Enhancer Kit (Thermo Scientific, USA) according the manufacturer recommendations.

Whole-mount in situ hybridization

Whole-mount *in situ* hybridization was performed by a standard procedure (14). Plasmids pBS-SK carrying *Vox* cDNA and pCS2+ carrying *Vent* cDNA were used for synthesizing of digoxigenin-RNAs (Roche) by T3 RNA polymerase (Fermentas).

Results and discussion

Here we present our results on spatiotemporal distribution of the *Vox* and *Vent* mRNAs and proteins in zebrafish WT and MZ*spg*^{793} mutant embryos at early developmental stages (*Figure 1*). We suppose that the *Vox* mRNA revealed at the stage of 8-16 blastomeres in both WT and mutants had been maternally prestored, then after the ZGA the new-synthesized mRNAs appeared only in the WT. The MZ*spg* mutants failed to synthesize it *de novo* and the prestored molecules gradually degraded. Whole-mount immunostaining with the anti-Vox antibodies could not reveal any difference in staining of the WT and mutant embryos at the gastrula (shield stage) (*Figure 1E,F,K,L*).

These results were confirmed with the Western-blot analysis of protein extracts of the WT and mutant embryos (*Figure 2*).

At the stage of 8–16 blastomeres, there are seen several protein bands (theoretically calculated the Vox protein molecular mass is 28 kDa) recognized by the anti-Vox antibodies in the case of the WT, and only slight shadows on the track with the extract of the mutants. At the later stages (64 cells and sphere) the Western blotting revealed the increasing amount of the Vox protein in mutants. It became almost comparable with that in the WT. Hence, at these stages the Vox protein is actively synthesized on the disappearing mRNAs. Interestingly, the Vox protein migrated as a doublet. This situation had been already described (15). The ^{35}S-labeled proteins were translated *in vitro* from capped mRNA. The authors noted that Vox and Pou5f1 artifactually migrated in electrophoresis as a doublet. However, Lippok and coauthors (16) investigated zebrafish Pou5f1 posttranslational modifications, and found dynamic patterns of phosphorylation during development. Pou5f1 in Western blots showed several discrete bands. The higher molecular mass appeared to be predominantly caused by phosphorylation. During gastrulation stages, the higher molecular mass forms of Pou5f1 prevailed. The authors proposed the possibility that Pou5f1 function may get modulated posttranslationally by phosphorylation, and that embryonic signaling pathways may contribute globally or in a region-specific manner to control of Pou5f1 activity. We suppose the doublet Vox band not to be an artifact but, like that of Pou5f1, it shows differently modified forms.

The *Vent* mRNA whole-mount *in situ* hybridization analysis of the WT and mutant zebrafish embryos revealed no sufficient differences. At the stage of 8–16 blastomeres there was no *Vent* mRNA staining in either WT or MZ embryos (*Figure 1M,S*). Then it appeared as a result of the ZGA at the sphere and shield stages. Perhaps, it seems to be slightly weaker in MZ embryos (*Figure 1N,O,T,U*). The

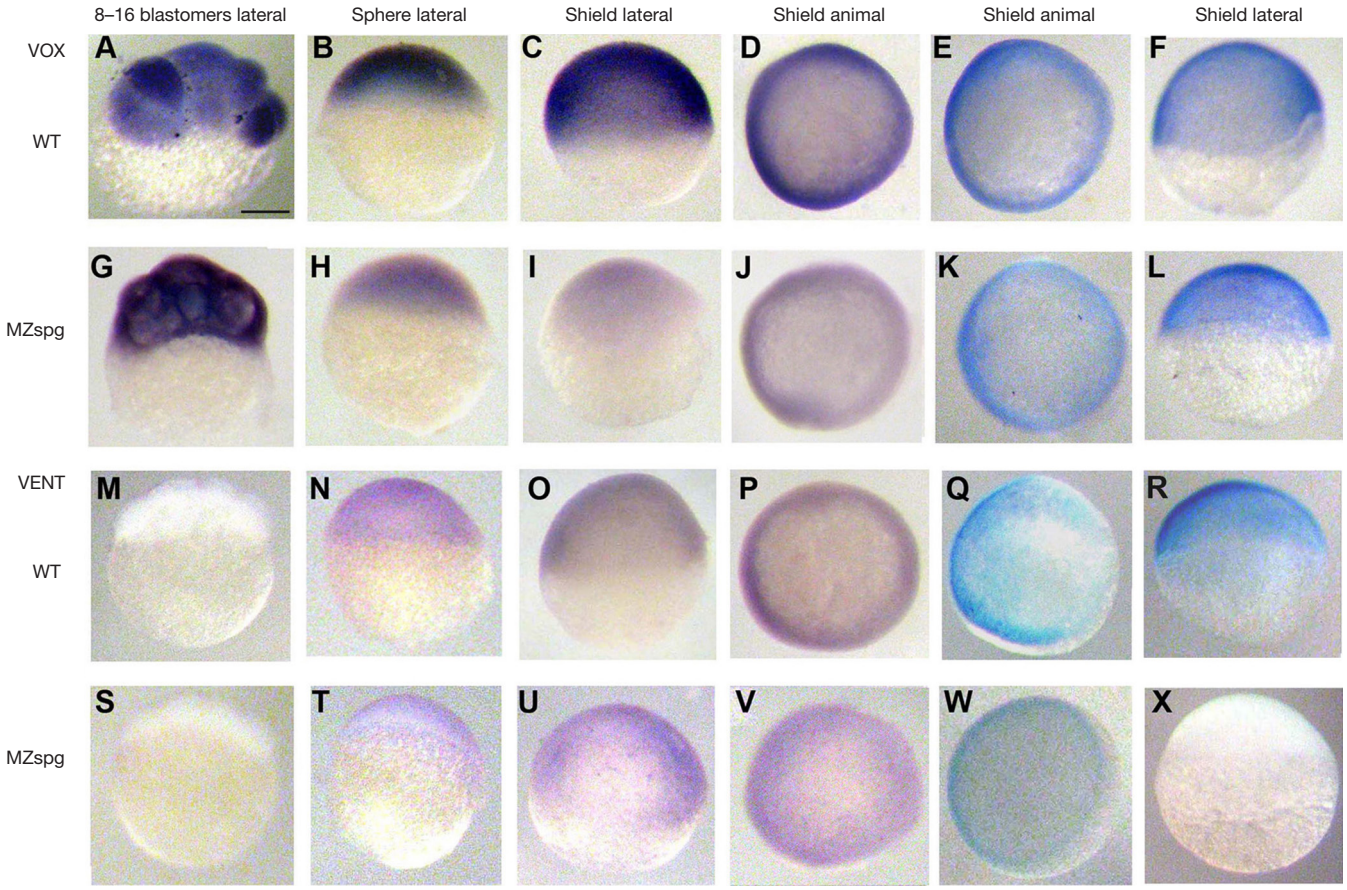

Figure 1 Expression of *Vox* and *Vent* mRNA and protein in the WT embryos and MZspg mutants. Spatial distribution of *Vox* mRNA (A-D and G-J), Vox protein (E, F, K, L), *Vent* mRNA (M-P and S-V) and Vent protein (Q-W) detected by whole-mount *in situ* hybridization and whole-mount immunostaining, respectively. (X) Control immunostaining of embryos by anti-Ig antibodies. Developmental stages are indicated above. WT, wild type, MZspg, maternal-zigotic mutant of *Pou5f3* gene. Scale bar: 200 μm.

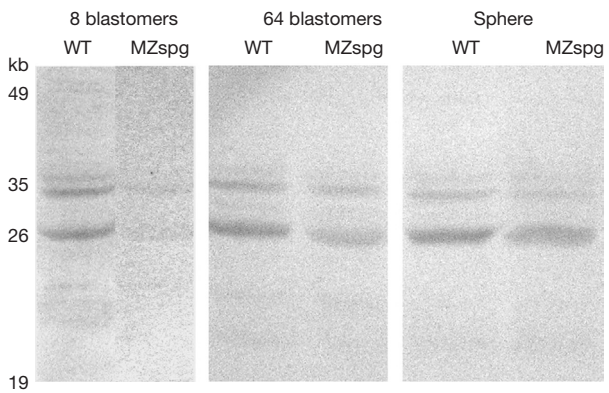

Figure 2 Western-blot of the extracts of WT and *MZspg* embryos with anti-Vox antibodies. Developmental stages are indicated above. The position of molecular weight markers is shown at the left. WT, wild type, *MZspg*, maternal-zigotic mutant of *Pou5f3* gene. 10 deyolked embryos per a track.

whole-mount immunostaining by the anti-Vent antibodies also could not reveal any difference in staining of the WT and mutant embryos (*Figure 1Q-W*). The Western blot with anti-Vent antibodies revealed faint Vent double bands in the WT and MZ (data not shown).

Summarizing the results obtained we can see that *Vox* mRNA is maternally stored and can be revealed at the 8-16 blastomeres stage in both WT and MZ embryos. After ZGA in the WT embryos the new *Vox* mRNA was synthesized. The MZspg mutants lacking Pou5f3 failed to synthesize the new *Vox* mRNA while the maternally prestored mRNA was degrading. Hence, the maternal synthesis of *Vox* mRNA did not depend on the Pou5f3 but the zygotic synthesis did. The Vox protein in WT seemed to be prestored or/and synthesized on the maternal and zygotic *Vox* mRNA. In MZspg mutant, Vox protein had not been prestored

maternally but was synthesized on the stored *Vox* mRNA. Actually, the amount of the protein does not depend directly on the amount of its mRNA. There are many factors affecting the rate of protein synthesis. Concerning the *Vent* mRNA, we can say it was not maternally prestored and then its synthesis slightly depended on the Pou5f3. This suggestion was confirmed by almost similar data of the whole-mount immunostaining of the WT and MZ*spg* embryos. Our results do not contradict with the idea of Belting and coauthors (10), who concluded that the zygotic *Vox* was a direct transcriptional target of Pou5f3, while *Vent* was upregulated to a lesser extent. Pou5f3 is not a single regulator of *Vent* family gens. It was also shown (17) that the zygotic synthesis of *Vox* and *Vent* mRNA were regulated by maternal Runx2, a transcription factor essential for bone formation. The data obtained suggest the existence of mechanism sustaining a required Vox and Vent proteins level, but this mechanism was not directly dependent on the Pou5f3. The regulation of translation, posttranslational modifications (16) and direct protein-protein interactions (15) may play a role in this process.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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