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Establishment of Transgenic *Xenopus Tropicalis* Line with EGFP Reporter Driven by S100Z Promote

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Abstract

S100Z, a member of S100 family proteins, is involved in a calcium-dependent control of several intracellular signaling pathways due to its helix-loop-helix EF-hand type structure which is highly conservative among family members. However, its expression patterns and functional importance in Xenopus tropicalis (X. tropicalis) remains largely unclear. To provide experimental animal model for explore the function of S100Z protein in X. tropicalis, a transgenic X. tropicalis line with EGFP reporter driven by s100z promoter was conducted in this study. About 5 kb promoter sequence upstream of TSS in s100z gene was cloned using the cDNA library from X. tropicalis heart tissue. The transgenic plasmid ps100z-EGFP was constructed by inserting the promoter sequence into the upstream of EGFP. In vitro data demonstrated that X. tropicalis s100z promoter is not a tissue and/or cell type specific promoter. In consistent with in vitro data, a Filial generation 0 of transgenic line (Tg: s100z-EGFP) constructed using microinjection showed that EGFP reporter gene systemically expresses in tadpoles. Taken together, this transgenic X. tropicalis line will provide a useful animal model to further explore the functional importance of s100z during development and related disease.

Keywords

S100Z; *Xenopus tropicalis*; Transgenic line.

1. Introduction

S100 calcium binding protein, an important protein in the organisms, can combine to Ca^{2+} and participate in cell signaling pathway and various life activities. S100 protein family is a low molecular weight protein consist of 25 member that have been reported [1]. Its function works due to the helix-loop-helix EF-hand type structure which is highly conservative among family members [2,3]. S100 family are involved in a calcium-dependent control of several intracellular signaling pathways [4] and closely related to many diseases. Many S100 family members show up-regulation or down-regulation in oral squamous cell carcinoma as compare with healthy control, indicating that it may be a diagnostic marker in OSCC [3]. S100A1 was showed to play important roles in the maintenance of normal myocardial function and related to heart failure and other heart diseases [5,6], as well as Kawasaki disease [9]. Recent study shows that some S100 protein showed the characteristics of tissue and cell type specific expression [2,7], while S100Z can be found in different expression of various tissue [2]. The crystal structure of Human S100Z and zebrafish S100Z is inconsistent [4,8]. However, many characteristics of S100Z, a member of S100 family, remains largely unknow..

Xenopus tropicalis (*X. tropicalis*) is a powerful animal model because of its following advantages including smaller sizes, shorter maturity cycle, diploid and higher homology with human [10-13]. *X. tropicalis* can spawn in large quantities at any time if it is experimental require and its whole body is transparent during the development to adult [10-12]. Therefore, X. tropicalis is extremely convenient

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for microinjection, transgenic construction and developmental biology studies. In the present study, about 5 kb promoter sequence in *X. tropicalis* s100z gene was amplified to construct transgenic plasmid (ps100z-EGFP). Using this transgenic plasmid, a transgenic *X. tropicalis* line (Tg: s100z-EGFP) was established through embryo microinjection. This line will provide a powerful animal model to further explore the function of s100z during development and in related disease.

2. Materials and methods

2.1 Experimental Animals

X. tropicalis used in this article raise in 24 hours circular water system with 14/10 light cycle at room temperature 25℃. The animal use and care protocols for these experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Jinan University, Guangzhou, China.

2.2 Cloning of s100z promoter and plasmids construction

The s100z promoter from wild type X. tropicalis' genome was cloned into pEASY-Blunt tool plasmid (TransGen). The promoter sequence was then subcloned into pFSP1B-EGFP plasmid (our lab) to replace FSP1B promoter in the upstream of EGFP. All primers are listed in Table 1.

Table 1. PCR primer sequence

Primer name	Sequence（5'-3'）	Product length (bp)
Ts100z-F	TGATTCCCCTACTGTGTCACTTTG	5150
Ts100z-R	GCTTGTATTTATCCCCTTCTTTGC	
ps100z-F	CACTATAGGGCGAATTGGGTACCTGATTCCCCTACTGTGTCACTTTG	5065
ps100z-R	TCGCCCTTGCTCACCATATCGATGGTGATCAAAGGGTACCTGGCATG	

2.3 Mammalian cell culture and transfect with expression vector

The vector expression experiments were performed in HEK 293T cell line and NIH 3T3 cell line (Laboratory Animal Center of Sun Yat-sen University). Cells were cultured in Dulbecco's Modified Eagle Medium with high glucose (4.5 g/L, GBICO) and supplemented with 10% fetal bovine serum (GBICO) and 1× penicillin–streptomycin (Thermo Fisher Scientific). Cell were plated into 24-well plates at 10^5 per well and cultured overnight. Lipofectamine 3000 (Thermo Fisher Scientific) were performed with expression vector. For each well, 500 ng plasmids and 1 μL P3000 were diluted with Opti-MEM I Reduced Serum Medium (GBICO) to a total of 25 μL, and 1.5 μL Lipofectamine 3000 diluted with 23.5 μL Opti-MEM in different tube. Stand for 5 min，then mixed them and incubate for 15 min. After that, slowly pipetted it onto cells and jiggle it gently.

2.4 *In vitro* fertilized egg preparation and microinjection

Twelve hours before experiment, 20 U Human Chorionic Gonadotropin (HCG, Beijing Tigsun diagnostics CO. Ltd) per frog were injected subcutaneously into a male and a female *X. tropicalis*, and raise overnight at 25℃, respectively. Three hours before microinjection, 180 U HCG were subcutaneous injected into *X. tropicalis* again, and put them into a plastic box containing a half volume of 0.1× MBS (5× MBS: 25.90 g NaCl, 11.90 g Hepes, 1.00 g $NaHCO_3$, 0.40 g KCl, 0.50 g $MgSO_4$, 0.80 g $Ca(NO_3)_2$, 0.20 g $CaCl_2$ dissolved in 1 L ultrapure water and adjust pH to 7.4, all of compounds is from Sigma unless otherwise noted). Fertilized eggs were collected into a beaker, 3% L-Cysteine hydrochloride (6.00 g L-Cysteine hydrochloride dissolved in 200 mL ultrapure water and adjust pH to 8.0) were put into with sufficient shaking 3 min. Then rinse eggs 6-8 times with 0.1× MBS to remove membrane of eggs. Plasmid preparation for microinjection: 100 ng plasmid, 2 μL I-SceI, 1 μL CutSmart Buffer were diluted with ultrapure water to a total of 10 μL (NEB), incubate in

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37℃ for 30min then centrifugate with 13,000 rpm for 3 min [17]. 20 pg linear plasmids were injected into each one-cell-stages' egg. After microinjection, fertilized eggs will raise in 0.1× MBS [14].

3. Results.

3.1 Analysis of s100z protein homology among different species

To understand the relationship of S100Z protein between *X. tropicalis* and other species, we compare amino acid (AA) sequence of S100Z with *X. tropicalis* and other species. Through sequences comparison and phylogenetic tree analysis, we can find that there are 97% similarity of protein encoding between *X. tropicalis* and *X. laevis*. Their EF-hand functional region (AA 28-76) are absolutely consistent while about 80% similarity between *X. tropicalis* and others species (Fig. 1), indicating that S100Z protein's function may high conservation during evolution.

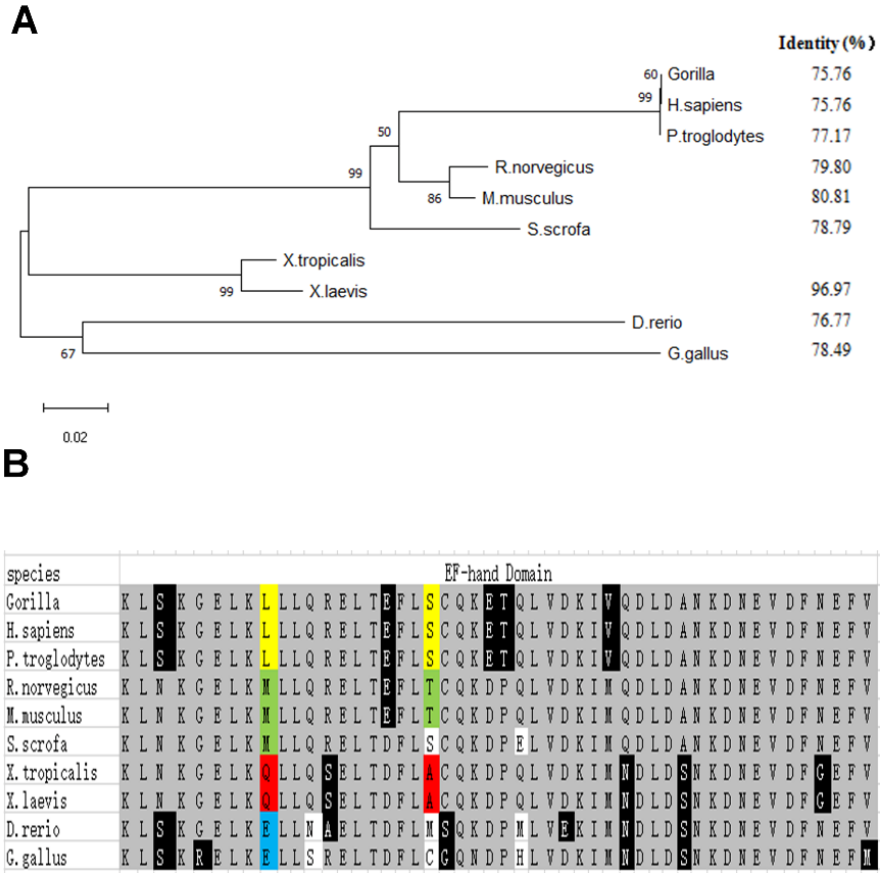

Figure 1. Homology analysis of S100Z protein among different species.

A, Left: the phylogenetic tree analysis of S100Z protein among species, right: identity of *X. tropicalis* between others species. B, EF-hand domain of S100Z protein sequence alignment among species. The identical amino acid sequences of all species are marked with gray shading, others are marked with different pigments.

3.2 Construction of transgenic plasmid

To explore the expression features of s100z protein, we design an expression vector using s100z promoter to drive enhance green fluorescence protein (EGFP) which can monitor the expression of s100z protein. Using the specific primer pairs, a 5 kb promoter sequence upstream TSS in s100z gene was amplified from *X. tropicalis* genomic DNA library (Fig. 2A). To construct transgenic plasmid, the s100z promoter was subcloned into pFSP1B-EGFP plasmid to replace mFSP1B promoter. Thus, the transgenic plasmid ps100z-EGFP was constructed in which the reporter gene EGFP was driven by s100z promoter (Fig. 2B). DNA sequencing using the EGFP-N sequencing primer further confirmed that the construction of ps100z-EGFP plasmid is successful (Fig. 2C).

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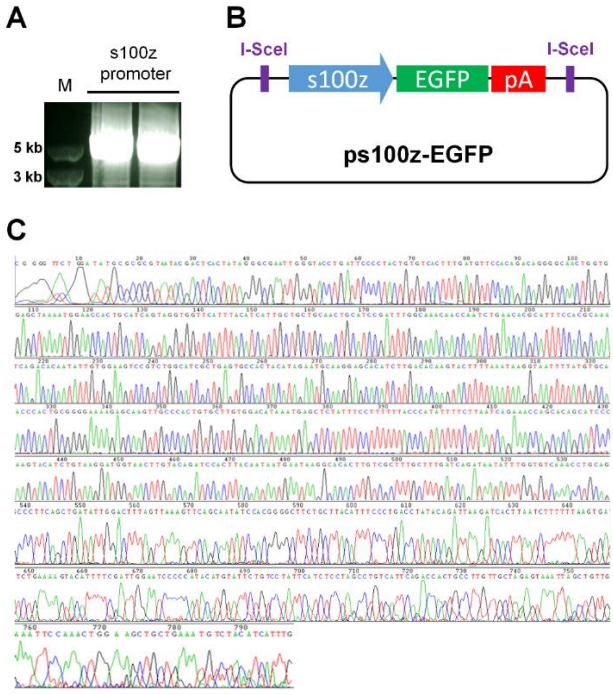

Figure 2. Construction of transgenic plasmid.

A, Gel electrophoresis of PCR production of *X. tropicalis* s100z promoter. B, Schematic of transgenic plasmid ps100z-EGFP. C, DNA sequencing peak map of ps100z-EGFP plasmid using EGFP-N sequencing primer.

3.3 Transfection of ps100z-EGFP plasmid in mammal cell lines

In order to verify EGFP can be absolutely expressed by the plasmid and explore the expression pattern of s100z promoter in vitro, we design a simple cell transfection experiment. Two plasmids: ps100z-EGFP and pEGFP-N1 (CMV promoter, as a positive control) were transfected into HEK 293T and NIH 3T3 Cell, respectively. 24 hours post transfection, we can find that both of two plasmids can express EGFP in two cell line perfectly, indicate that s100z promoter we cloned retained functional integrity (Fig. 3). These results also indicate that the activation of *X. tropicalis* s100z promoter does not depend on cell types.

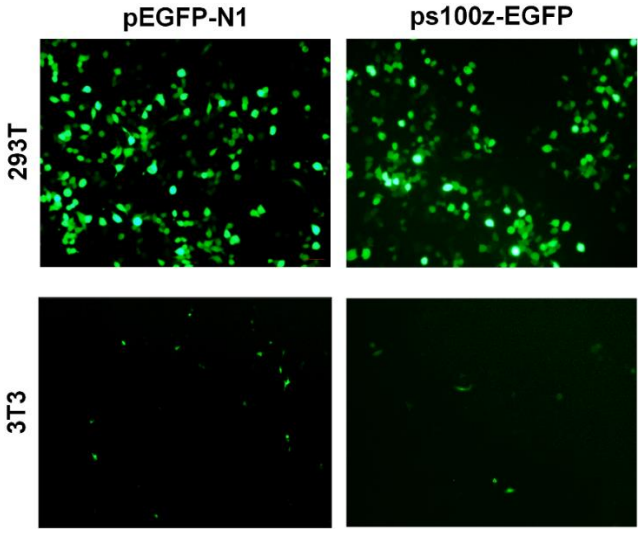

Figure 3. *In vitro* expression of the ps100z-EGFP plasmid.

Both 293T and 3T3 cell lines were transfected with ps100z-EGFP plasmid for 24 hours. pEGFP-N1 plasmid was used as positive control. Upper panel denotes 293T cells transfected with ps100z-EGFP plasmid. Lower panel denotes 3T3 cells transfected with ps100z-EGFP 2plasmid. Scale bars = 50 μm.

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3.4 Establishment of transgenic *X. tropicalis* line

After *in vitro* confirmation of the transgenic plasmid, we construct the transgenic line of *X. tropicalis* (Tg: s100z-EGFP) via fertilized ovum microinjection. We can observe that small amount of green fluorescence in the embryo at 24 hours post microinjection (Fig. 4A). In addition to abdominal spontaneous fluorescence, there are some green fluorescence are observed at tadpoles' head, chest, spine or tail 4 days post microinjection (Fig. 4B). Moreover, a few tadpoles showed systemic expression of EGFP (Fig. 4B, upper panel).

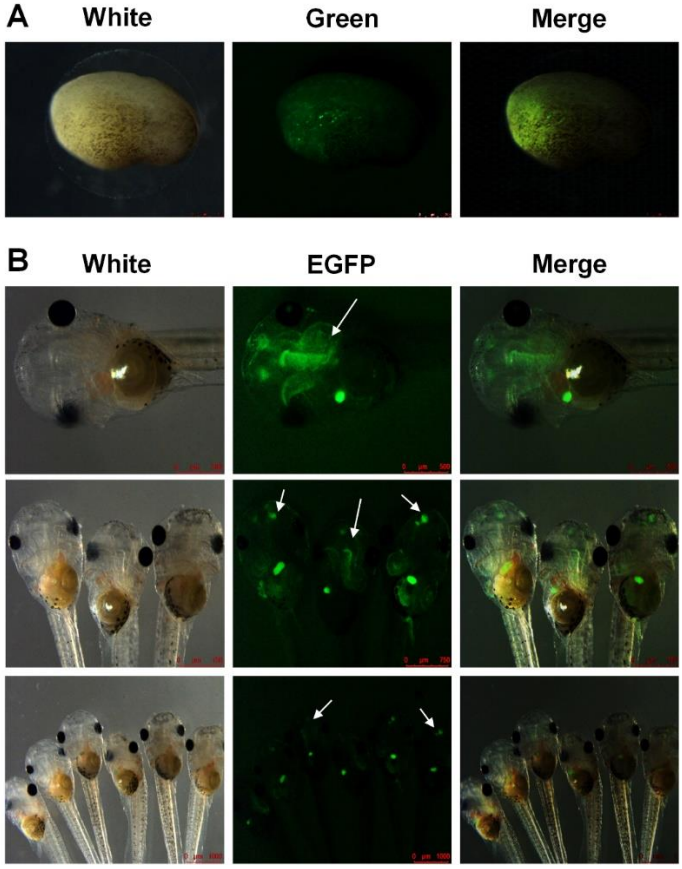

Figure 4. Establishment of transgenic *X. tropicalis* line (Tg: s100z-EGFP). The transgenic plasmid ps100z-EGFP was injected into the fertilized embryos at 1 cell stage. EGFP expression was then captured at indicated time points.

A, EGFP expression in embryo 24 hours post microinjection. Scale bars = 250 μm. B, EGFP expression in tadpoles 4 days post microinjection. Scale bars = 500, 750, or 1,000 μm.

4. Discussion

In the present study, we have compared *X. tropicalis* S100Z protein sequence with others species and found that the EF-hand type structure region is highly conservation in different species during evolution, indicating that S100Z protein function is similarly in various species. In addition, through the phylogenetic tree analysis, we find that zebrafish and human were not on a same root while *X. tropicalis* was, confirmed the previous research that the crystal structure of Human S100Z and zebrafish S100Z is inconsistent [4,8] and supposed that *X. tropicalis* S100Z protein is more similar with human than zebrafish.

To explore the s100z promoter's expression pattern, we design an expression plasmid using *X. tropicalis* s100z promoter to promote EGFP expression. We have tested the plasmid function *in vitro* and *in vivo*. For *in vitro* experiment, we transfected the plasmid into 293T and 3T3 cells separately. The results suggest that *X. tropicalis* s100z promoter can promote EGFP expression in two cell lines, indicating that *X. tropicalis* s100z promoter is not a tissue and/or cell type specific promoter, which

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is consistent with humans S100Z protein's expression pattern [2]. For *in vivo* experiment via microinjection, we found that EGFP rarely express at *X. tropicalis* embryos and increased during developing. It may suggest that S100Z may play a role in developing. The transgenic tadpoles showed a variety of EGFP expression feature due to the Filial generation 0 of transgenic line is chimera when using I-SceI enzyme, the s100z expression pattern is not necessarily consistent with wild type *X. tropicalis* [15-17]. Despite all this, we could find some tadpoles shows systemic EGFP expression, which is consistent with our *in vitro* results and the recent study [2]. When tadpoles grow into adult, they can be mated with wild type *X. tropicalis* to generate a hereditary transgenic line (Tg: s100z-EGFP) which will be an important experimental animal model for explore S100Z protein's expression patterns and functional importance in *X. tropicalis*.

Acknowledgments

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