Generation and Confirmation of Human TAL2 Transgenic Drosophila Model

Zhihao Wei [1], Hongchao Liu [1,*], Yajun Yang [2], Mengting Liu [2]

[1] Department of Pathology, The Yiluo Hospital of Luoyang, The Teaching Hospital of Henan University of Science and Technology, Luoyang, China

[2] Department of Neurology, The Yiluo Hospital of Luoyang, The Teaching Hospital of Henan University of Science and Technology, Luoyang, China

Abstract. TAL2 is a transcription factor of the basic helix-loop-helix (bHLH) family. *TAL2* may be an oncogene that is overexpressed as a consequence of the chromosomal translocation in T-cell acute lymphoblastic leukemia. Recent studies have indicated that TAL2 involved in development of many tumors. Although aberrant TAL2 expression was associated with tumorigenicity, TAL2 has been shown to play a crucial role in normal brain development. However, its role in neural development is unknown. In this study, molecular cloning and microinjection technology were used to construct UAS-*TAL2* transgenic *Drosophila* model. The coding sequence of human *TAL2* gene was achieved by gene synthetic technology and subcloned into pUAST expression vector. The pUAS-*TAL2* recombinant vector was microinjected into the embryos of wild-type flies. The UAS-*TAL2* transgenic flies with red eyes were screened out with *mini-white* marker, then the balancing and mapping of these strains were conducted. The integration of transgene into fly genome was confirmed through PCR amplification and the expression of *TAL2* gene was examined by quantitative PCR. The results showed that pUAS-*TAL2* recombinant plasmid was successfully constructed, and four independent transgenic strains were obtained after the screening and balancing. PCR analysis showed that the P[*mini-white*, UAS-*TAL2*] had integrated into genomes of transgenic strains and could express under regulations. The expression level of *TAL2* gene in adult brains was upregulated significantly under elav-GAL4 driver. In conclusion, the UAS-*TAL2* transgenic *Drosophila* model has been successfully generated, which lays the foundation for further studies of the function and regulation of human *TAL2* gene in the neural development with GAL4/UAS system.

Keywords: *TAL2* gene; neural development; overexpression; transgenic *Drosophila*; GAL4/UAS system.

1. Introduction

The basic helix-loop-helix (bHLH) protein family is widely distributed in the biological community with high conservation[1-3]. Studies have shown that most of the known bHLH proteins act as transcription factors, which regulate the cell process by forming homodimers or heterodimers with other proteins, and then activating or inhibiting genes related to differentiation and proliferation[4-6]. bHLH family plays an important role in various developmental processes, including the development of skeletal muscle, pancreas, hematogenesis, neural stem cell differentiation, and the development of vertebrate spinal cord and telencephalon cortex[7-11].

TAL2 is a transcription factor of the bHLH family. The oncogene *TAL2* was overexpressed at the junctions of t(7;9)(q34;q32)chromosomal translocations associated with T-cell acute lymphoblastic leukemia (ALL)[12-14]. Recent studies have indicated that TAL2 involved in development and poor prognosis in many tumors, such as TAL2 was overexpressed in lung adenocarcinoma tumor tissues and conversely, the expression was significantly downregulated in ovarian carcinoma tissue than that in normal tissues[15-17].

Although aberrant TAL2 expression was associated with tumorigenicity, TAL2 has been shown to play a crucial role in normal brain development. Tal2 is expressed during embryogenesis and tissues of Tal2 expression include specific regions of the mesencephalon, diencephalon, and metencephalon[18]. Tal2-null mutant mice are viable at birth. However, targeted disruption of the Tal2 locus leads to dysgenesis of the midbrain tectum and to hydrocephaly, which ultimately causes the death within 32 days after birth without this gene[19]. In zebrafish embryos, tal2 is strongly

expressed in the mesencephalon and diencephalon. However, in addition to this conserved brain expression, tal2 expression was also detected in the floor plate of the spinal cord[20]. These findings indicate that TAL2 plays a pivotal role in development of the central nervous system; nevertheless its role in neural development remains unknown.

The GAL4/UAS expression system uses tissue-specific promoters or enhancers to activate the expression of yeast transcriptional activating factor (GAL4), and then the GAL4 protein specifically binds the upstream activating sequences (UAS) fused with the target gene[21]. GAL4/UAS is an ideal tool for studying gene function because it controls the spatiotemporal specificity of target gene expression[22, 23]. In this study, molecular cloning and microinjection technology were used to construct UAS-*TAL2* transgenic *Drosophila* model, which laid a foundation for overexpression of *TAL2* gene with GAL4/UAS system and further studies of the function and regulatory mechanism of *TAL2* gene in neurodevelopment.

2. Materials and methods

2.1 Materials

The wild-type W1118, elav-GAL4, and B1/CyO; TM2/TM6B strains and pUAST expression vector 21 were preserved in our laboratory. Trizol reagent were purchased from Invitrogen. The restriction enzymes Xho I and EcoR I, T4 DNA ligase, DNA molecular weight marker, high-fidelity PCR amplification kit, PrimeScript reverse transcriptase kit, and SYBR Premix Ex Taq II kit were provided by TaKaRa company. DH5α competent cells, DNA gel recovery kit, plasmid DNA extraction kit were obtained from the CWBIO company. High purity plasmid extraction maxi-prep kit was purchased from Qiagen and Halohydrocarbon oil 700 from Sigma. All other chemical reagents used were of analytical grade.

2.2 Construction of pUAS-TAL2 Recombinant Plasmid

The coding sequence of human *TAL2* gene was synthesized by GENEWIZ Biotech company, and the HA-tag sequence TACCCATACGATGTTCCTGACTATGCG was introduced before the termination codon TAG. At the same time, enzyme sites *EcoR* I and *Xho* I were added to the 5’ and 3’ ends of the synthetic fragment respectively. The synthetic products with high quality and pUAST expression plasmid were respectively digested with *EcoR* I and *Xho* I at 37°C for 2 h and the digested products were separated by 1% agarose gel electrophoresis. Then the target fragments were recovered via gel extraction kit and ligated with T4 ligase at 20 °C for 4 h to generate a recombinant vector pUAS-*TAL2*. The ligated products were transformed into DH5α competent cells by heat shock method, then smeared on a lysogeny broth (LB)/ampicillin (AMP) plate, and cultured at 37°C overnight. Colonies were picked and grown in 2 ml LB/AMP media with shaking at 200 rpm for 2 h, then colony PCR was preformed with bacterial culture as a template. The objective colonies were transferred to a conical flask, and cultured in LB/AMP overnight at 37°C. The plasmids were extracted by a plasmid extraction kit and verified by double enzyme digestion. Finally, the recombinant plasmid was confirmed by DNA sequencing (GENEWIZ). After confirmation by sequencing, the correct pUAS-*TAL2* plasmid with high concentration was purified by using plasmid extraction maxi-prep kit, which was used for embryo microinjection.

2.3 Embryo Microinjection

Two days prior to embryo collections, about 200 W^{1118} fruit flies were transferred to collection cages, supplied with grape-juice agar plates coated with yeast paste for adaptation. On the third day, embryos were collected on fresh grape juice plates at 25°C for 40 minutes in the dark. At room temperature, embryos were dechorionated with 50% bleach for 2 minutes, and rinsed thoroughly with ddH_2O[24]. Then the eggs were glued on the slide by double-sided adhesive and covered with halohydrocarbon oil 700 to keep moisture. Under an inverted microscope, microinjection needle was inserted into the polar cells at the tail end of the embryos, then the needle was rapidly

withdrawn after the pressure was released[25]. The preparation of microinjection included 25 μg recombinant plasmid pUAS-*TAL2*, 5 μg helper plasmid Δ2-3 and 50 μl buffer for injection.

2.4 Screening and Balance of Transgenic Drosophila

After microinjection, embryos were cultured on agar media at 18℃, then the larvae were picked up between 36-72 hours and transferred to cornmeal media. They were raised at 18℃ for 3-4 days, then transferred to 25℃ for another 5 days. In this period, the eclosion male flies and virgin flies were picked out in time and crossed with the virgin flies and male flies of W^{1118} strain respectively. After hybridization, their offspring were kept at 25℃ and observed after about 10 days. The color of compound eyes changed to red due to the expression of *mini-white* marker gene. Therefore, we can infer whether the transgene was integrated into the genome of fly by observing the color of compound eyes of offspring. The selected UAS-*TAL2* transgenic *Drosophila* was crossed with B1/CyO; TM2/TM6B double balancer, and a stable transgenic strain was obtained after location and balance according to the process shown in Figure 1.

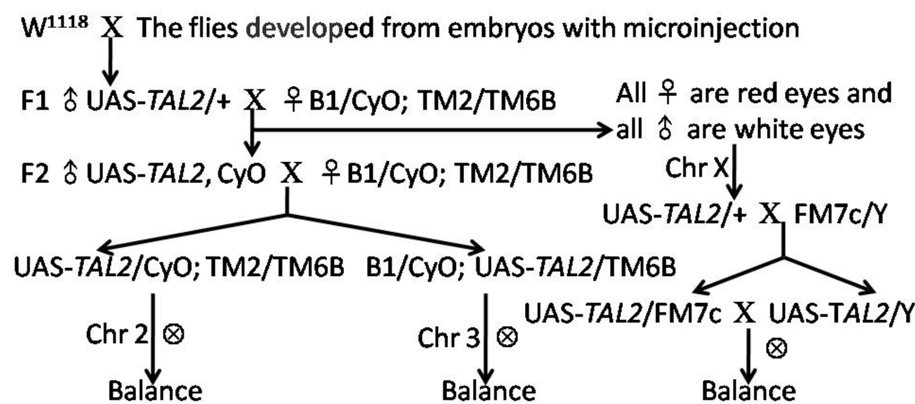

Fig. 1 Screening and balance of UAS-*TAL2* transgenic *Drosophila*

2.5 PCR Validation of Transgenic Drosophila

The genomic DNA of transgenic *Drosophila* and W^{1118} flies were extracted as the reaction template respectively. The PCR validation was conducted by using a universal primer pair of pUAST plasmid with forward primer GCTTCGTCTACGGAGCGACAATTCAATTCAAAC and reverse primer GCAGTAGCCTCATCATCACTAGATGGCATTTCTTC, which was synthesized by GENEWIZ company. PCR reaction system included 10 × buffer (containing Mg^{2+}) 5μL, dNTP mixture (2.5 mmol/L) 4 μL, forward primer (10 μmol/L) 1μL, reverse primer (10 μmol/L) 1μL, template (150 ng/μL) 1μL, DNA polymerase (5 U/μL) 0.25μL, and sterile distilled water 37.75 μL. The PCR amplification procedure was 94 ℃ for 4 min; 94 ℃ for 30 s, 60 ℃ for 30 s, 72 ℃ for 1 min, 35 cycles; 72 ℃ for 8 min. PCR product was detected by gel electrophoresis and purified by gel recovery kit, and then sent to GENEWIZ company for sequencing.

2.6 The Expression of TAL2 Detected by Quantitative PCR

The *TAL2* gene was selectively overexpressed in the nervous system when UAS-*TAL2* transgenic *Drosophila* crossed with elav-GAL4 strain[26]. 50 adults of UAS-*TAL2* and elav-GAL4/UAS-*TAL2* strains were selected respectively. The brain tissue was cut off and immediately washed in cold PBS. The total RNA of adult brain was extracted according to the instruction of Trizol reagent and stored at -80℃. Nanodrop 2000 was used to detect the concentration and purity of RNA samples and 1% agarose gel electrophoresis was preformed to evaluate the integrity. The first strand cDNA was synthesized according to the instruction of PrimeScript reverse transcription kit, then the cDNA reaction solution was used as a template for subsequent quantitative PCR following the instruction of SYBR Premix Ex Taq II kit. The reaction system consisted of SYBR Premix (2×) 12.5 μL, template 2 μL, upstream and downstream primers 0.5 μL respectively, adding sterile distilled water to 25 μL. The primers were synthesized by TaKaRa company with the sequence *TAL2*-F：

AAAGCCTGCAACAAACGGGA, *TAL2*-R: TGGACCAGGTGAAGGAACCT; *rp49*-F: CGGTTACGGATCGAACAAGC, *rp49*-R: CTTGCGCTTCTTGGAGGAGA. The reaction procedure was 95 ℃ 30s, 40 cycles: 95 ℃ 5S, 60 ℃ 30s. *Rp49* gene was used as internal reference to evaluate the relative expression level of *TAL2* gene by $2^{-\triangle\triangle Ct}$ method[27]. Three replicates were made for each sample, and three multiple pores were set for each reaction.

2.7 Statistical Analysis

SPSS17.0 software was used for statistical analysis, and the measurement data was expressed as $\bar{x} \pm s$. The one-way ANOVA followed by Tukey's test was used to analyze the between-group differences. The test level was α = 0.05, and $P<.05$ was considered statistically significant.

3. Results

3.1 pUAS-TAL2 Recombinant Plasmid Was Constructed

The coding sequence of *TAL2* gene was synthesized and *EcoR* I and *Xho* I digestion sites were added at both ends (Figure 2). The synthesized product was inserted into pUAST vector at *EcoR* I / *Xho* I restriction sites. The plasmid DNA extracted from positive colony was analyzed by enzymes digestion and results of gel electrophoresis showed that the target fragment with 360 bp was obtained after recombinant plasmid was digested with the same restriction enzymes used for subcloning (Figure 3). Furthermore, no point mutation or frame shift mutation was found in the DNA sequencing results compared with the database.

GAATTC*ATGACCAGGAAGATCTTCACAAATACCAGGGAGCGGTGGAGGCAGC*

AGAATGTCAACAGCGCCTTTGCCAAGCTGAGGAAGCTCATCCCCACTCACCCT

CCAGACAAAAAGCTGAGCAAAAATGAAACGCTTCGCCTGGCAATGAGGTATA

TCAACTTCTTGGTCAAGGTCTTGGGGGAGCAAAGCCTGCAACAAACGGGAGT

GGCTGCTCAGGGGAACATTCTGGGGCTCTTCCCTCAAGGACCCCACCTGCCAG

GCCTGGAGGACAGAACTCTGCTTGAGAACTACCAGGTTCCTTCACCTGGTCCA

*AGCCACCACATTCCT*TACCCATACGATGTTCCTGACTATGCG*TAG*CTCGAG

Fig. 2 The nucleotide sequence of synthesized human *TAL2* gene

5' EcoR I highlighted in Yellow, 3' Xho I highlighted in Blue, Initiation codon ATG highlighted in Green, Termination codon TAG highlighted in Red, Open reading frame of *TAL2* highlighted in Italics, HA-tag sequence highlighted in Pink

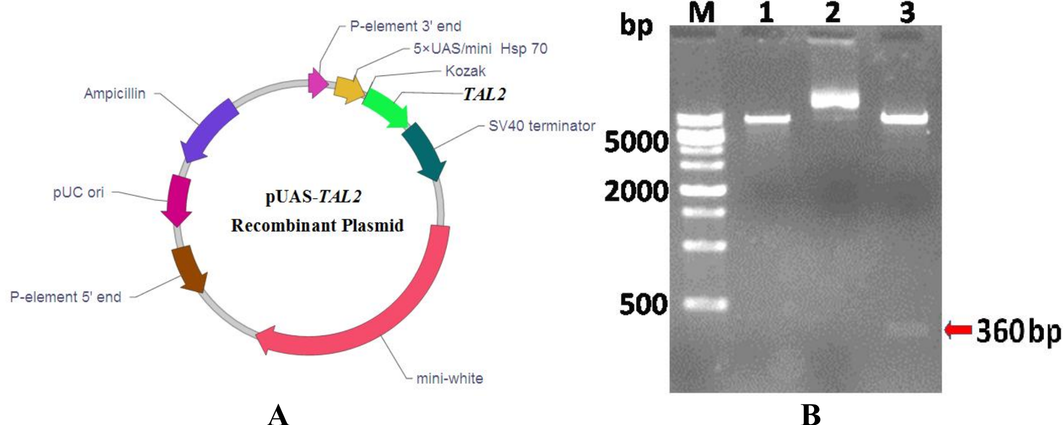

Fig. 3 Identification of pUAS-*TAL2* recombinant plasmid by enzyme digestion

A: Schematic diagram of pUAS-*TAL2* recombinant plasmid. B: Enzyme digestion analysis .M: DNA marker; Lane 1: pUAST vector digested with *EcoR* I and *Xho* I; Lane 2: Recombinant plasmid undigested; Lane 3: Recombinant plasmid digested with *EcoR* I and *Xho* I

3.2 Screening and Balance of Transgenic Drosophila Model

In this experiment, about 300 W^{1118} strain embryos were microinjected, about 30% of them hatched into larvae, and finally 36 developed into adults. According to whether compound eyes turned red or not, four transgenic strains were screened out from their offspring. After cross with double balancer to locate and balance, the results showed that P[*mini-white*, UAS-*TAL2*] of four transgenic strains were integrated into chromosome 3, and stable genetic stocks were established after purification.

3.3 PCR Verification of Transgenic Drosophila Model

The genomic DNA of W1118 and transgenic strains, as well as pUAS-TAL2 plasmid were used as template for PCR amplification. The target fragment with 861 bp was observed with the genome of transgenic Drosophila and recombinant plasmid as the template (Figure 4). Further sequence analysis showed that the PCR product contained the coding sequence of TAL2 gene, and no point mutation and frame shift mutation were found.

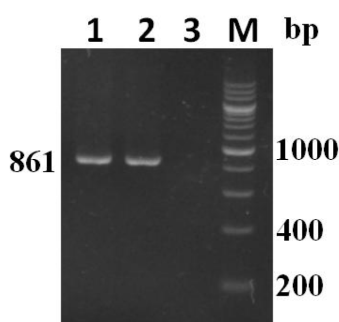

Fig. 4 PCR verification of UAS-*TAL2* transgenic *Drosophila* model
M: DNA marker; Lane 1: transgenic *Drosophila*; Lane 2: pUAS-*TAL2* recombinant plasmid; Lane 3: W^{1118} strain

3.4 The Expression of TAL2 Gene in Nervous System

The total RNA of adult brain tissue of UAS-TAL2 and elav-GAL4/UAS-TAL2 strain was extracted. The concentrations were 130.01 ng/μl and 117.10 ng/μl with OD260/280 2.03 and 1.98 respectively. The results of agarose gel electrophoresis showed that total RNA samples were intact without degradation and genomic DNA incorporation. Therefore, the extracted total RNA samples can be used for subsequent experiments. First, reverse transcription PCR was used to detect the specificity of the primer pairs. After amplification with primers for target gene TAL2 and internal reference gene rp49, it was found that the PCR products had no primer dimer or non-specific heteroband. Then the relative expression level of TAL2 gene was detected by real-time quantitative PCR. Compared with UAS-TAL2, the expression level of TAL2 gene in the brain of elav-GAL4/UAS-TAL2 flies was significantly increased (Figure 5).

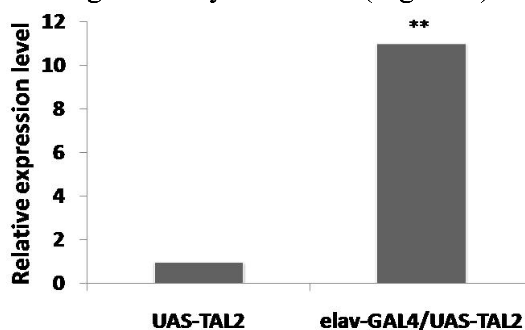

Fig. 5 The relative expression level of *TAL2* gene in the adult brain by quantitative PCR
Note: $^{**}P < 0.01$

4. Discussion

Upregulating or downregulating gene expression levels can provide valuable clues for gene function. The GAL4/UAS system was first established in *Drosophila* and widely used to create transgenic flies by combining a driver (GAL4) and responder (UAS) line based on the properties of GAL4[28-30]. This binary system provides a highly flexible platform to drive gene expression in vivo. This system can be used for gene overexpression, RNA interference, gene expression pattern description, genetic mutation rescue, etc., and widely adopted in the development research of nervous system, cardiovascular system, retina and muscle[22, 31, 32]. At present, GAL4/UAS system has been applied to many model organism, including mice, zebrafish, Arabidopsis, etc., which has proved to be an efficient way to identify gene function[33-36].

The closely related bHLH transcription factors Tal1, Tal2 and Lyl1 are important regulators of normal development and differentiation. Tal1 is essential for primitive hematopoiesis and plays a role in erythrocytic/megakaryocytic gene expression regulation[37]. Nevertheless, the Lyl1 is not needed for early hematopoietic development and may have overlapping and independent functions with Tal1[8, 38]. Tal2 protein is smaller than Tal1 and Lyl1, because of lacking N-terminal transactivation or repression domains. Despite these differences, Tal1, Lyl1 and Tal2 are associated with human T-ALL[39, 40]. Tal2 was found at the t(7;9) (q35;q34) chromosomal translocations[12]. No overt defects in hematopoiesis were observed in *Tal2*-null mutant mice. However, the brain of mice and the lateral floor plate of zebrafish *Tal2* is expressed[18-20]. Targeted disruption of Tal2 in mouse leads to developmental defects of the central nervous system and to early death after birth[19]. However, the function of TAL2 during brain development is unclear.

Courtial *et al* found that *Tal2* was expressed in hematopoietic cells of the myeloid lineage and *Tal2* was upregulated during oesteoclastogenesis[41]. *Tal2* was a direct target gene of PU.1, a key transcription factor for osteoclast gene expression. These findings revealed a regulatory function of *Tal2* during hematopoietic cells and osteoclast differentiation. Achim *et al* showed that *Tal2* and *Tal1* were specifically and sequentially activated during mice midbrain GABAergic neurogenesis. Similar to zinc-finger transcription factor Gata2, a selector of GABAergic neuron identity, *Tal2* expression was activated very early during GABAergic neuron differentiation and the majority of midbrain GABAergic neurons switch to a glutamatergic-like phenotype without *Tal2*[42]. Yang *et al* also found *tal2* knockdown embryos lacked expression of GABA-synthesizing enzyme glutamic acid decarboxylase 67 gene (*gad67*) in the zebrafish lateral floor plate, whereas more dorsally located *gad67*-expressing cells were unaffected by the knockdown of *tal2*. This suggests that Gata2 is necessary for expression of the Tal2 that acts upstream of *gad67* in Kolmer-Agduhr" cells[43]. Kobayashi *et al* found that *Tal2* was induced by the complex of all-trans retinoic acid (atRA) and retinoic acid receptor α (RARα) during neural differentiation in mouse P19 cells. Kobayashi *et al* found a RARE-like element in the intron of *Tal2* and a TATA-box-like element in the 5' region. The TATA- box binding protein bound to TATA-box-like element upstream of *Tal2* and RARα was bound to RARE-like element responded to atRA signaling that activated transcription in P19 cells[44, 45]. These results suggest that the transcription of *Tal2* is coordinately mediated by two distal regulatory elements.

The results showed that we had successfully constructed pUAS-*TAL2* recombinant plasmid and microinjected it with Δ2-3 helper plasmid into the embryos of wild-type *Drosophila*, and then screened out the UAS-*TAL2* transgenic *Drosophila* by *mini-white* marker. The GAL4/UAS system is derived from the P-element transposon of *Drosophila* and combined with the strong inducible promoter GAL4 to regulate transgenic expression. P transposase mediated gene insertion or GAL4-dependent expression of the target gene could be effectively realized by using the pUAST plasmid. pUAST plasmid consists of five tandemly arrayed UAS sites followed by heat shock

protein 70 (Hsp70) minimal promoter and transcriptional start, a polylinker containing unique restriction sites and the SV40 small T intron and polyadenylation signal. These features are included in a P-element vector (pCaSpeR3) containing the P-element ends (P3' and P5') and the *mini-white* gene which acts as a dominant marker for successful incorporation into *Drosophila* genome in a white mutant background. Δ2-3 helper plasmid is a modified defective P factor of *Drosophila*, which can encode transposase[46, 47]. When the target cells were cotransfected with the pUAST and helper plasmid, the transposase generated by helper plasmid recognized the 3’ and 5’ P-element terminal repeats on the pUAST plasmid, and then inserted the transposable region into the host genome. Transgenic flies can be produced by coinjection of pUAST and helper plasmid into early embryos of *Drosophila*. P transposase mediates the recombination between two P-element terminal repeats, resulting in the generation of transgenic progenies carrying target genes. P transposase is only transiently expressed, thus the integration of transposons into host genome becomes permanent with the loss of helper plasmid. The expression of *mini-white* gene can change the eye color of adult fly, which is a reporter to identify the success of transgenic events.P[*mini-white*, UAS-*TAL2*] in the recombinant plasmid of pUAS-*TAL2* can transpose and insert into genome of *Drosophila* under transposase. PCR analysis confirmed that P[*mini-white*, UAS-*TAL2*] had integrated into the genome of four independent transgenic strains, so that their compound eyes showed red. In addition, we crossed the UAS-*TAL2* transgenic strains with elav-GAL4, and found that human *TAL2* gene was overexpressed in nervous system under the regulation of elav-GAL4.

The GAL4/UAS is an efficient system for the preparation and expression control of transgenic *Drosophila*. The UAS-*TAL2* transgenic *Drosophila* model provides a research tool for us to further explore the function and mechanism of *TAL2* gene. First, we can cross the UAS-*TAL2* transgenic *Drosophila* with different GAL4 strains, and express the *TAL2* gene in specific tissue and cell development process. By observing the abnormal phenotype, we can preliminarily hypothesize the biological function of *TAL2* gene. We introduced HA-tag sequence before termination codon of *TAL2* gene, so we can use anti-HA antibody to evaluate the expression of *TAL2* gene by immunohistochemistry or protein electrophoresis. In addition, immunocoprecipitation and mass spectrometry were used to screen proteins interacting with TAL2 protein[48, 49]. At the same time, chromatin immunoprecipitation and high-throughput sequencing technology were employed to screen the specific DNA region of TAL2 protein bound in the whole genome, and identify the downstream target genes of TAL2 transcription factor[50].

Several deficiencies need to be pointed out. First of all, the random integration of P-element makes it difficult to locate the insertion site, and the genomic location may also affect the expression of transgenic gene. The endogenous genes may be affected when their regulatory elements were inserted and destroyed. Second, the efficiency of P-element mediated generation of transgenic progenies is usually lower than that of the φC31 integrase mediated system (pUAST-attB). In addition, the production of transgenic *Drosophila* requires embryo injection and flight feeding, which has certain technical complexity.

5. Conclusion

In conclusion, the successful construction of UAS-*TAL2* transgenic *Drosophila* laid a foundation for further investigating the function and regulatory mechanism of *TAL2* gene in the development process with GAL4/UAS system, and also provided a reference for the related research of other mammalian bHLH proteins.

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