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ANALYSES OF β-CELL DEVELOPMENT IN TRANSGENIC ZEBRAFISH WITH A CONSTRUCT OF INSULIN PROMOTER AND GREEN FLUORESCENT PROTEIN

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Abstract With many advantageous features such as small size, high production and fertilization in vitro, zebrafish has become a kind of the model creature for the investigation of vertebrate development and human diseases. So we set up a transgenic zebrafish model to investigate the β-cell development. Firstly, we obtained an INS: GFP construction that contains the zebrafish insulin (INS) promoter and green fluorescent protein (GFP). Secondly, we injected the construction into the cytoplasm of one-cell-stage embryos. Finally, we gained germ-line INS transgenic zebrafish that displayed highly specific β-cell expression of GFP in both larvae and adult. By following GFP expression, pancreas formation was detected at the 18h post-fertilization in the earliest time points between the second bilateral somites, a group of GFP-positive cells located from ventral to the notochord. From day 1 to 5 post-fertilization, the number of insulin/GFP expressing cells migrated and formed a right organ. Thus, our works demonstrated that the transgenic line provided a convenient and direct experimental tool in analyzing endocrine pancreas development, injury and recovery.

Key words: Zebrafish; β-cell; Green Fluorescent Protein (GFP); Insulin

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At present, diabetes mellitus has become the third largest chronic disease after cardiovascular disease and tumor. And the number of patients suffering from diabetes keeps increasing all over the world. In both diabetes of type 1 and type 2, the insufficient number of insulin-producing beta cells is a major cause of defective control of blood glucose and its complications. Despite the help of the existing therapies with exogenous insulin or hypoglycemic agents for the diabetes of type 1 and type 2, most individuals with diabetes are unable to maintain a blood glucose level in the normal range at all times. Repeating hyperglycemia unawareness and long-term hyperglycemia will lead to capillary vessel complication of renal, eyes and nervous system which may result in the poor health and shorter life-span of the sufferer. Stem cells and islet transplantation both suggest that diabetes can be cured by the replenishment of deficient β-cells in the future. Stem cell for the treatment of diabetes is not only limited by ethical questions, but also by the ability to differentiate stem cells and to acquire fully functional islets, so it is still only tested in trials. Edmonton's protocol[1] of islet transplantation has limited the clinical utility of the availability of donors as well as the need for continued

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immunosuppression with its risks and side effects. Accordingly, therapies increasing functional β-cell mass may offer a cure for diabetes. Based on the realization that β-cells are capable of significant proliferation throughout adult life[2], the enhanced proliferation of β-cells *in vivo* is pursued as a strategy for regenerative medicine for diabetes[3].

Zebrafish has advantages for scientific research such as small size, transparency of embryos, rapid development and free-feeding larvae within 5d of fertilization, et al. Over the past decade, the zebrafish has come to the forefront of vertebrate model research and eventually offered the unique combination of invertebrate and mammalian for genetic studies and pharmacological screens. The zebrafish embryo fertilizes in vitro and generation time is as in mice, with adults reaching sexual maturity within 3 months old, however, fecundity is much higher. A zebrafish pair can yield hundreds of embryos in a single clutch every week. Furthermore, embryos and larvae are optically transparent, allowing easy visualization of cellular morphology and movement.

By taking the fish as an object, researchers use DNA constructs containing regulatory sequences of the zebrafish insulin genes and germline transgenic zebrafish expressing the GFP reporter gene in the β-cell, which were generated to detect islet rise, migration and maturity in a living creature. With the purpose to create such a model, we tried to induce β-cell ablation using Streptozotocin (STZ) which causes apoptosis of the β-cells in mammals. We could observe fluorescent change to identify information about β-cells apoptosis or regeneration. In conclusion, this model was good for research β-cells development, injury and recovery.

1 Materials and methods

1.1 The zebrafish (*Danio rerio*) were raised in our lab and maintained according to the Zebrafish Book.

1.2 Promoter isolation and transgenic constructs

The zebrafish gene insulin (INS) was selected from GenBank (GenBank accession No. AF036326) of *Danio rerio* nucleotide sequencing and the literature[4]. Then we amplified the promoter by PCR from *Danio rerio* genomic DNA using the primers shown below. Coding regions were capitalized with restriction sites underlined and endonucleases shown in parentheses.

Forward primer (SalI) 5′-TTTGACAGAGATCGGCATTTTGAGGC-3′

Reverse primer (EcoRV) 5′-GGTCACACTGACACAAACACACAC-3′

INS promoter was ligated into IsceI-GFP fusion vector as a SalI/EcoRV Fragment which the vector flanks with the special I-sceI site. The clone was transformed into JM109 bacteria. Finally, the plasmid DNA was sequenced and compared with the database in GenBank by BLASTN.

1.3 Microinjection and generation of INS: GFP transgenic zebrafish

We microinjected plasmid DNA linearized by I-sceI meganuclease (NEB, Germany) into fertilized eggs at the one-cell stage[5]. Fish hatched from the injected eggs were raised to sexual maturity. Transgenic founder fish were selected by pair mating the individual fish with nontransgenic fish and by testing fluorescence expression. The progeny which expressed GFP were then selected as real germline insulin transgenic zebrafish.

1.4 Analysis of transgenic zebrafish

1.4.1 Detection of GFP gene by PCR Genome DNA was isolated from 24h post fertilization by using the conventional proteinase K method[6]. For the detection of the GFP gene, polymerase chain reaction (PCR) was performed by using a GFP-gene-specific primer pair, which yielded a 750-bp fragment. The GFP forward primer sequence used was 5′-TCAATGGATATGGTGAGCAAGGGCGAG-3′, and the reverse primer sequence used was 5′-CACGAATTCCTTGTACAGCTCGTCC-3′. Likewise, the plasmid of construction and wild type fish genomic DNA was amplified as a negative control. The PCR amplification cycles consisted of 95 ℃ for 15s, 60 ℃ for 30s, 72 ℃ for 50s and 30 cycles.

1.4.2 Whole-mount RNA in situ hybridization and immunofluorescence Antisense digoxigenin-labeled RNA probes were generated from cDNA clones of the insulin genes, using a DIG RNA Labeling kit. Whole-mount RNA in situ hybridization conditions were described as in Zebrafish Book[7]. After hybridization,

embryos were manually stained until the color was clearly developed. Images were obtained by using Nikon SMZ1500 digital camera. The embryos which are mosaic after a 24h fertilization, though, were fixed, and whole-mount immunofluorescence embryos were incubated overnight as well as divided in Anti-GFP-Rabbit conjugated antibody and anti-Rabbit-Alexa (Molecular Probes™).

2 Results

2.1 INS promoter clones

The zebrafish INS promoter clones, used in zebrafish genomic DNA template and analyzed by gel electrophoresis, were used to provide one band of 1400bp (Fig. 1. lane 3). Furthermore, we claimed that the fragments were recombined within IsceI-GFP fusion gene and the construct was assayed by restriction digestion as detected on agarose gel electrophoresis. The electrophoretic pattern yield digested by SalI and EcoRV, which was cut twice within the insulin promoter or the IsceI-GFP fusion gene and revealed two intense hybridizing bands (Fig. 1, lanes 1 and 2). Finally, the DNA construct was named INS: GFP as the sketch showed below (Fig. 2).

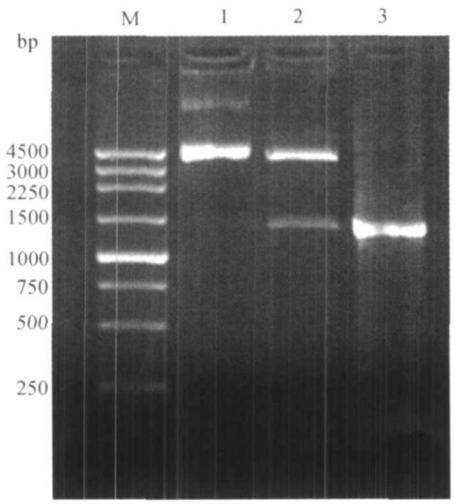

Fig. 1 Restriction analysis of INS: GFP
M: DNA marker; 1: INS: GFP; 2: INS: GFP digested with SalⅣ and EcoRV; 3: PCR product

2.2 Development of endocrine pancreas in zebrafish

The embryos, injected with INS: GFP plasmid digested by I-sceI enzyme, were raised to sexual maturity (3 months old), and outcrossed with wild type fish to carry F1 stable transgenic lines. A weak GFP expression was initially detected in the pancreas around 18 hpf (data not shown). By following GFP expression from day 1 to 5 post-fertilization, transgenic embryos were imaged by using Nikon digital camera DXM1200F. We found that the insulin/GFP cells in early development were individually aligned into a single flattened layer in the center of the embryo ventral to the notochord at the level of the second somite (Fig. 3, A and B). From day 2 to day 5 post-fertilization, the number of insulin/GFP expressing cells increased and formed a spherical structure. At 72hpf (hour post fertilization) insulin/GFP positive cells were inclined to the right side (Fig. 3, C), then the number of insulin/GFP expressing cells migrated, formed a right organ (Fig. 3, D) and located dorsally to the duodenum at 120hpf.

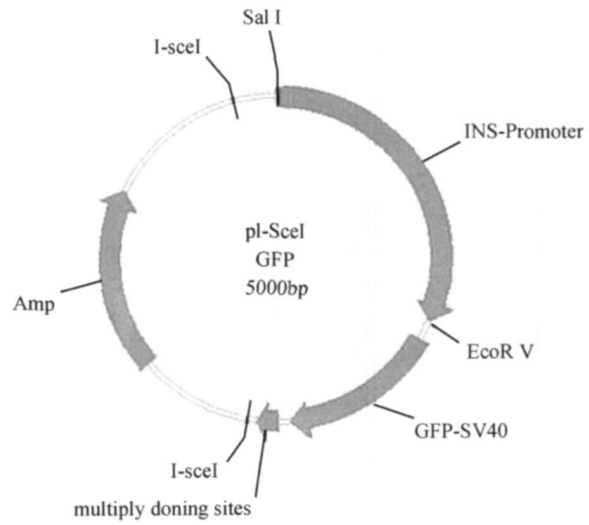

Fig. 2 The sketch map of INS: GFP

2.3 Stable transgenic INS: GFP lines

2.3.1 Analysis of GFP integrated into genome by PCR

Genomic DNA was isolated from transgenic and non-transgenic zebrafish. Primer sequences were designed within the coding region of the GFP gene to produce a 750 bp amplification product. We detected 750 bp band in transgenic fish genome and equal band with plasmid template (Fig. 4 land 1 and 3). Simultaneous-

ly, we could not find any band in the wild type fish template (Fig. 4 and 2) in sight since foreign GFP DNA in the fluorescence expression of injected fish was integrated into genomic DNA and stably inherited.

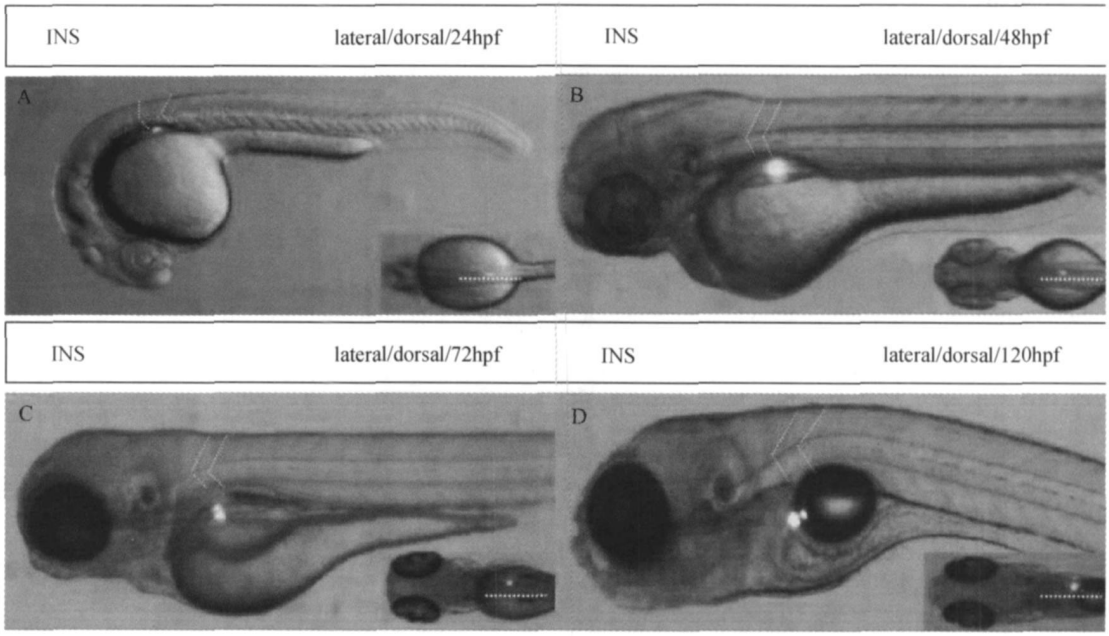

Fig. 3　Expression of GFP in germ line transgenic zebrafish embryos directed by INS promoter

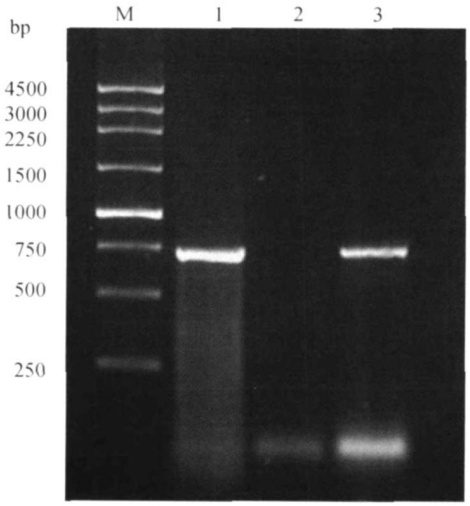

Fig. 4　Amplification of green fluorescence protein (GFP) from germ line transgenic zebrafish genome DNA

M: DNA marker; 1: PCR product (750bp); 2: Negative control; 3: Positive control

2.3.2 Whole-mount RNA in situ hybridization (WISH)

Using in situ hybridization to detect insulin transcripts and expression localization, we similarly observed no difference between transgenic and wild type embryos (Fig. 5A, B and C). At the 18-somite stage, insulin expression was firstly detected in the central region of the embryo in a right and a left longitudinal row of cells situated ventrally to the notochord under the second somite (Fig. 5A).

As shown in transgenic mosaic embryos, GPF expresses in the same way, while few cells appeared green within the region of the islet. By increasing the strength of the laser, weakly green fluorescent cells could be identified within the pancreas of mosaic embryos at 18hpf (Data not shown). Subsequently, the level of insulin mRNA increased in the pancreas. By WISH, the expression of INS mRNA was the same with those of INS: GFP transgenic expression at 72 hpf and 120 hpf in the pancreas region. Usually, this expression domain was detected on the right side of the anterior gut approximately at the level of the second somite (Fig. 3C and D, Fig. 5B and C).

To verify the fluorescence was to localize specifically β-cells, we performed immunofluorescent staining for insulin on INS: GFP embryos at the 24 hpf (Fig. 5D, E and F). Our results showed that the onset of GFP expression coincided with the insulin spatial and temporal expression patterns as detected by RNA in situ hybridization.

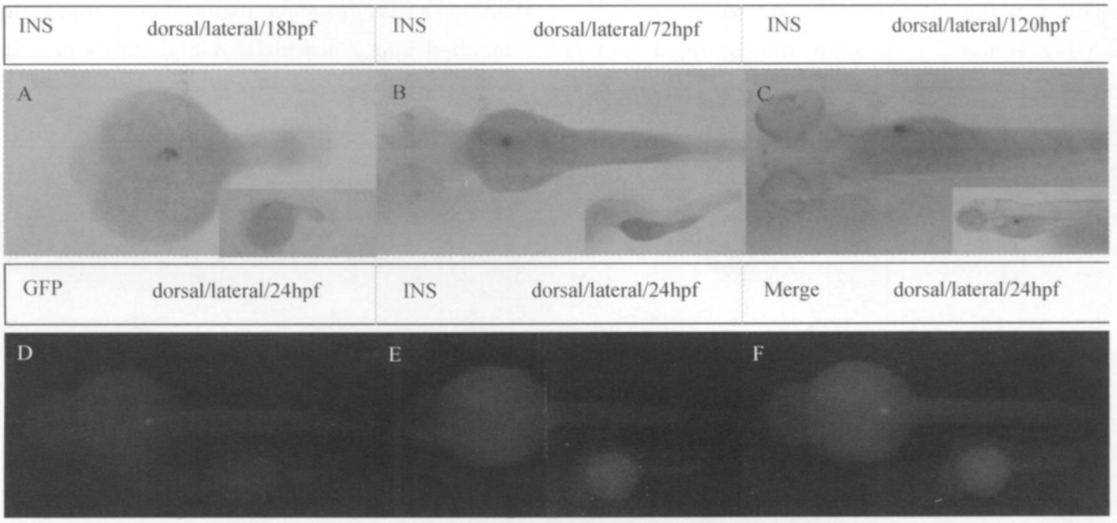

Fig. 5 Expression of INS and GFP in zebrafish embryos detected by whole amount in-situ hybridization

A、B、C: whole amount in-situ hybridization with the digoxingenin labeled RNA probe. INS beging to express dispersely in 18hpf; Increasing expression and according to transgenic line in 72hpf; Pancrease islet lie in right. D、E、F: Whole amount in-situ hybridization with the digoxingenin labeled RNA probe and green fluorescence protein antibody, they are detected according with spatial and temporal

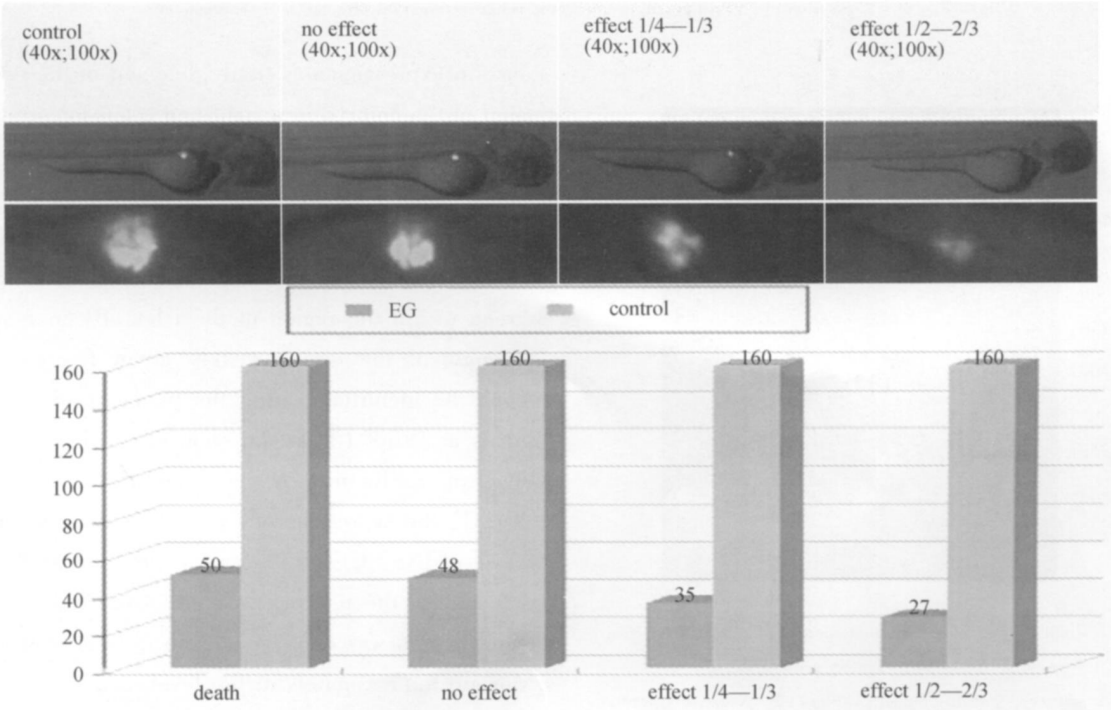

Fig. 6 Induction experimental diabetes in transgenic zebrafish by streptozotocin

EG: Experiment group

2.4 Application of living color transgenic fish

The zebrafish is one of the teleosts with endocrine islets tightly embedded inside the exocrine tissue to form a pancreas quite like a mammalian pancreas[8]. In our study, we used the GFP transgenic fish system to investigate gene expression patterns and tissue/organ development, to analyse tissue-specific promoters, to trace cell lineage and migration and to analyse cellular localization, etc. Based on these researches, we tried to ablate β-cells by streptozotocin which was widely

used to induce experimental diabetes in animals[9]. After addition of the drug to the water, embryonic zebrafish was screened for phenotypes by facilitated and availability of the INS: GFP transgenic line. 24 hpf embryos were incubated in STZ concentrations (2.3 mM) at 28 ℃ in the dark. After 24h of incubation, we had found detectable different effects on β-cell mass as judged by GFP expression amounted of 160 transgenic fish (Fig. 6). However, the phenotype is not stable and difficult to repeat, so we could not use this method to induce of experimental diabetes in the zebrafish.

3 Discussion

Diabetes is a disease which is characterized by a near-absolute (type 1) or relative (type 2) deficiency in the number of pancreatic β-cells. The pancreatic β-cells are a vital organ controlling glucose homeostasis and food digestion in vertebrates. In mammals, birds, and amphibians, both exocrine and endocrine pancreatic tissues are of endodermal origin and develop from dorsal and ventral epithelial evaginations, arising from the gut tube caudal to the stomach that fuse to form a single structure. Every islet is composed of four major cell types: insulin-producing β-cells form the core while somatostatin-producing γ-cells, glucagon-producing α-cells, and pancreatic polypeptide secreting *PP*-cells are located at the periphery[10]. Pancreatic development is well studied in the zebrafish. As mammals, it consists of both an endocrine component and exocrine component. While molecular events appear highly conserved between zebrafish and mammals[11, 12].

In mammals, it has been difficult to study islet morphogenesis because of the in vivo development of mammalian embryos. And in zebrafish, the pancreas is also difficult to recognize in living transparent embryos. To overcome this obstacle, we generated germline transgenic zebrafish expressing the GFP reporter gene under the control of regulatory sequences from insulin. At last, we gained further insight into the process of pancreatic islet morphogenesis in living. In zebrafish, insulin expression is initially detected in cells located on both sides of the midline. With development, insulin positive cells were reorganized into an oval shaped cluster without a significant increase in cell population, then the number of insulin expressing cells increased and formed a spherical structure and migrated a right organ[13]. It is a different mechanism with mammals, whose pancreas arises from the endoderm as a dorsal and a ventral bud as well as fuses together to form the single organ.

According to our generation of transgenic zebrafish, we have observed left/right (L/R) asymmetry during embryonic development for pancreas positioning. An asymetrical process is also necessary for properly functioning of many organs. One striking example is the heart, whose organization into left and right chambers is essential; if L/R asymmetry was abnormal, the heart ventricles and atria would not develop perfectly asymmetrical, thus leading to congenital heart diseases in humans. Defective L/R asymmetry causes a variety of other abnormalities including liver mispositioning, asplenia or polysplenia, bilateral lobation of lungs, gut misrotation, and anomalous coronary connections et al[14, 15]. Our model provided an useful tool to research the mechanism and pathway of asymmetry in vivo.

Meanwhile, we tried to induce of experimental diabetes in the zebrafish using chemicals, which are very convenient and made it easy to observe by GFP expression by selectively destroying pancreatic β-cells. Perhaps, this way is useful to mammalian but fails to be repeated in zebrafish. In our results, we could gain a part of phenotype of β-cell disappeared, but a stable and duration effect was not arrested. Recently, a new technology of metronidazole(Met) dependent cell ablation has been found in zebrafish[16—18]. The model used the bacterial Nitroreductase (NTR) enzyme to convert the prodrug Metronidazole (Mtz) into a cytotoxic DNA cross-linking agent to conditional targeted cell ablation. Combining the advantages of zebrafish, this way would provide an opportunity to conduct large-scale screens for pharmacological and genetic modifiers of β-cell regeneration.

In the past 5 years, the sequence of the zebrafish genome has increased the profile of zebrafish research even further, expanding into other areas such as pharmacology, cancer research and drug discovery[19]. It is clear that the zebrafish is a good model for vertebrate endocrine function. We anticipate that the use of these

techniques will make the zebrafish a prominent model in endocrine research in the coming years.

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转基因斑马鱼分析胰岛 β-细胞发育情况

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摘要: 斑马鱼的个体小、高产和体外受精等特点使其已经迅速成为研究脊椎动物器官发育和人类疾病的模式生物之一。我们建立了一个转基因斑马鱼动物模型来研究胰岛 β-细胞的发育。首先, 构建了斑马鱼胰岛素 (Insulin, INS) 启动子与绿色荧光蛋白 (GFP) 组成的表达载体, 命名为 INS: GFP。其次, 将质粒在斑马鱼 1-细胞期注射到细胞质内。最后我们成功获得了生殖系稳定遗传胰岛素转基因斑马鱼, 在成鱼和幼鱼期均可以通过 GFP标记 β-细胞。通过方便的荧光筛选, 我们观察到胰岛在受精后 18h开始形成, 1— 5d后由初始的脊索中线两侧向右迁移。从我们构建的胰岛素转基因斑马鱼, 可以直观判断胰岛的发育情况, 为研究胰岛的发育、损伤和再生提供了一个简便和直观的新型工具。

关键词: 斑马鱼; β-细胞; 绿色荧光蛋白; 胰岛素