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建立誘導式開啟心臟專一性基因的轉殖魚(1/2)

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中文摘要

Gossen (1992)利用大腸桿菌(*Escherichia coli*)對四環黴素(tetracycline)抗藥性基因操作子之 tetracycline repressor (TetR) DNA 序列，與單純疱疹病毒(Herpes simplex virus) virion protein16 (VP16)之 C-terminal domain 相互連接所形成的 tetracycline-controlled transcativator (tTA)，研發出 tetracycline－controlled transcription system。並進一步利用突變型的 TetR 序列衍生出 reverse Tc-controlled transactivactor (rtTA)，在加入 doxycycline 時，rtTA 可與 tetracycline operator (*tet*O)結合而活化其後的報導基因，而稱為 Tet-On system。這套系統廣泛應用於各種細胞株與模式動物中，但迄今尚未在魚類中經過測試。為了在斑馬魚(*Danio rerio*)體內建立穩定表現的 Tet-On system，利用可在斑馬魚心臟專一表現的 zebrafish cardiac myosine light chain (*cmlc2*) promoter 來啟動、在斑馬魚纖維母細胞能良好表現的 rtTA，經顯微注射後的斑馬魚胚胎雖帶有少許非專一的溢出性螢光，但再經 Doxycycline (Dox)處理後，能穩定表現心臟專一性綠螢光。針對帶有 Tet-On system 的斑馬魚基因轉殖品系 F1 進行測試，發現不同濃度的 Dox (10 贡g/ml、1贡g/ml、100 ng/ml、10 ng/ml、and 1ng/ml)具有不同誘導效果，並認為 1贡g/ml 是最適切的誘導濃度。授精後 72 小時的 F1 胚胎經 1贡g/ml Dox 誘導後，於第六小時開始出現肉眼可見的心臟專一性綠螢光；綠螢光的強度隨誘導時間增加，並在誘導後第 36~48 小時到達最高值。F1 與野生型交配所產下的 F2 胚胎顯示也擁有類似的誘導趨勢，但 F1 雄雌互配產下的 F2、誘導後心臟專一性綠螢光的最高亮度稍有增加，達到最高亮度的時間也稍有延遲至誘導後第 48~60 小時；與 F1 性狀相較之下，伴隨著更顯著的誘導效果，F1 互配產生的 F2 胚胎亦呈現較強的非專一溢出性綠螢光。也期待在斑馬魚體內建立的 Tet-On system，能應用未來在基因功能的研究上。

Abstract

The tetracycline–controlled transcription system is well developed in many transgenic organisms. Yet, this system has not been reported in zebrafish. In order to develop tet-on regulatory system to allow the transgenic GFP to be expressed specifically in the zebrafish heart, we use the zebrafish cmlc2 promoter to drive the reverse-transactivator(rtTA). We constructed vectors which presented GFP after Dox induction in zebrafish fibroblast cell line. Then, we applied this system *in vivo*. Although fluorescence appeared slightly leaky, transgene showed that rtTA in the transient embryos and drive GFP specific in the heart. After we got the germ-line F1 embryos, we found that the Dox dosage depended induction efficiency (10 μg/ml、1μg/ml、100 ng/ml、10 ng/ml、and 1ng/ml). After 6 hr induction under treating with 1 μg/ml Dox, the rtTA-derived heart-specific GFP expression appeared in the transgenic lines embryos at 72-hpf with a relative low leakiness. The higher GFP levels, the longer induction time: the highest level was reached within 36~48 hr after induction. The germ-line transmitted F2 from F1 crossing wild-type has the same tendency of induction, and the F2 from F1 crossing F1 shows stronger GFP intensity of highest level within 48~60 hr after induction These results were consistent with cell line induction. The leaky GFP is also stronger than those of F1 and F2 from F1 crossing wild-type. This is the first tet-on system developed in transgenic fish, which should be very useful to study gene function at any stage whenever gene is induced.

Introduction

A transgenic mouse with tTA regulatory system was reported by Yu (1996). When this mice strain crosses with strain containing tTA-responsive target genes, luciferase or β-galactosidase, they find a 300-fold increasing of the luciferase activity between presence and absence of tTA, the location of target gene specific expression in heart with non-specific expression in kidney, lung and tongue, and the degree of tetracycline-dependent repression were examined in progeny. As visualized by X-gal stain and using luciferase for quantification, target gene expression is reduced to the levels indistinguishable from those observe in the absence of transactivator. However, there are some disadvantage of using transgenic mice： (a) few embryos are available; (b) *in vivo* direct observation of the dynamical expression is extremely limited, and (c) it costs higher. Therefore, we attempt to establish a conditional gene regulatory system *in vivo* in the heart of zebrafish model both that may reveal the mechanisms of some disease-link genes of heart.

Zebrafish (*Danio rerio*) is a good model animal because it's fecund, embryos are transparent, developmental processes are rapid, and dynamical changes in embryos can be observed in a non-invasive and high-throughput manner. Based on previous study (20), the 1.6 kb heart-specific cMLC2 promoter of the zebrafish could drive GFP expressed specifically in the heart of transgenic embryos. Since there is no *in vivo* inducible system, such as tet-on system, has been developed in zebrafish, in this communication we use zebrafish *cmlc2* promoter to drive rtTA expression in the heart and generate a germ-line transmitted zebrafish. Results show that tet-on system works well in zebrafish. Therefore, this inducible system can be applied to carry out genomic function analysis of many tissue-specific genes.

Materials and Methods

Generation of Transgenic Constructs

The six constructs used in this study are illustrated schematically in Figure 1. The cardiac-specific rtTA construct pICML1-rT16-1b was generated by modifying the previously described pICMLE-(-870/787) plasmid (Huang et al., 2003), in which the upstream region (-870/-1), exon 1, intron 1, and a part of exon 2 of zebrafish *cmlc*2 were included. The EGFP segment of pICMLE-(-870/787) was replaced by rtTA-M2. The resulting pICML1-rT16-1b consisted of the regulatory region of *cmlc*2, rtTA-M2, and AAV-ITR. The rtTA-dependent bidirectional *tetO* (Clontech) was cloned into pIEGFP (Chou et al., 2001) to generate pIBIE, resulting in TRE being located upstream of the EGFP gene. Plasmid pITSBIE followed a similar construction to that of pIBIE, except that a Tet-controlled transcriptional silencer (Clontech) was included. Plasmids pBILE and pBILd2 were generated by cloning EGFP and d2EGFP cDNA into pBIL (Clontech), respectively. Luciferase and EGFP were engineered downstream on each side of bidirectional *tetO* in the absence of AAV-ITR.

Another strategy of plasmid construction used in this study was the cointegrated form of a plasmid containing the cardiac-specific rtTA and a plasmid containing the rtTA-dependent GFP reporter gene. Plasmid pICML-rT16-1b was fused with plasmid pIBIE or pBILE to generate a single dual plasmid of pICMLM2_IBIE or pICMLM2_BILE, respectively.

Experimental fish and GFP observation

Zebrafish (AB strains) were maintained according to procedures described by Westerfield (1995). Prior to the initial cleavage, fertilized eggs were collected and microinjected with linearized constructs (Wang *et al*., 2002). The concentration of upstream and downstream plasmid was taken equally to carry out microinjection. Injected eggs were incubated at 28.5°C. Transgenic embryos were observed under a stereo dissecting microscope (Leica MZFIII) equipped with a fluorescent module and enhanced GFP filter cube (Kramer Scientific). Photographs were taken with coolpix 990 digital camera (Nikon). Zebrafish developmental stages were determined according to criteria described by Kimmel *et al* (1995).

Results and Discussion

Effect of bi-directional elements on the leaky and heart-specific expressions

When the downstream plasmid pIBIE, in which tTS was absent, was injected into the zebrafish embryos, we found that the rate of leakiness increased, and the intensity of

green fluorescent signal was also slightly increased in the transgenic embryos. The heart-specific expression rate of transgenic embryos did not increase greatly after induction with Dox. However, we noticed that the expression level of GFP level in the heart increased remarkedly (from *** to *****) after Dox induction for transgenic embryos.

When the plasmid pBILE, in which AAV-ITR was absent, was injected, we found that both the leakiness and the intensity of fluorescence decreased significantly in the transgenic embryos. The level of heart-specific expression after Dox induction was slightly down to ****.

When the plasmid pBILd2, which was as same as pBILE except that GFP was replaced by d2-GFP, had been injected, we found that there was no leaky expression in the transgenic embryos. However, the rate of heart-specific expression of the green fluorescence decreased greatly (only 3.5% of injected embryos) and the intensity of green signal in the heart was a little faint.

Inducible GFP expression in the transmitted germ-line zebrafish embryos

The F1 embryos aged 3-dpf from line T03 were induced by Dox at the concentrations of 10 μg/μl、1 μg/μl、100 ng/μl、10 ng/μl and 1 ng/μl. Results showed that the GFP was induced to express in the heart specifically after treating with 10 μg/μl and 1μg/μl Dox for 6 hours. The intensity of green fluorescent signals became stronger and stronger rapidly. The fluorescence reached to the maximum level after induction for 36-48 hours. Whereas, in the 100 ng/μl -treated group, the heart-specific expression of induced GFP in F1 embryos was not detected until 12 hours after Dox treatment. The intensity of fluorescence increased relatively slow and did not appear quite strong even after 48 hours treatment. For groups treated with lower dosages of Dox (10 ng/μl and 1 ng/μl), the heart-specific expression of GFP was not observed in the F1 embryos until 18 hours later, and the intensity was extremely faint and did not increase afterward.

Compared to the inducible expression of GFP from the transient assay on F0 generation, the rate of the leaky expression before induction in F1 embryos was lower (only 1% of embryos). In addition, the intensity of heart-specific expression after induction in F1 embryos became stronger when the concentration of Dox increased. The highest level of intensity was reached at 48-hour after induction.

In the F2 derived embryos 3-dpf derived from T03 F1 male crossing wild-type female or from F1 male crossing F1 female were induced by Dox at the concentrations of 10 μg/μl、1 μg/μl、100 ng/μl、0 ng/μl. After the induction, all the F2 embryos show similar tendency to those occurred in F1 embryos. We noticed that the F2 embryos from

F1 male crossing F1 female expressed higher signal at rates but with a higher leakiness. The time point needed reach the max GFP intensity was somewhat degree of delay, 48~60 hours (Table5 , Fig 4, and Fig 5). Unexpectedly, we observed leakiness occurred in the gill cover and the dorsal chest of F2 embryos.

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計畫成果自評

本計畫執行期為兩年，主要目的為建立組織專一表現的誘導系統於斑馬魚活體中。在這第一年度中，我們順利進行了心臟專一、四環黴素誘導的質體構築，且進行了基因轉殖，也成功挑選到基因轉殖品系。目前正進行更細部的研究，以探討四環黴素誘導系統應用在斑馬魚活體上時，表現的情形，以及未來可應用的發展性。

附錄

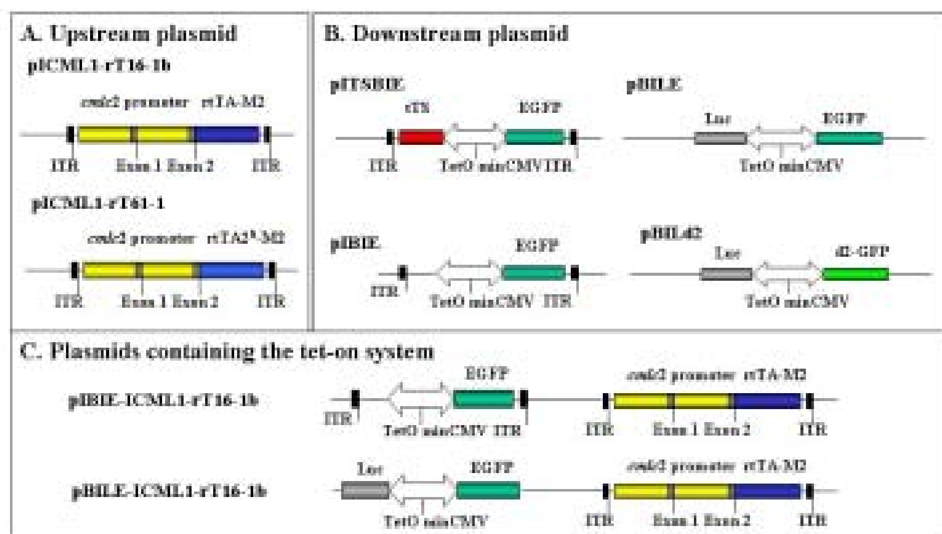

Fig 1. Plasmid construction. (A) The upstream plasmid include the Zebrafish *cmlc2* promoter and transactivators (rtTA-M2 & rtTA2^{S}-M2). (B) The downstream plasmid contain the bi-directional TRE and reporter gene. (C) The combination of upstream and downstream plasmid in single plasmid. *cmlc2*:cardiac myosin light chain, Luc:luciferase, ITR:inverted terminal repeat sequence of AAV, TetO minCMV:The TRE element is tween two minimal CMV promoter, tTS:tetracycline-controled transcriptional silencer.

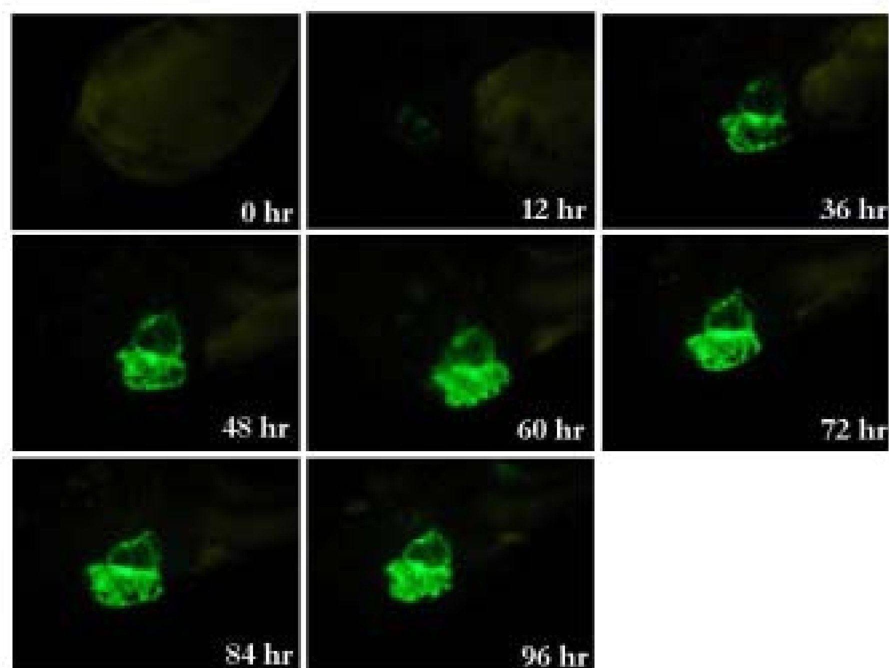

Fig 2. Photo-images of F2 embryos after induction. Induction:10 mg/ml Dox ,Stage: 3-dpf