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活體條件式誘導 VEGF 基因在視網膜表現：建立以斑馬魚為後天性視網膜新生血管之模式動物
研究成果報告(精簡版)

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In vivo conditional induction of VEGF gene expression in retina: establishing zebrafish as an animal model of acquired retinal neovascularization

中文摘要

目的：視網膜及脈絡膜新生血管是許多嚴重眼睛疾病的作用機轉，包括糖尿病視網膜病變、老年性黃斑病變、視網膜血管阻塞疾病、高度近視視網膜病變等等，它會對視力造成嚴重的破壞，是導致已開發國家人民失明的主要原因，也是目前亟待解決的問題。現在已知有許多能夠刺激血管增生的因子，其中血管內皮生長因子（vascular endothelial growth factor，VEGF）扮演了極重要的角色，所以抑制 VEGF 的訊息傳遞就成為一個重要的治療方向。要研究在視網膜中 VEGF 的訊息傳遞以及它對各種視網膜細胞間交互作用的影響，就必須先建立能夠在視網膜過度表現 VEGF 的模式動物。雖然從文獻得知已經有基因轉殖鼠可以在視網膜中持續產生 VEGF 而造成視網膜內及視網膜下新生血管，但是這種太早而且持續產生 VEGF 的致病機轉是類似先天性的病變，並不適合用來模擬後天性的視網膜疾病，例如糖尿病視網膜病變及老年性黃斑病變。此時條件式誘導 VEGF 過度表現的系統就是最好的解決辦法。

方法：我們建構了一個名為 pTet-ON-Opsin-VEGF 的質體，它可以在暴露到四環素後製造 VEGF 及紅螢光蛋白，並藉由視網膜專一之 *rhodopsin* promoter 使它只在視網膜內作用。我們將此 10,000-bp 的質體剪下並線性化後，顯微注射到一個細胞時期的 *TG(fli1:EGFP)*轉殖斑馬魚胚胎中。受精後 72 小時的幼魚會被放在四環素溶液（10 μg/ml）中飼養 2 天，在 5 天大時挑出在眼睛產生紅色螢光表現的幼魚，然後等到 3 個月後讓它們互相交配，再挑出能在眼睛表現紅色螢光的子代 F1，以後再讓它們互相交配，以期能培育出 homozygous 世代。

結果：我們將此質體顯微注射到數千個 *TG(fli1:EGFP)*斑馬魚胚胎中，在 5 天大時有 2%可在眼睛表現紅色螢光，另有 63%會在其他部位表現紅色螢光。把這些具有眼睛紅色螢光表現的幼魚飼養 3 到 4 個月後互相交配，得到數個子代，目前仍需進行研究來確定注射的序列是否已經傳給這些子代。

討論：我們可能建立了 *TG(Tet-ON-Opsin:VEGF)*轉殖斑馬魚，但是仍需更多的實驗來證明它的作用，包括 VEGF 的表現型態及其受到四環素出現及消失的影響。

關鍵詞：Tet-ON，斑馬魚，血管內皮生長因子，rhodopsin

Abstract

Purpose: Retinal and choroidal neovascular diseases, including diabetic retinopathy (DR), age-related macular degeneration (AMD), retinal vascular occlusive diseases and myopic retinopathy, are tremendously harmful to vision, especially in the developed countries with many aged people. Many angiogenic factors are important for neovascularization. Vascular endothelial growth factor (VEGF) plays an essential role, and interruption of VEGF signaling is an important therapeutic strategy. To study on signal transduction of VEGF and its influence on cellular interaction in retina, an animal model of retinal VEGF over-expression should be extremely useful. Early and constitutive over-expression of VEGF in the retina of transgenic mice causes intra-retinal and subretinal neovascularization. However, such pathogenesis is like congenital retinal degenerations rather than acquired retinal diseases such as AMD and DR. Conditional induction system is a good way to solve the problem of too early over-expression of VEGF.

Methods: We constructed the plasmid, pTet-ON-Opsin-VEGF, which can express both VEGF and reporter (red fluorescent protein) with doxycycline (Dox) exposure. Linked to a retina-specific promoter (*rhodopsin* promoter), it should function in the retinal rod cells only. This 10,000-bp construct was linearized and microinjected into 1-celled *TG(fli1:EGFP)* embryos. After incubated in the Dox solution (10 μg/ml) for 48 hours, the 5 days post-fertilization (dpf) larvae with red fluorescence in eyes were sorted out. The transgenic *TG(Tet-ON-Opsin:VEGF)* founder fish were raised and incrossed with each other after at least 3 months old. Their offspring (F1) with red fluorescence in retina were raised and incrossed with each other later to generate possible homozygotes with more homogeneous gene expression.

Results: Thousands of *TG(fli1:EGFP)* embryos were microinjected with the pTet-ON-Opsin-VEGF. After 2-day incubation in Dox solution, 2% of these 5-dpf larvae expressed red fluorescence in eye, whereas 63% presented ectopic red fluorescence. After these putative founders were cultured for 3 to 4 months, several lines of F1 progeny were collected. Further researches are in progress to confirm whether the injected sequence is transferred to the F1 or not.

Discussions: We possibly generated the *TG(Tet-ON-Opsin:VEGF)* zebrafish. However, further studies are necessary to confirm the expression pattern of VEGF, including the responses to exposure and subsequently elimination of Dox.

Keywords: Tet-ON, zebrafish, VEGF, rhodopsin

Introduction

The newly generated blood vessel is fragile, prone to bleed, and therefore cause severe functional disturbance. That plays a very important role in many major ocular disorders such as diabetic retinopathy, retinal vascular occlusive diseases, retinopathy of prematurity, age-related macular degeneration (ARMD) and myopic retinopathy.[1] Even in the developed countries, neovascularization in retina or choroid is the most common cause of severe visual loss.[2] Good animal models of ocular neovascularization are essential for studying their pathogenesis and treatment.

Many angiogenic factors participate in ocular neovascularization. Among them, vascular endothelial growth factor (VEGF) plays an essential role, and interruption of VEGF signaling is an important therapeutic strategy.[3] VEGF promotes neovascularization, increases vascular permeability and enhances the recruitment, activation and adhesiveness of inflammatory cells.[4] Over-expression of VEGF in transgenic mouse photoreceptors resulted in extensive immature thin-walled blood vessels from deep retinal capillary bed and extended to retinal pigment epithelium and subretinal space.[5]

Zebrafish, *Danio rerio*, is a fresh water teleost. It is easy to care and has fecund, big, transparent and extra-uterine eggs that are suitable for studying embryonic development and gene modulation.[6-8] The remarkable evolutionary conservation of vertebrate eyes provides the basis for using zebrafish as a model system for detection and analysis of genetic defects potentially related to human eye disorders.[7] Zebrafish retina is simple and develops very fast. It becomes functional between 60 and 80 hours post-fertilization (hpf).[9-11] At 72 hpf, the emmetropization of eye and lens achieves and most embryos have hatched out.[10,12] There is also significant homology in the molecular and signaling pathways that drive blood vessel development in vertebrates.[13,14] Zebrafish treated with VEGF receptor inhibitor lacks all major blood vessels, while concomitant over-expression of an effector of VEGF signaling allows blood vessels to form.[15]

Angiogenesis researchers have faced the difficulty of finding suitable methods for assessing angiogenic response.[16] The ideal assay should be reliable, straightforward, easily quantifiable and physiologically relevant. Current methods of visualizing blood vessels of zebrafish include whole mount in situ hybridization,[17,18] detection of endogenous alkaline phosphatase activity,[19] microangiography[20] and transgenic line with fluorescent blood vessels.[21-23] The former 2 methods require fixation of specimen. Microangiography is labor-intensive and does not allow the observation of actively growing vessels that are unable to carry flow.[21,22] By contrast, transgenic zebrafish expressing enhanced green fluorescent protein (EGFP) driven by the promoter for *fli1* in vascular endothelium helps demonstrate that angiogenic blood

vessels had dynamic filopodial activity and pathfinding behaviors.[22]

The tetracycline (Tc)-controlled transcriptional regulation system is the most widely used externally regulatable transgenic system and has been well developed in many organisms including zebrafish.[24,25] When Tc or Doxycyline (Dox) is introduced, the selected sequences are transcribed and then functioning. Together with a tissue-specific promoter, this inducible system could provide both temporal and spatial control of the target gene.[24] Rhodopsin promoter is specifically confined to retinal rod photoreceptor cells. Accordingly, we can establish a Tet-On system to express VEGF in retina and then may induce acquired retinal neovascularization.

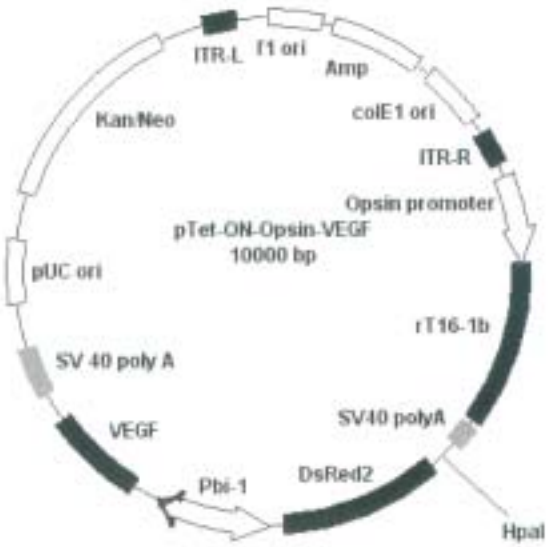
Material and Methods

Animals and embryos

Adult *TG(fli1:EGFP)* zebrafish line obtained from the Zebrafish International Resource Center (Eugene, OR) were bred with a controlled light cycle of 14-hour light / 10-hour dark at 28.5°C.[22,26,27] Fertilized eggs were collected in about 3 hours after the lights turned on, and sorted into petri dishes.[26] The first division occurs at 0.75 hpf, and 3 divisions every 0.25 hour thereafter.[26,28] So the post-fertilization time can be estimated by the cell number observed with a light microscope. Embryos and larvae were raised in the incubators at 28.5°C.

Plasmid construction

The new construct, pTet-ON-Opsin-VEGF, is about 10,000 bp. The important primers during plasmid construction are 5'-GAGGAAGGTCCCGCATAAGCGAACGC (*opsin* promoter, forward), 5'-GGCTGCGGTTGGATGTGGCGCTCAGC (*opsin* promoter, reverse), 5'-ACCGGTATGAACTTGGTTGTTTATTTGATAC (VEGF, forward), and 5'-TCATCTTGGCTTTTCAC (VEGF, reverse). The construct was ligated to pGEM-T Easy vector (Promega, Madison, WI) and transformed into *Escherichia coli* DH5α competent cells.



Microinjection of plasmid and germ-line establishment

At 1-cell stage, *TG(fli1:EGFP)* embryos were microinjected with the linearized

construct, and bred in incubators under the same environmental settings mentioned above. The hatched-out 72-hpf larvae were raised in Dox solution (10 μg/ml) for 48 hours, and then were observed under fluorescein microscope. We sorted out the 5 days post-fertilization (dpf) larvae with red fluorescence in eyes under dissecting microscope equipped with proper fluorescent modules and filters. The transgenic *TG(Tet-ON-Opsin:VEGF)* founder fish were raised and incrossed with each other after at least 3 months. Their offspring (F1) with red fluorescence in retina were raised and incrossed with each other again to generate possible homozygotes with more homogeneous gene expression.[27]

Results

We microinjected the construct, pTet-ON-Opsin-VEGF, into thousands of *TG(fli1:EGFP)* embryos. After 2-day incubation in Dox solution, 2% of these 5-dpf larvae expressed red fluorescence in eye (Figure 1), whereas 63% presented ectopic expression of red fluorescence.

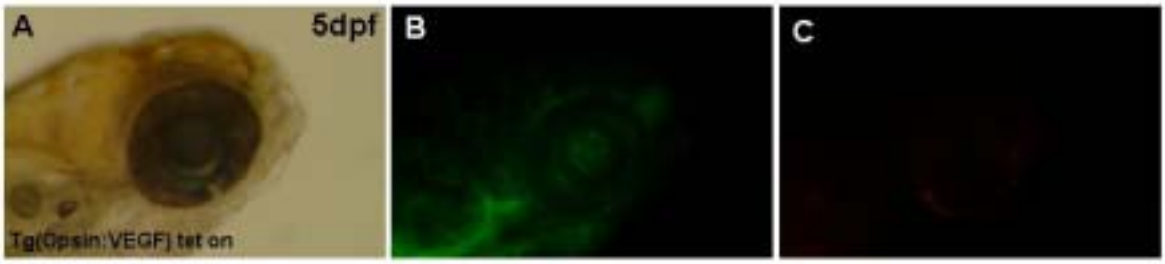


Figure 1. The 5-dpf *TG(Tet-ON-Opsin:VEGF)* founder fish after induction with Dox for 2 days does not show any difference with other *TG(fli1:EGFP)* fish when observed under light (**A**). However, it shows diffuse green fluorescence in eye (**B**), and red fluorescence in eye (**C**).

We raised tens of the P0 larvae expressing red fluorescence in retina. After these putative founders were cultured for 3 to 4 months, several lines of F1 progeny were collected. Further researches are in progress to confirm whether the injected sequence is transferred to the F1 or not. And then their expression pattern will be studied.

Discussions

Although over-expression of VEGF in transgenic mouse photoreceptors resulted in extensive immature thin-walled blood vessels from deep retinal capillary bed and extended to retinal pigment epithelium (RPE) and subretinal space,[10] such pathogenesis is congenital rather than acquired. Conditional induction system is a good way to solve the problem of too early over-expression of VEGF. We successfully constructed the complex Tet-ON plasmid for zebrafish retinal rod cells to

simultaneously produce VEGF and reporter after Dox exposure. It took more than half a year to establish the *TG(Tet-ON-Opsin:VEGF)* P0 founders. We need more time and studies to confirm the inheritance, expression pattern of VEGF, and the responses to exposure and subsequently elimination of Dox.

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