

行政院國家科學委員會專題研究計畫 成果報告

Autosomal Dominant Retinitis Pigmentosam 基因轉殖斑馬 魚

計畫類別： 個別型計畫

計畫編號： NSC92-3112-B-002-004-

執行期間： 92 年 05 月 01 日至 93 年 04 月 30 日

執行單位： 國立臺灣大學醫學院眼科

計畫主持人： 陳慕師

共同主持人： 陳裕芳，王安國，蔡懷楨，王一中

計畫參與人員： 陳裕芳，王一中，陳慕師

報告類型： 完整報告

報告附件： 出席國際會議研究心得報告及發表論文

處理方式： 本計畫可公開查詢

中 華 民 國 93 年 9 月 20 日

基因體醫學國家型科技計畫

National Research Program for Genomic Medicine
National Science Council, the Executive Yuan, ROC.

Autosomal Dominant Retinitis Pigmentosam 基因轉殖斑馬魚

Autosomal Dominant Retinitis Pigmentosam Model in
Transgenic Zebrafish

報告類別：☐ 新進研究計畫 ☐ 修正後計畫書 ☒ 年度成果報告
(New Proposal) (Revised Proposal) (Progress Report)

計畫類別：☒ 個別型計畫 ☐ 整合型計畫
(Individual Project) (Program Project)

計畫編號：NSC 92-3112-B-002-004

計畫主持人 (Principle Investigator)：陳慕師

共同主持人 (Co-Principle Investigator)：王一中、陳裕芳

執行單位 (Institution)：臺大醫學院 眼科部

中華民國 92年 12 月 日

National Research Program for Genomic Medicine
National Science Council, the Executive Yuan, ROC.
Progress Report—Research Project

基因體醫學國家型科技計畫

國科會延續性計畫進度報告

個別型計畫 (Individual Project)

Program Classification:

- ☒ Genomic Medicine ☐ Bioinformatics
☐ Proteomics & Structural Genomics ☐ ELSI

Project Number: NSC 92-3112-13-002-004 (計畫編號)

NSC Funding Number: _____ (93 年度國科會預核編號)

Title of Project 計畫名稱	(in Chinese) 中文 Autosomal Dominant Retinitis Pigmentosam 基因轉殖斑馬魚
	(in English) 英文 Autosomal Dominant Retinitis Pigmentosam Model in Transgenic Zebrafish
Institution 研究（執行）單位	(in Chinese) 中文 台大醫學院 眼科部
	(in English) 英文 National Taiwan University Hospital Ophthalmology
Principle Investigator 計畫主持人	(in Chinese) 中文 陳慕師
	(in English) 英文 Chan Muh-Shy

FY	2002	2003	2004	Total
Budget	4,650,515	3,599,782	4,066,343	12,956,640

(in NT dollars: 1USD = 34 NTD)

Signature of the PI : _____ Date : _____

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Progress Report

1. Response to previous reviewers' critiques

Please describe the previous reviewers' critiques and how based on the critiques, you made modifications to specific aims, experimental design, or resource allocation etc.

Critique from the reviewers are further elucidated as the following:

The PI does not have much experience in zebrafish; however, he is outstanding and well-known in research of ophthalmology, especially in field of retinal diseases. Besides, technical support from the zebrafish expert, Dr. Tsai, can be a strong approval for the success of this project. Dr. Tsai has been studies in the field of zebrafish for long, and he also has reported many useful preliminary data from his published work. Hence, cooperation between the PI and Dr. Tsai can give unique contribution to the field of ophthalmology and zebrafish biology.

- 1. Although the mutation characteristics of RP patient in Taiwan are not identified in this project, hundreds of mutations has been reported worldwide. We believe that the animal disease model established in this project is helpful to prove the causal-result relationship between RP and the mutation genes associated with RP, since the specific aim 1 in this project is focused on the retina-specific gene expression of EGFP in zebrafish. The retina-specific gene expression is essential for further comparison of regulated or over-expressed opsin. With this model, we can study the mutations responsible for RP in Taiwan more detail.**
- 2. Rational for the selection of the two mutants: P23H and V20G/P23H/P27L:
For P23H: Rhodopsin gene defects account for 25% of cases with ADRP in the USA. The most common rhodopsin mutation is P23H, which is found in about one third of patients with rhodopsin mutations (IOVS 2000; 41:3124-3127).
For V20G/P23H/P27L: An independent transgenic mouse line expression the rhodopsin mutations V20G, P23H, and P27L producing slowly progressing photoreceptor degeneration resembling human adRP has been documented (IOVS 2001; 42: 826-833). Stimulation of human adRP with the same triple mutants was also been studied in transgenic mice before (Proc Natl Acad Sci USA 1993; 90: 5499-5503, Neurobiology)**

3. The concern that the human rhodopsin mutants may not inhibit the function of the endogenous zebrafish rhodopsin can be answered as the following:

First, we believe that over expression of mutation gene can cause disease phenotype expression. Since P23H is regarded as an AD mutation, it can be expressed in phenotype, theoretically, whether or not endogenous genes exist.

Second, we believe that human rhodopsin gene can affect the phenotype of zebrafish. Although preliminary data from zebrafish is lacking, a morphometric study in transgenic rat models of retinitis pigmentosa has been reported (IOVS 2003; 44: 848-855). Besides, rhodopsin accumulation at abnormal sites in retinas of mice with a human P23H rhodopsin transgene has also been documented previously (IOVS 1994; 35: 4049-4062).

4. As the reviewers' suggestion, if the p23, V20, P27 residues are conserved, we will consider constructing the same mutations into the zebrafish opsin gene using the zebrafish opsin cDNA, and by using the zebrafish opsin mutant cDNAs to generate transgenic zebrafish lines. At present, human rhodopsin mutants are considered first because blood sample of RP patients is more easily accessible to us.
5. Functional analysis of retina will be performed by ERG. This is challenging indeed; however, detail methodology and technology has been published elsewhere (cited in application proposal). We believed that same procedure can be established and repeat in our laboratory.
6. Since after differentiation, opsin does not express in tissues other than the retina, it is unpredictable when it is expressed elsewhere. Hence, we think that creation of a retina-specific mutation is mandatory.
7. Dr. Tsai has established a 'carp model' for rhodopsin study (FEBS Letters. 508(2): 265-71, 2001 Nov 16. and Comparative Biochemistry & Physiology. Part B, Biochemistry & Molecular Biology. 125(1): 37-45, 2000 Jan.). Since zebrafish has many advantages over carp, we think it is worthwhile to generate another 'zebrafish model' transgenic line which is tissue specific.
8. The environment and resources of this application can be divided into two parts. Rhodopsin will be constructed in the laboratory of Ophthalmology in NTUH (the PI's laboratory). Pronuclear injection and gene expression

will be performed in Dr. Tsai's laboratory. Procedures to be performed in both laboratories are well prepared and designed. Except for ERG, we also have much experiences in these procedures in these two laboratories, respectively.

- 9. We hope that by establishing the zebrafish model we can provide a better way of research in ophthalmology. We also hope the causal-result relationship between mutant genes and diseases as well as the genetic mechanism of the diseases can also has further understanding. Hence, we believe this project is important in this field.**

In this year, we have set up our transgenic zebrafishes facility. The deletion and mutant construct were ready for injection since this May. At this moment, the fusion constructs of EGFP and mutant rhodopsin cDNA were also injected in May, so we have F1 progenies for these constructs now.

Before these transgenic experiments, we have transfected deleted constructs into Cos1 cells, the expression of these constructs were expected as our initial concerns. Our findings were that the N-terminal extracellular domains and C-terminal cytoplasmic domains did not affect the trafficking of rhodopsin protein in the cells. However, the transmembranous domains actually directed the trafficking of rhodopsin protein in the Golgi and ER. We also presented our findings in the annual meeting of Genome Project of NSC

Signature/ Date :

2. Specific Aims

Please state the overall goals of the project, and specific aims, as reviewed and approved by the Study Section and actually awarded. If these specific aims as actually funded did not differ in scope from those actually pursued during the grant period, and if the aims have not been modified, state this. If they have been modified, give the revised aims.

Retinitis pigmentosa (RP), the group of hereditary conditions involving death of retinal photoreceptors, represents the most prevalent cause of visual handicap among working populations in developed countries. Genetically, RP is the most heterogeneous disorder; it has been estimated that autosomal dominant RP (adRP) and autosomal recessive RP (arRP) each accounts for approximately 20% of the RP cases, and that X-linked RP (xRP) accounts for 10% of the cases. RHO encodes opsin (the apoprotein of rhodopsin), the light-sensing molecule in the rod photoreceptor cell. To date, over 100 RHO mutations have been reported. Most RHO mutations cause adRP. However, the pathophysiologic and molecular mechanisms underlying dominant RHO mutations remain unclear (Wang Q, et al., 2001 and Farrar GJ, et al., 2002).

The zebrafish, *Danio rerio*, has emerged as a novel vertebrate model system that is amenable to mutagenesis and transgenesis. High fecundity, rapid oviparous development, and a translucent embryo make zebrafish a prolific experimental model. Furthermore, the zebrafish eye possesses distinct advantages for studying the development, function, and inherited diseases of the retina.

Because the rhodopsin EGFP transgenic zebrafish had already been established well in Dr. Tsai's laboratory, we will continue his model to establish a human autosomal dominant retinitis pigmentosa disease analogue (Lim J et al., 1997; Hsiao CD, et al., 2001; Ma GC, et al., 2001)

Specific Aim 1: To create retina-specific expression of EGFP in zebrafish

The retina-specific gene expression is essential for further comparison of regulated or over-expressed opsin. We will identify a 1.2 kbp fragment containing the putative zebrafish rod opsin promoter, the transcription start site, and 52 bp of 5'-untranslated sequence from zebrafish genomic DNA. We will construct pZOP-EGFP by insert the DNA fragment into upstream of the EGFP cassette and to generate tissue-specific transgenic zebrafish. Whole-mount and frozen sections of larval and juvenile transgenic G1 fish will be labeled by polyclonal antirhodopsin. Finally, we wish to evaluate temporal and spatial patterns of EGFP and endogenous rhodopsin in zebrafish.

Specific Aim 2: To determine the function role (ERG) of wild type and mutated rhodopsin in the pathogenesis of retinitis pigmentosa of zebrafish

Normal and P23H mutated human rhodopsin gene will be obtained from patients' WBC. The over expressed rhodopsin will be calculated by Western blotting. The 'regulated'-rhodopsin will be driven by zebrafish opsin promoter. The morphology (apoptosis) and the function (ERG) of retinal photoreceptor cells will be compared.

Progress Summary

Summarize concisely the results obtained for **each specific aim during the past year (or reporting period)**. Negative results, if any, should also be included and approaches taken to improve the prospects of the project discussed. (Do not exceed **5 pages**, not including figures and references.)

I. To create a retina-specific expression in zebrafish

We identified a 2.1-kb fragment containing the putative zebrafish rhodopsin promoter, and the rhodopsin gene. This fragment contains the region from nucleotide 2 to 2154 and was amplified from zebrafish genomic DNA by PCR mix reaction (ABgene). The polymerase chain reaction was using a forward primer (5'-cgggatcccGAGCTCGGGATAGATCCTTCTG-3') and a reverse primer (5'-cgggatcccgTTACGCCGGAGACACGGAG-3'). BamHI restriction site was attached to 5'-edges of both the forward and reverse primers. The resulting 2.1 kb fragment was cloned into pEGFP-1 vector obtained from Clontech Laboratories, Inc. The GFP-expression construct was also digested with BamHI. This 2.1 kb DNA fragment was separated in agarose gel and cloned into pIRES2-EGFP-CMV⁻ vector. The pIRES2-EGFP-CMV⁻ vector was modified to lack Human cytomegalovirus (CMV) immediate early promoter by deletion of 584 nucleotides .

II. Zebrafish rhodopsin promoter and human rhodopsin gene expression construction.

1. We clone zebrafish rhodopsin promoter by PCR method with a forward primer (V2-1~1105-F+LINKER- gaagatcttcGAGCTCTGGGATAGATCCTTCTG) and reverse primer (V2-1~1105-R+LINKER- cggaattccgCCTCTGTACCGTTCATGGCT) and we can get a 1.089 kb fragment. This fragment is digested and cloned into vector pEGFP-1 by restriction enzyme BglII and EcoRI, and we can get a 5.8 kb clone (pEGFP-1-Zp) with pEGFP-1 vector.
2. Human rhodopsin cDNA (1.067 kb fragment) was cloned from RT-PCR reaction with a forward primer (v2-hu gene-96-EcoRI-F- cggaattccgATGAATGGCACAGAAGGCCCTAAC) and a reverse primer (v3-hu gene-1163-SmaI-R-tccccgggggaCCACAGAGTCCTAGGCAGGT). This fragment can be digested and cloned into pEGFP-1 vector by restriction enzyme EcoRI and XmaI, and resulted into a 5.8 kb clone (pEGFP-1-Hg).
3. The human rhodopsin cDNA in pEGFP-1-Hg was further cloned into pIRES2-EGFP vector by restriction enzyme EcoRI and XmaI, and resulted in a 6.4 kb vector (pIRES-CMV⁺ -Hg).
4. The 1.089 kb zebrafish rhodopsin promoter was further cloned into

- pIRES-CMV⁺ -Hg vector from pEGFP-1-Zp by restriction enzyme BglII and EcoRI, and the resulting clone will be a 6.4 kb vector (pIRES-CMV⁺ -Zp+Hg).
5. The pIRES-CMV⁺ -Zp+Hg can be reached by restriction enzyme BglII and XmaI, and the 2.156Kb zebrafish rhodopsin promoter and human rhodopsin gene fragment to ligate in the pIRES-CMV⁺ vector can be ligated into a 7 kb vector (pIRES-CMV⁺ -Zp+Hg).

III. A human rhodopsin promoter directs EGFP expression

1. The complete sequence of human rhodopsin promoter will be obtained from NCBI web site. The 5.7 kbp human rhodopsin promoter was amplified by polymerase chain reaction.
2. The polymerase chain reaction was using a forward primer(5'-tccccgcggAGCTGGCCCTCCCTACCT-3') and a reverse primer(5'-catcccgggCCCAGGGTGGCAAAGAAG-3'). SacII and XmaI restriction sites were attached to 5'-edges of the forward and reverse primers, respectively. A 5.7 kbp DNA fragment was located in the nt 2 to 5792 region. This human rhodopsin promoter fragment was cloned upstream of the EGFP- coding sequence in pEGFP-1

4. Projected Timeline & Brief Summary of Plans for Next Year

Provide a short paragraph to describe the plans for next year including a realistic timetable and appropriate milestones for the project, based on the progress reached so far. (Do not exceed **1 page**.)

We hope to identify the promoter sequence of zebrafish opsin gene, which can drive EGFP gene well. Specifically, the EGFP should be expressed with spatially and temporally correction. The Gene transmission through germ line will be confirmed to make sure that it is possible to create rhodopsin promoter-driven transgene in zebrafish.

We will screen the patients with clinically autosomal dominant retinitis pigmentosa to check their genotype. We will focus on the nucleotide mutation, which will direct a P23H mutation in amino acid sequence. Besides, the complete gene with or without P23H mutation will be cloned separately into the amplifying plasmid. Finally, the different transgenic zebrafish will be created as the work in the first year. Genotype will be confirmed.

The well transgenic zebrafish will be inbred for phenotype comparison. The details of morphological change, protein expression pattern, amount and time-dependent course will be documented. The cell apoptosis will be compared. We will set the specific ERG system to detect the function change of the retina of transgenic zebrafish.

5. Personnel

Summarize the **personnel involved in the project during the grant period**. List the personnel in accordance to the following categories: (1) senior investigators, including visitors; (2) postdoctoral fellows; (3) graduate students; (4) technicians or research assistants. Specify for each individual the period of involvement and the percentage commitment of effort.

Name		Position Title	Education Degree	% of personal effort on this project	Job Description or Responsibilities
In Chinese	In English				
陳慕師	Muh-Shy Chan	Professor	Ph.D.	30%	To creat retina-specific expression in zebrafish
王一中	I-Jong Wang	Physician	Ph.D.	30%	Zebrafish rhodopsin promoter and human rhodopsin gene expression construction
陳裕芳	Yu-Fung Chen	Physician	M.D.	30%	A human rhodopsin promoter directs EGFP expression

6. Publications and/or Patents

6a. Publications

List the title and complete references (author(s), journal or book, year, page number) of all publications **directly resulting from studies supported by the project (i.e., with citation of this grant in the acknowledgement section)**. List the publications for the project in accordance to the following categories: (1) manuscripts published and accepted for publications; (2) manuscripts submitted; and (3) conference proceedings. Provide one copy of each publication not previously reported to the National Science Council in the Appendix.

6b. Patents

List all inventions disclosed, patents filed, and patents granted. Please note the inventors, assignee, title of patent, country or area where patent applied for, filing or issued number and date.

Appendix