行政院國家科學委員會專題研究計畫 成果報告

利用斑馬魚系統進行藥物篩選
研究成果報告(精簡版)

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研究計畫內容：

(一）研究計畫之背景及目的

INTRODUCTION

Recently, researchers have created assays based on the zebrafish (*Danio rerio*), a small freshwater teleost . Zebrafish embryos are transparent and develop externally from the mothers, permitting direct assessment of drug effects on internal organs and tissues in vivo. The fish is easy to maintain and breed, and their embryo product is high: Each female can produce 100~200 embryos per mating, providing large numbers of animals for high-throughput assays. Because of the embryos size are very small, zebrafish embryos and early larvae can be raised in only 100μL of water in the wells of a 96-well plate for whole-animal assays requiring only small amounts of compounds. Drug administration is also simple because researchers can dissolve small-molecule compounds in the water, where they diffuse into the embryos.

The rapid external development of the transparent zebrafish embryo permits easy visual analysis of phenotypic defects. Currently, the simplest method for generating a loss of-function in a particular gene in the zebrafish is the use of antisense technology using morpholino oligonucleotides to induce a translational block in gene function (Nasevicius and Ekker, 2000; Ekker, 2000). Although important information can be obtained by morpholino injections, this method, similar to the use of null mutations, blocks gene function at the earliest stage.

The major regulators of angiogenic signaling, the vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs), promote endothelial cell differentiation, survival, and migration (Carmeliet, 2000; Ferrara and Alitalo, 1999). The chemically induced VEGF receptor defect can be prompted by up-regulating the activity of a putative downstream effector, AKT/ PKB, allowing for normal endothelial cell functions in migration and survival. In this project, we also used this strategy to study angiogenic signaling in the transparent zebrafish embryo.

Angiogenesis

In recent years, understanding how vessels are formed has attracted considerable interest. This is mainly due to the hope that, in future, can be treated by inhibiting a common process occurring in most tumors, namely the formation of new vessels from existing ones (angiogenesis) and disrupting the nutrient flow to the cancer cells. The optical transparency and its ability to survive without functioning circulation for 3–4 days make the zebrafish especially amenable to in vivo vascular cell biology. (Weinstein, 2002) In contrast, in mammals, most vascular cells are deep within the animal and not accessible for optical imaging. The vascular system of zebrafish larvae can be imaged through fluorescence microscopy, using transgenic animals or by injecting fluorescent dextran beads into the vascular system (microangiography), or through an enzymatic assay, achieved the vascular localization of alkaline phosphatase in young zebrafish.(Habeck, et al., 2000) A number of genes involved in vertebrate angiogenesis, e.g., vascular endothelial growth factors, angiopoietins, ephrins and their respective receptors, have been identified in zebrafish and been shown to have similar functions to those in mammals.((Weinstein, 2002; Habeck, et al., 2000) Knockdown of VEGF, for example, induced by injecting antisense oligonucleotide

morpholinos, targeting VEGF, into zebrafish embryos (Nasevicius, et al., 2000) leads to a complete lack of all vessels. A phenocopy can be obtained by bathing embryos in medium containing PTK787/ZK222584 (an anilinophthalazine compound), supposedly selective for VEGF receptors (VEGFR). (Chan, et al., 2002) The specificity of the compound has been shown by the lack of destructive effects on the general morphology and by a significant reduction of endothelial cells in the region of the dorsal artery and posterior cardinal vein at 24 hpf. When the compound was administered relatively late, only the intersegmental vessels failed to formand washout of the drug induced a slight recovery. (Chan, et al., 2002) These data indicate a major advantage of drugs in comparison to morpholinos, the former can be added at any stage during embryo development and induction of chemotypes might be reversible. The high similarity of the human and zebrafish VEGFR tyrosine kinase domain is the likely structural basis for the observed specific effects of the human VEGFR inhibitors on zebrafish embryos. Screenings for new anti-angiogenic drugs appears possible in zebrafish. Embryos are put into 96-well plates, bathed with compounds and, after a few days of development, a fluorescent readout for endothelial alkaline phosphatase activity is used as an indicator for anti-angiogenic activity of the compounds tested.

Zebrafish

Zebrafish is a good model organism for the study of vertebrate development (Penberthy et al., 2002; Rubinstein, 2003). The embryos develop outside the mother and are optically transparent, allowing direct observation of their embryonic development that takes only 48 hours for completion at 28°C. We have cloned several zebrafish tissue-specific promoters including pancreatic-, neuron-, and muscle-specific promoters. Their tissue specificies of gene expression were confirmed by expression of GFP in zebrafish embryos. Therefore, these tissue-specific promoters could be used to drive GFP or RFP expression in zebrafish embryos. In general, it is common to investigate the function of known or novel genes by gain-of-function and loss-of-function in zebrafish. To achieve gain-of-function, genes of interest are driven by tissue-specific promoters and injected into one-cell zebrafish embryos (Gong et al., 2001). Alternatively, the expression constructs under the control of either ubiquitous or tissue-specific promoter were co-injected with tissue-specific promoter/GFP construct. On the other hand, to achieve loss-of-function, genes of interest are knockdowned by injection of morpholino antisenseoligomnucleotides (MAO) or coinjection of MAO with tissue-specific promoter/GFP construct (Nasevicius and Ekker, 2000; Urtishak et al., 2003). The suitable transgenic GFP/RFP zebrafishes also can be used to inject MAO or expression constructs, respectively.

（二）研究方法及進行步驟

Materials and Methods

All the restriction enzymes were purchased from the Promega Biosciences, Inc. (Madison, WI, USA) or New England Biolabs, Inc. (Beverly, MA, USA). Chemicals were purchased from Merck (Darmstadt, Germany) or Sigma (MO, USA).

Fish

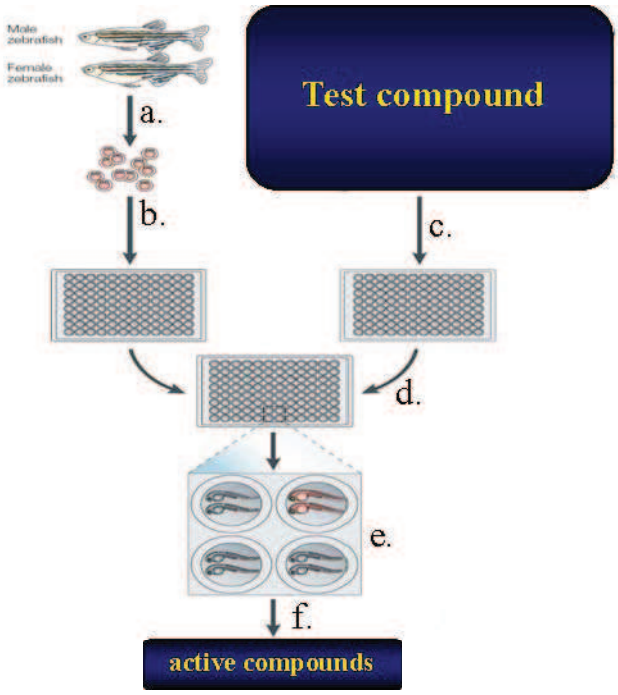
Zebrafish (*Danio rerio*) were maintained at 28ºC on a 14 h-light/10 h-dark cycle. Embryos were incubated at 28ºC and different developmental stages were determined according to the description in Zebrafish Book (Westerfield, 1995).

Maintenance of zebrafish and drug treatments

Embryos were collected by natural spawning, raised in embryo media (Westerfield, 1995) and staged up to 24 hr (30 somites) according to Kimmel et al. (1995); beyond this point, embryo stage is given as hours post fertilization (hpf). The test compounds were dissolved in DMSO at stock concentrations of 2~50 mM; then diluted in embryo media (Westerfield, 1995) and added to live zebrafish embryos at the stages indicated (described in below). Control embryos were treated with the equivalent amount of DMSO solution. All embryos are incubated at 28°C.

Zebrafish drug screens.

a. Adult zebrafish are mated, producing 200~300 embryos per female. **b**. Embryos are distributed to 96-well assay plates. Typically, three embryos are placed in each well.**c**. A small-molecule library that contains potentially biologically active compounds is synthesized or acquired. **d**. Test compound are added to the water surrounding the zebrafish. A single small molecule or combinations can be added to each well. **e**. After a period of incubation, the phenotypic effects of the compounds on the zebrafish are determined visually read-out. **f**. referral to the library database reveals the identities of the biologically .



Total RNA isolation and first-stranded cDNA synthesis

Total RNA was isolated from the fertilized eggs at different stages (0, 5, 12, 24, 48 and 72 h postfertilization) and from various tissues (brain, gill, intestine, muscle and ovary) of zebrafish using the RNAzol reagent (Tel-Test, Inc.) according to the instructions of the manufacturer. After treatment with RQ1 RNase-Free DNaseI (Promega), 50-100 μg of total RNA from each tissue was used for the first strand cDNA synthesis in a 25 μl reaction mixture containing 10 pmole of oligo(dT) primer and 100 ng of random primer (Promega), 30 units of RNasin (Promega), 1 mM dNTP, 10 mM dithiothreitol, and 300 units of Superscript II RT (Invitrogen Life technologies Co., CA). The reaction mixture was incubated at 42oC for 1 h. Two μl of the

cDNA products was used for subsequent PCR amplification.

Microinjection of expression clones into zebrafish embryos

The test compounds were microinjected into the zebrafish embryo at one-cell stage by using Narishige IM 300 microinjector system (Narishigi Scientitific Instrument Lab., Tokyo, Japan). Embryos at 48 h and 72 h postfertilization were observed under an Olympus IX70-FLA inverted fluorescence microscope. Images were taken by using the SPOT system and assembled by PhotoShop program.

（三）文獻探討

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（四）結果與討論

收集受精後 72 小時 （72 hpf）未經藥物處理的胚胎，以螢光顯微鏡進行觀察，利用綠色螢光蛋白的表現，可清楚觀察和記錄斑馬魚血管發育和分佈（如圖 1 所示）。

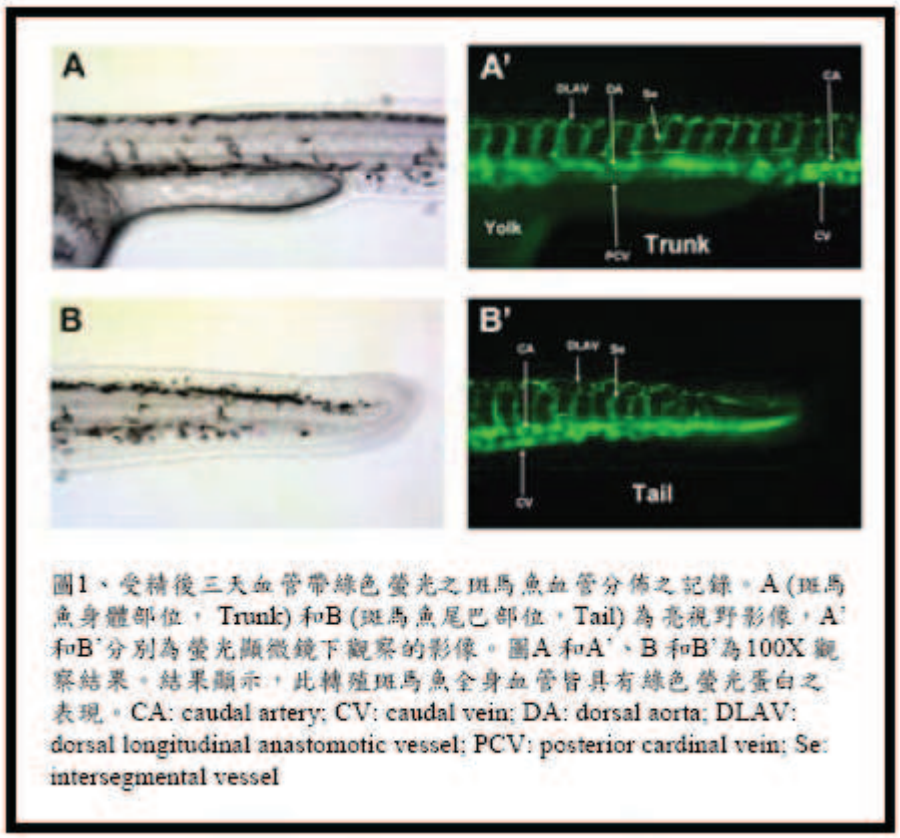

圖1、受精後三天血管帶綠色螢光之斑馬魚血管分佈之記錄。A (斑馬魚身體部位，Trunk) 和B (斑馬魚尾巴部位，Tail) 為亮視野影像，A' 和B' 分別為螢光顯微鏡下觀察的影像。圖A 和A'、B 和B' 為100X 觀察結果。結果顯示，此轉殖斑馬魚全身血管皆具有綠色螢光蛋白之表現。CA: caudal artery; CV: caudal vein; DA: dorsal aorta; DLAV: dorsal longitudinal anastomotic vessel; PCV: posterior cardinal vein; Se: intersegmental vessel

本計畫由「生技製藥國家型科技計畫辦公室」提供藥物利用斑馬魚的系統進行藥物篩檢，於計畫執行期間，目前總共分析 120 種藥品，結果如圖，其中 9 種藥物造成心臟發育異常 （圖2、3）、6 種藥物造成體節發育異常（圖2、4 和 5）、6 種藥物會造成腦組織死亡（圖 5）、4 種藥品影響體節發育造成魚體彎曲（圖2）、15種藥物影響胚胎發育造成死亡（圖 5）、16 種藥物影響心跳（圖3）、2 種藥物除了影響心跳外同時血液循環亦受影響，甚至無血液循環（圖3）。

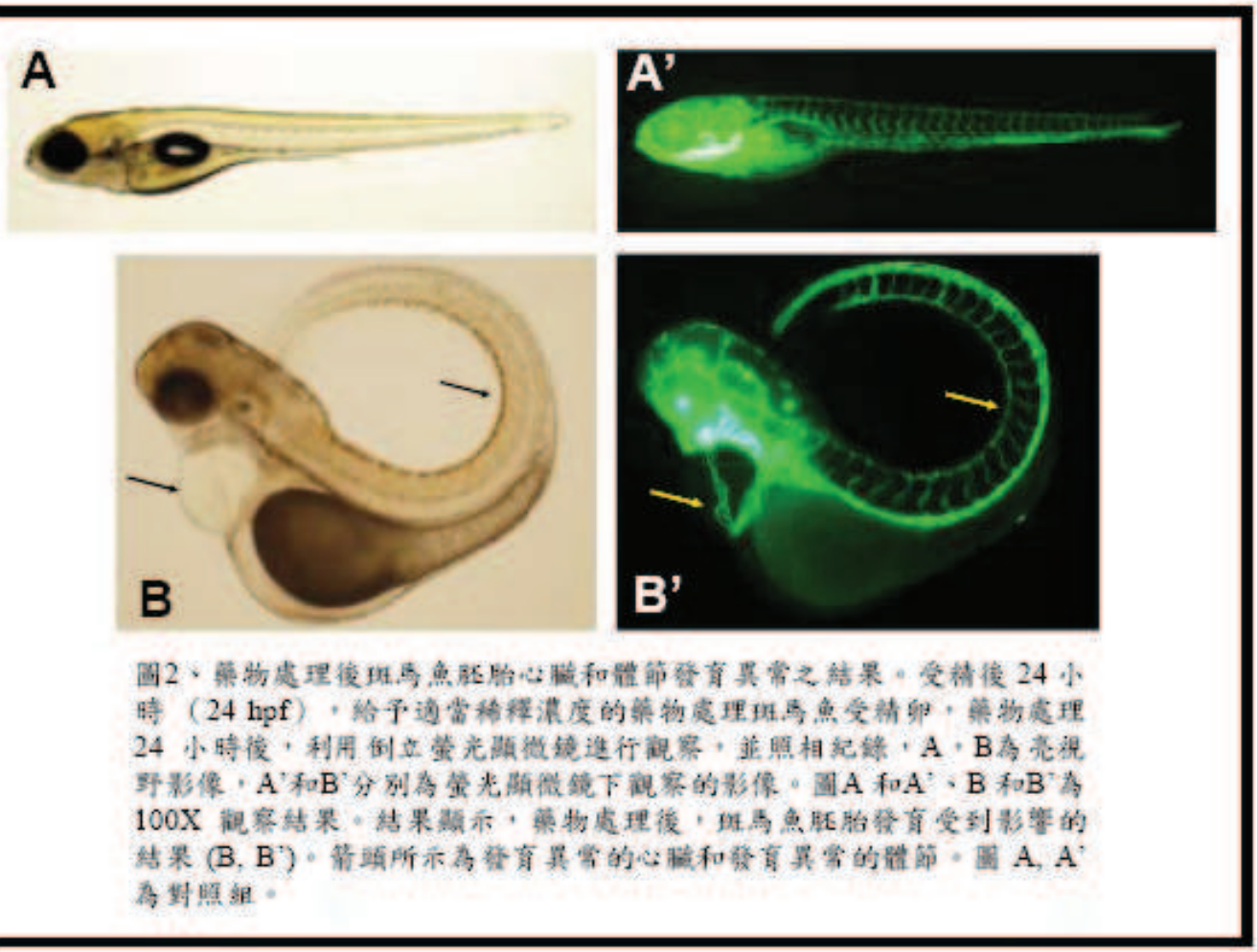

圖2、藥物處理後斑馬魚胚胎心臟和體節發育異常之結果。受精後 24 小時 （24 hpf），給予適當稀釋濃度的藥物處理斑馬魚受精卵，藥物處理 24 小時後，利用倒立螢光顯微鏡進行觀察，並照相紀錄，A，B為亮視野影像，A'和B'分別為螢光顯微鏡下觀察的影像。圖A 和A'、B 和B'為 100X 觀察結果。結果顯示，藥物處理後，斑馬魚胚胎發育受到影響的結果 (B, B')。箭頭所示為發育異常的心臟和發育異常的體節。圖 A, A' 為對照組。

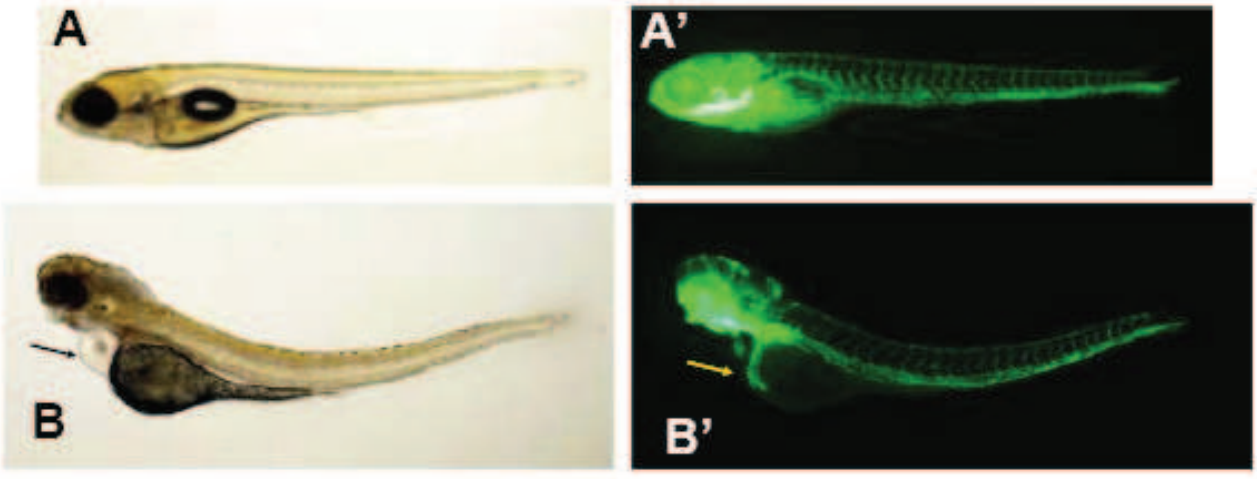

圖3、藥物處理後斑馬魚胚胎心臟發育、心跳和血液循環異常之結果。受精後 24 小時 （24 hpf），給予適當稀釋濃度的藥物處理斑馬魚受精卵，藥物處理 24 小時後，利用倒立螢光顯微鏡進行觀察，並照相紀錄，A，B為亮視野影像，A'和B'分別為螢光顯微鏡下觀察的影像。圖A 和A'、B 和B'為100X 觀察結果。結果顯示，藥物處理後，斑馬魚胚胎心臟發育、心跳和血液循環異常 (B, B')。箭頭所示為發育異常的心臟和發育異常的體節。圖 A, A'為對照組。

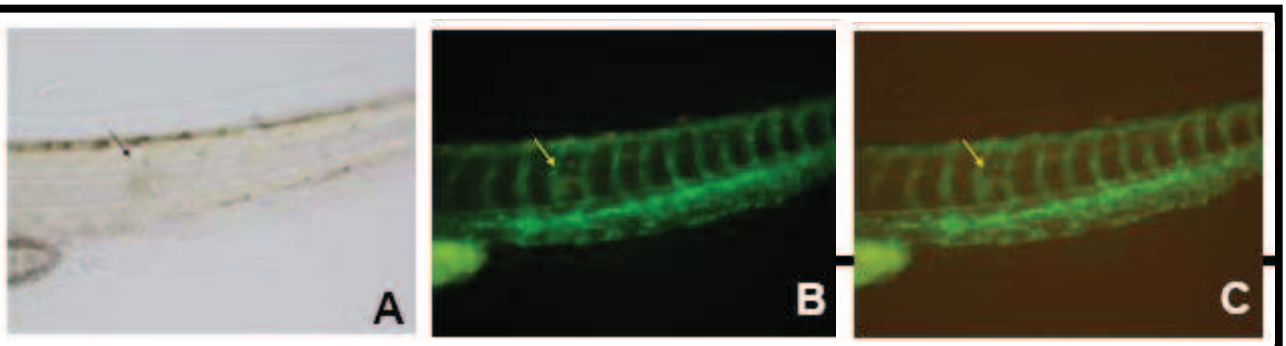

圖4、藥物處理後斑馬魚胚胎脊索發育異常之結果。受精後 24 小時（24 hpf），給予適當稀釋濃度的藥物處理斑馬魚受精卵，藥物處理 24 小時後，利用倒立螢光顯微鏡進行觀察，並照相紀錄，A為亮視野影像，B 為螢光顯微鏡下觀察的影像，C 則為合併後的圖形。圖A，B 和 C 為 200X 觀察結果。結果顯示，藥物處理後，斑馬魚胚胎脊索發育異常，箭頭所示為脊索發育異常的區域。

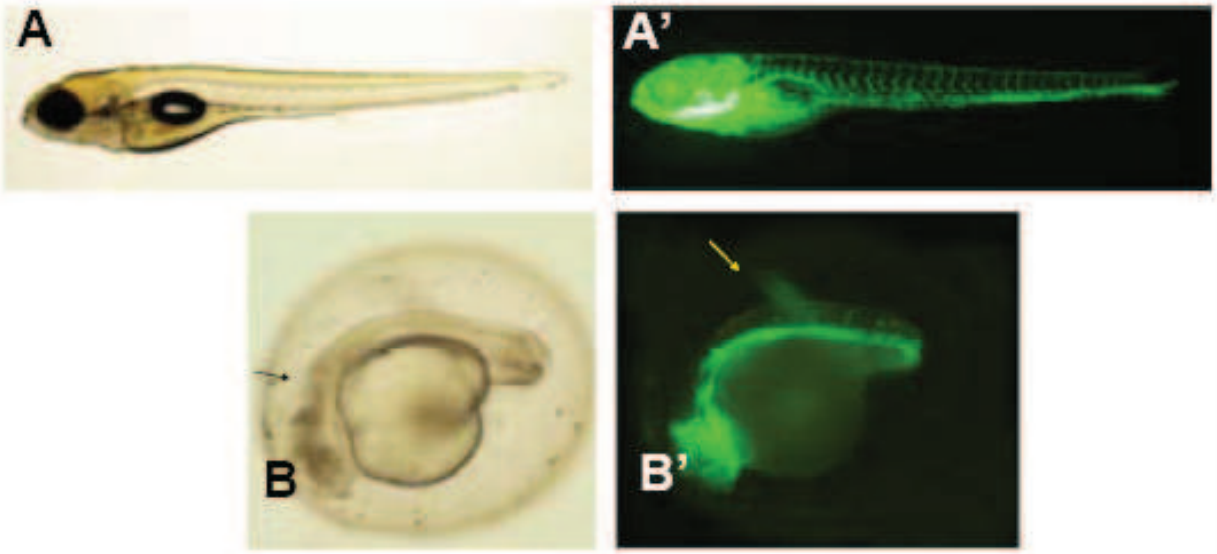

圖5、藥物處理後斑馬魚胚胎腦組織壞死、體節異常和血管發育異常之結果。受精後 24 小時（24 hpf），給予適當稀釋濃度的藥物處理斑馬魚受精卵，藥物處理 24 小時後，利用倒立螢光顯微鏡進行觀察，並照相紀錄，A，B為亮視野影像，A'和B'分別為螢光顯微鏡下觀察的影像。圖A 和A'、B 和B'為100X 觀察結果。結果顯示，藥物處理後，斑馬魚胚胎腦組織壞死、體節異常與血管發育異常 (B, B')。箭頭所示為血管發育受到抑制（黃色箭頭所示）和壞死的腦組織（黑色箭頭所示）。圖 A, A'為對照組。

經過初步篩選後，將會影響斑馬魚胚胎發育或血管形成的藥物選出，利用 RT-PCR 方法進行相關基因表現之分析，例如斑馬魚 *vegf* 基因在胚胎發育過程之表現，結果顯示如圖 6，斑馬魚 *vegf* 基因有兩個不同的轉錄剪接形式 (alternative splicing form)，*vegfA* 和 *vegfA-b*。目前主要以分析促進血管形成或抑制血管形成的基因，來進行 PCR primer 的設計和測試 （例如 VEGFR-1、VEGFR-2 等）。

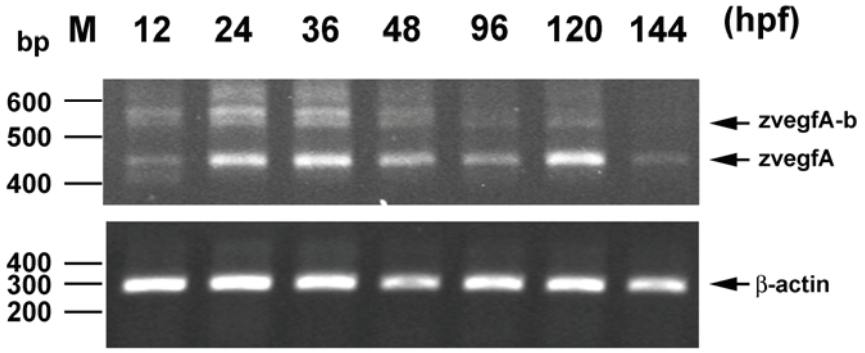

圖 6、利用 RT-PCR 分析斑馬魚 *vegf* 基因在胚胎發育時期表現的情形。

在本計畫執行中發現有些藥物對斑馬魚體節、血管和心臟發育會造成影響，與 *heart-and-soul* 基因變異的斑馬魚有相似的表現型，過去研究指出在 serine/threonine kinase inhibitors (例如 PKC412 和 U0126) 處理後，斑馬魚胚胎也有相同的表現型。未來將利用這些篩選出來的藥物處理斑馬魚，並以 RT-PCR 進行相關基因表現之分析和 morpholino oligonucleotide (MO) 的技術將特定基因的功能抑制 (knock-down)，來研究影響斑馬魚體節、心臟和抑制血管形成可能的分子機轉，進而用來評估這些藥物的生物活性。

計畫成果自評：

本計畫「利用斑馬魚系統進行藥物篩選」，以轉殖斑馬魚來進行藥物的篩選，可提供快速的藥物檢測系統，同時又是活體分析系統，可用來分析藥物之生物活性。本計畫中所篩選的 120 種藥物中，發現有些藥物對斑馬魚胚胎的體節、血管和心臟發育會造成影響，這些活體的結果，在 in vitro 細胞的研究中是無法觀察到的。而未來將可利用這些篩選出來的藥物處理斑馬魚，來研究影響斑馬魚體節、心臟和抑制血管形成可能的分子機轉，進而用來評估這些藥物的生物活性。