行政院國家科學委員會專題研究計畫　成果報告

斑馬魚中 FOX05 轉錄因子的選殖、表現模式與定性分析

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國科會專題研究計畫成果報告撰寫格式

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行政院國家科學委員會補助專題研究計畫 ☑ 成 果 報 告
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斑馬魚中 FOXO5 轉錄因子的選殖、表現模式與定性分析

計畫類別：☑ 個別型計畫　□ 整合型計畫
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計畫主持人：許立松
共同主持人：謝錦源
計畫參與人員： 碩士研究生李慧敏及張智雯

成果報告類型(依經費核定清單規定繳交)：☑精簡報告　□完整報告

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執行單位：

中　華　民　國　95　年　10　月　31　日

摘要

第 O 型 forkhead 轉錄因子(FOXO)家族可以調控許多細胞的功能如：代謝、細胞分化及細胞凋亡等。在此研究，我們發現了斑馬魚的 FOXO5 基因與哺乳類動物的 FOXO 基因有高度的相似性。FOXO5 的轉錄起始於受精後兩小時並且持續表現製程於階段。利用定位雜合實驗，FOXO5 的表現於受精後 24 至 48 小時慢慢的表現在心臟區域而於腦部慢慢的消失。利用微注射 FOXO5 專一性的反義核苷酸至斑馬魚胚胎會造成生長、細胞色素及血球生成被抑制。被 AKT 蛋白質激酶磷酸化後，FOXO5 會停留在細胞質中。我們的實驗顯示斑馬魚的 FOXO5 對於細胞色素及血色素的行成扮演一個重要角色。

關鍵詞：斑馬魚、FOXO5 基因、反義核苷酸

Abstract

Forkhead transcription factor subclass O (FOXO) family proteins regulate a wide range of cellular functions such as metabolism, cell differentiation, and apoptosis. In this study, we identified the zebrafish FOXO5 gene which shared high similarity to mammalian orthologs. FOXO5 transcripts were found 2 hours post fertilization (HPF) and persistently expressed thereafter. Using in situ hybridization, FOXO5 was observed dramatically increased in heart and decreased in brain region during 24 to 48 HPF. Injection of zebrafish embryo with morpholino antisense oligonucleotides against FOXO5 resulted in inhibiting of growth, pigment formation, and erythropoiesis. Through phosphorylation by AKT leaded to cytoplasma retention of FOXO5. Taken together, our results suggested that zebrafish FOXO5 may play an important role in zebrafish pigmentation and erythropoesis.

Keywords: zebrafish, FOXO5, morpholino

Introduction

Fox class O (FOXO) proteins are a sub-group of Forkhead transcription factors and play an important role in regulating a wide range of cellular processes such as cell cycle progression, DNA damage/repair, apoptosis, oxidative stress responses, and metabolism (Accili et al. 2004, Greer et al. 2005). In mammals, at least four FOXO subclasses of the Forkhead transcription factors including AFX (FOXO4), FKHR (FOXO1), FKHR-L1 (FOXO3a) and FOXO6 were identified and shown to be highly homologous with Daf-16, a regulator of longevity in *Caenorhabditis elegans,* and which represents the mammalian orthologus of Daf-16 (Furuyama et al. 2000, Jacobs et al. 2003). In *C. elegans*, the *daf-2/age-1/daf-16*, an insulin-like pathway, controls the life-span extension (Vanfleteren et al. 1999). Previous studies have revealed that growth factors regulate the sub-cellular localization and activity of the FOXO family mediated through the PI3K/PKB pathway (Gan et al. 2005, Matsuzaki et al. 2005, Srinivasan et al. 2005). PKB can phosphorylate FOXO1 at three PKB consensus phosphorylation sites including Thr24, Ser256 and Ser319 (Gan, Zheng 2005). In the unphosphorylated state, these transcription factors increased the expression of proteins such as p27 Kip1 (Dijkers et al. 2000), p130-Rb2 (Kops et al. 2002) and Bim (Stahl et al. 2002). Upon phosphorylation by PKB,

A previous report has demonstrated that each member of the FOXO family (FOXO1, FOXO3, FOXO4 and FOXO6) shows a tissue-specific and developmentally specific expression pattern (Furuyama, Nakazawa 2000, Jacobs, van der Heide 2003). The expression pattern of the three genes was similar in embryos, whereas a distinct expression pattern was found in adult tissues (Furuyama, Nakazawa 2000). FOXO1 mRNA is highly expressed in brown and white adipose

tissues and FOXO4 is expressed at a higher level in skeletal muscle while FOXO3 was highly expressed in adult tissue rather than in embryo tissue (Furuyama, Nakazawa 2000). Recently, Hoekman et al demonstrated a distinct spatial and temporal expression pattern of FOXO family proteins (FOXO1, FOXO3 and FOXO6) in the embryonic and adult mouse brain using in situ hybridization (Hoekman et al. 2006). FOXO6 was detected in E12.5 and was mainly expressed in the hippocampus, the amygdalohippocampal area and the shell of the nucleus accumbens (Hoekman, Jacobs 2006). FOXO1 and FOXO3 were found in E14, the former expressed in more restricted regions such as the striatum and neuronal subsets of the hippocampus, whereas the latter was transcripted in a diffused pattern throughout the whole brain (Hoekman, Jacobs 2006). Furthermore, genetic analysis using transgenetic animals has revealed that each FOXO gene plays a distinct role in development. Overexpression of constitutively active FOXO1 and FOXO3a impaired tube formation and VEGF-induced cell migration in human umbilical vein endothelial cells (HUVEC) (Potente et al. 2005). In addition, it has been reported that FOXO3a$^{-/-}$ possesses a malfunctioned ovarian follicles due to deregulation of ovarian follicle development (Castrillon et al. 2003). As a result of impaired vascular development, complete disruption of FOXO1 mice is embryonically lethal, which indicates that FOXO1 may play a role in angiogenesis (Hosaka et al. 2004). In addition, down-regulation of FOXO4 by siRNA increased myocardin activity and smooth muscle cell differentiation, however, it was found that no detectable phenotype defect in FOXO4-null mice compared to normal mice (Liu et al. 2005)

The super-family of Forkhead transcription factors consists of approximately 90 members, which are expressed in an array of species ranging from yeast to human (Carlsson et al. 2002).

However, the role of Forkhead transcription factors in embryogenesis of the zebrafish remains to be elucidated. Herein, the spatial expression and tissue expression of the zebrafish FOXO5 gene, using RT-PCR and in situ hybridization, was investigated. FOXO5 was continuously expressed during different developmental stages and all adult tissues with abundant expression in the brain and retina. Knockdown of FOIXO5 by anti-sense morpholino oligonucleotide caused heme and pigmentation was delayed in morphants.

Method and materials

Materials. All the chemical compounds were obtained from Sigma. The restrict enzymes and Taq DNA polymerase were purchased from Promega Biosciences and Viogene, respectively. Control and constitute activated AKT were kindly gifted from Dr. Chin-Wen Chi (Yang Ming University, Taiwan). Dominant negative AKT was obtained from Dr. Chiung-Tong Chen (NHRI, Taiwan).

Fish. Zebrafish (*D. rerio*) AB strain was raised and maintained at 28℃ on a 14h light/ 10 dark cycle according to previous described. Different develop stages were detected according to the Zebrafish book (Westerfield 1995).

RT-PCR of zebrafish FOXO5. Total RNA was isolated from different stages and from a various tissues using TRIzol Reagent (Invetrogen). The RNA yield and integrity was measured by spectrophotometry and 1 % agarose gel electrophoresis, respectively. Five μg of total RNA was converted into first strand cDNA by Moloney murine leukemia virus reverse transcriptase according to the manufacturer's recommendations (Superscript II, Life Techonologies). Briefly, Total RNA was annealed with 0.5μg random primers in a total volume of 12 μl at 70℃ for 5 min. Seven μl of reaction mix (4 μl of 5 X reaction buffer, 1 μl of ribonuclease inhibitor, and 2 μl of

10 mM dNTP) was added to the primer/RNA complexes and incubated at room temperature for 5 min. After annealing, 1 μl of reverse transcriptase was added and incubated at 42℃ for 60 min. The reaction was terminated by heating at 70℃ for 10 min.

Using 1 μl first strand cDNA as templates, PCR amplification was performed with primers (sense: 5'-CGTATAGGAGATGCTGGCAG-3' and anti-sense: 5'-ATGCAGTGAACGGTAGCTCTC-3') and program as: denaturation at 95 for 5 min followed by 35 cycles of 94℃ for 1 min, 50℃ for 1 min, and 72℃ for 1 min and the final extension at 72℃ for 5 min. The 400bp PCR products were separated by 3% agarose gel and visualized by ethidium bromide staining. Negative control using water instead of cDNA was concomitantly performed. Amplification of elongation factor will be used as an internal control by sense primer (5'-GCTCAAGGAGAAGATCG-3') and anti-sense primer (5'-TCAAGCATTATCCAGTCC-3').

Whole mount in situ hybridization and morpholino injection. DNA fragment corresponded to nucleotide 3741 to 4140 was PCR amplified then subcloned into pGEMT-easy vector (Promega) and transformed into JM109 competent cells. Colonies with insert were selected for producing probes. Sense and anti-sense riboprobes for FOXO5 was made from Pst I-digested linear DNA using in vitro transcription by T7 RNA polymerase in the presence of digoxigenin-labeled UTP. Whole-mount in situ hybridization was performed as previous described (Westerfield 1995).

Morpholino injection. Morpholino (MO) antisense oligonucleotide (Gene-Tools) targeted against FOXO was designed as following sequence: 5'-CCAGGGTTGTCTCTGCCATCTTTCC-3' corresponded to -6 to +19 of FOXO5. The MO was dissolved in 1 X Danieau solution containing 0.5% phenol Red and injected into embryos at 1 to 2 cell stage.

O-dianisidine stain. Embryos were stained with staining solution (0.6 mg/ml o-dianisidine, 10 mM sodium acetate, pH 5.2, 0.65 % H2O2, and 40 % ethanol) at dark room for 15 min followed by washing with PBS to stop reaction.

Cell culture and transfection. Human lung cancer cell line H1355 was maintained in RPMI medium supplemented with 5% fetal bovine serum. Transfection was performed using Lipofetamine (Invitrogene) as manufacturer's recommendation.

Plasmid construction. The full coding region of FOXO5 was amplified using primers (forward: 5'-CTCGAGAGATGGCAGAGACAACCCTG-3' and reverse: 5'-GGATCCCCATCAGCCTGGCACCCAAC-3') and zebrafish cDNA as template following conditions as: 30 cycle; 90℃ for 30 sec, 50℃ for 30 sec, and 72℃ for 1 min. The amplified fragment was ligated into pGEMT-easy vector (Promega) and transformed to JM109 competent cells. Plasmid from positive clone was digested with Xho I and EcoR I and subcloned into pEGFP-c1 vector to construct the pEGFP-FOXO5 fusion protein. All the plasmids were sequenced from both directions by ABI 3100 DNA sequencer (MISSION BIOTECH).

Sub-cellular localization of GFP-FOXO5. Human H1355 cells were co-transfected with wild type, dominant negative or constitutive active form of AKT and pEGFP or pEGFP-FOXO5 fusion protein using Lipofetamine (Invitrogen) according to the manufacturer's recommendation. Forty-eight after transfection, the sub-cellular localization of GFP or GFP-FOXO5 was determined using fluorescent microscopy.

Results and Discussion

Identification of the FOXO5 zebrafish gene

FOXO family transcription factors can regulate a wide range of gene expression including cell cycle-related genes, DNA repair genes, and energy metabolism genes (Accili and Arden 2004, Greer and Brunet 2005). FOXO family proteins have been isolated from fruit fly (Kramer et al. 2003), Xenopus (Pohl et al. 2004), and vertebrate (Anderson et al. 1998, Biggs et al. 2001, Hosaka, Biggs 2004) To address the role of FOXO5 protein in zebrafish development, we attempted to isolate zebrafish FOXO5. An in silico method was employed to identify the FOXO5 gene in zebrafish. Firstly, the human FOXO1 protein sequence was selected and a BLAST search using the TBLASTN program for sequence similarity analysis was performed. One EST (AF114262) clone containing the full length FOXO5 was obtained. The zebrafish FOXO5 consists of 4547 bp, which encode 651 amino acids and which shares 37%, 57%, and 31% homology to mouse FOXO1a, FOXO3a and FOXO4, respectively. The forkhead domain was located in amino acids 127 to 212 and displayed 75%, 77% and 75% homology to mouse FOXO1a, FOXO3a and FOXO4, respectively. Based on homology, it was speculated that the function of FOXO5 might be more similar to FOXO3a. Unlike mammalian orthorlogs, two putative AKT phosphorylation sites were found following a Scansite search and were localized within T30 and S223, whereas only the T30 residue fit the 14-3-3 binding motif (RXRXXT/S).

Expression patterns of zebrafish FOXO5 transcripts in different development stages and adult tissues

To determine the expression pattern of FOXO5 during zebrafish development, total RNAs, derived from 10 different stages (2–96 hour post fertilization) of zebrafish, were converted into

cDNA and PCR amplification was performed to determine the temporal expression patterns of FOXO5. As shown in Fig. 1, mRNA of FOXO5 was detected 2 h after fertilization and was substantially expressed thereafter, which suggests that the FOXO5 gene was maternally deposited.

To investigate the expression pattern of FOXO5 in zebrafish, RT-PCR and whole mount in situ hybridization were performed to determine the tissue and spatial expression patterns of FOXO5. Unlike the mammalian orthologues, which is expressed in more restricted tissues (Furuyama, Nakazawa 2000, Jacobs, van der Heide 2003), the zebrafish FOXO5 was ubiquitously expressed in all adult tissues with the highest level of expression observed in the brain and retina (Fig. 2). Compared to the mouse FOXO family, distinct expression patterns of different FOXO numbers in different species was observed. Higher expression levels of FOXO5 detected in the brain region was similar to that observed with mouse FOXO3a, whereas modest detection of FOXO5 in the heart was similar to mouse FOXO1. Nevertheless, all three FOXO genes remained undetectable in the mouse ovary, however, FOXO5 expression was detected in ovary tissue in the zebrafish.

To further define the exact expression patterns of FOXO5, whole-mount in situ hybridization was performed using anti-sense ribonucleotides, where the probe corresponded with nucleotides 3741 to 4140 of FOXO5. FOXO5 transcripts were found intact throughout the body in 12 hpf (Fig. 3). In 24 hpf embryos, FOXO5 mRNA transcripts were detected predominantly in the midbrain, hindbrain, and muscles. In 36 hpf, FOXO5 appeared in the anterior tectum, telecelophon, midbrain, somite, and was observed in the heart. However, FOXO5

was strongly expressed in the heart and aorta region, and was detected in brain regions in 48 hpf embryos. Interestingly, our data obtained from whole-mount in situ hybridization indicated that FOXO5 expression patterns alternated during the development stage.

Knockdown of FOXO5 delayed erythropoiesis

Morpholino (MO)-mediated blocking gene translation has been widely used to determine gene function *in vivo* (Corey et al. 2001). To investigate the role of FOXO5 in zebrafish development, a microinjection of MO anti-sense oligonucleotide was used to knockdown FOXO5 protein translation. Embryos that received MO (8 ng) developed abnormally compared to the normal phenotype. Based on the severity, the phenotype of morphants was divided into two categories. Severe type: at 48 hpf, growth retardation, small and dark brain, small eyes, pericardial edema, pig tail, disruption of somite formation, and reduced melanocye in body and retina was observed in the treated embryos. These morphants were almost died at 72 hpf. Mild type: the phenotype was like severe type except brain region was transparent and bent trunk instead of pig tail. At 72 hpf, reduced melanocytes number and differentiated red blood cells were found in morphants treated with 8 ng morpholino. Using *o*-dianicidine staining, reduced heme staining was observed in morphants injected with MO at 48 and 72 hpf (Fig. 4).

Several previous reports have demonstrated that FOXO family proteins are involved in angiogenesis and vascularization (Furuyama et al. 2004, Potente, Urbich 2005). Castrillon et al have shown that abnormal heamatologic defects such as mild compensated anemia and reticulocytosis are observed in FOXO3a null mice (Castrillon, Miao 2003). An increasing expression and activity of FOXO3a was observed during erythroid differentiation (Bakker et al.

2004). In addition, constitutively activated FOXO3a facilitated erythroid differentiation, but reduced FOXO3a expression inhibited differentiation (Bakker, Blazquez-Domingo 2004). Using the microarray assay, Bakker et al indicated that the B cell translocation gene 1 (BTG1) was the direct target gene of FOXO3a and played an important role in erythroid differentiation (Bakker, Blazquez-Domingo 2004). Based on our observation, zebrafish FOXO5 may involve in erythropoiesis.

Phosphorylation by AKT altered the sub-cellular location of FOXO5

Previous studies have indicated that phosphorylation of specific serine or threonine site by AKT contributed to the regulation of the sub-cellular localization of FOXO family protein in mammalian cells (Gan, Zheng 2005, Jacobs, van der Heide 2003, Matsuzaki, Ichino 2005). To address whether the activation of AKT leads to the translocation of zebrafish FOXO5, the human lung cancer cell line H1355 was co-transfected simultaneously with various forms of AKT and pEGFP alone or pEGFP-FOXO5. The GFP alone protein was distributed in both nuclear and cytoplasmic compartments in the presence of dominant negative or constitutively activated AKT. In the presence of dominant negative AKT, GFP-FOXO5 was expressed throughout the whole cell whereas GFP-FOXO5 was localized predominantly in the cytosolic compartment in the presence of constitutively activated AKT. Moreover, apoptotic bodies were found in cells transfected with dominant AKT. These results suggest that, similar to the orthorlogues of humans or mice, the activation of AKT is sufficient to induce cytosolic localization of zebrafish FOXO5. These data suggest that external signals may negatively regulate the biological function of FOXO5 via the PKB pathway.

Taken together, our results indicate that the zebrafish FOXO5 gene may play an important role both in pigmentation and erythropoiesis and may be negatively regulated by AKT phosphorylation.

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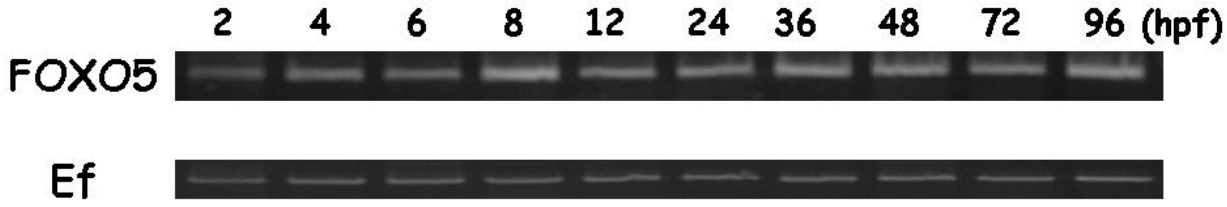

Fig.1. Expression of FOXO5 in different development stages of zebrafish. Total RNA derived from indicated stages of zebrafish was converted into cDNA and subjected to RT-PCR with specific primers for FOXO5. Elongation factor was used to normalize the amount of cDNA.

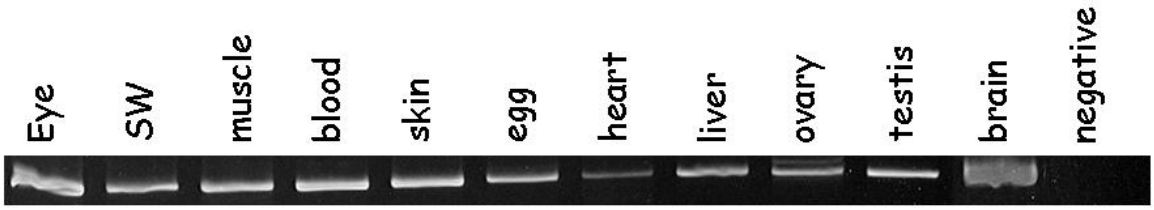

Fig. 2. Tissue distribution of FOXO5 in adult zebrafish. RT-PCR was performed using RNA from indicated tissues. Negative control was included using water instead of cDNA. FOXO5 was abundantly expressed in brain and eye and weakly expressed in heart.

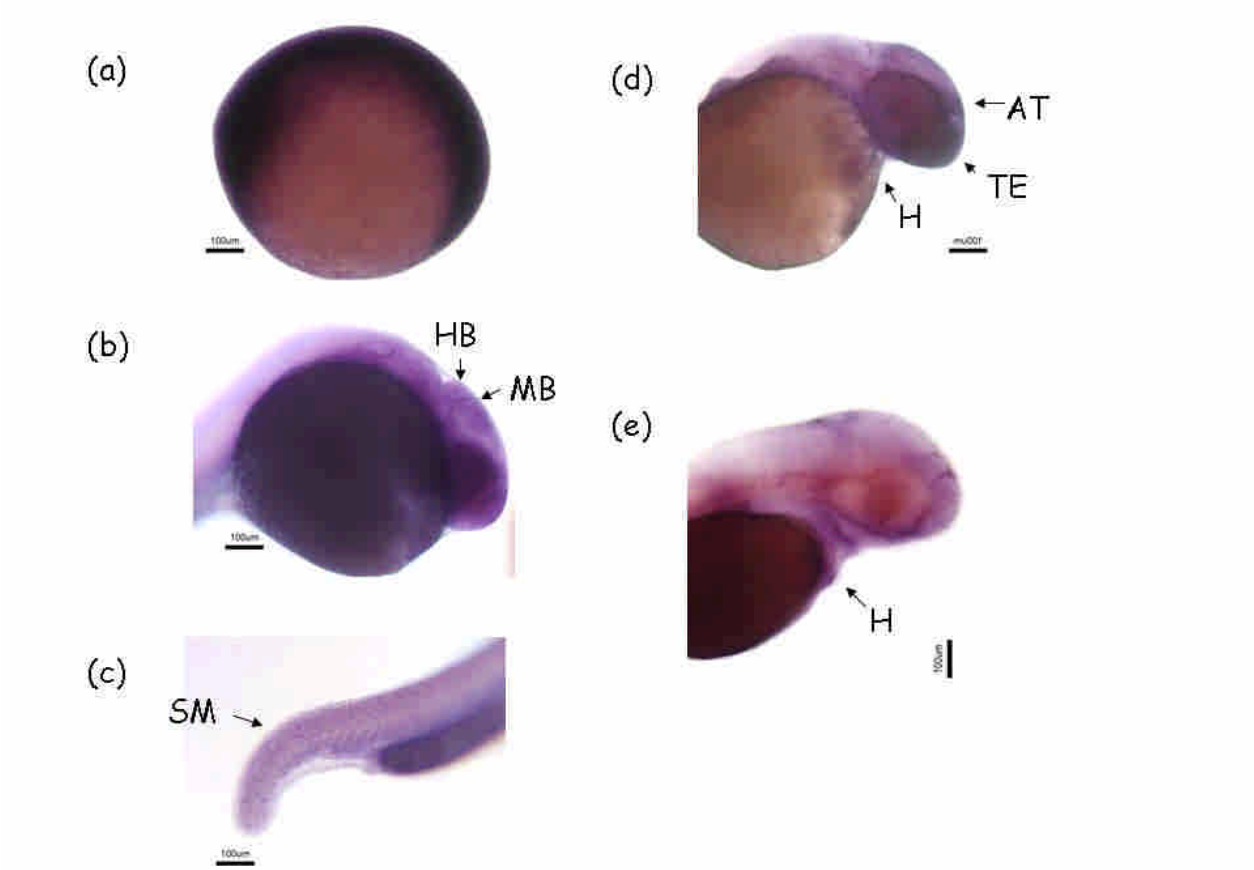

Fig. 3. Whole-mount in situ hybridization of FOXO5 during zebrafish development stages from 12 to 48 HPF. (a) 12HPF (b) and (c)24 HPF(d)36 hpf and (e)48 hpf. MB denoted midbrain, HB denoted hidbrain, sm denoted somite, AT denoted anterior tectum, TE denoted telecelophon, and H denoted heart.

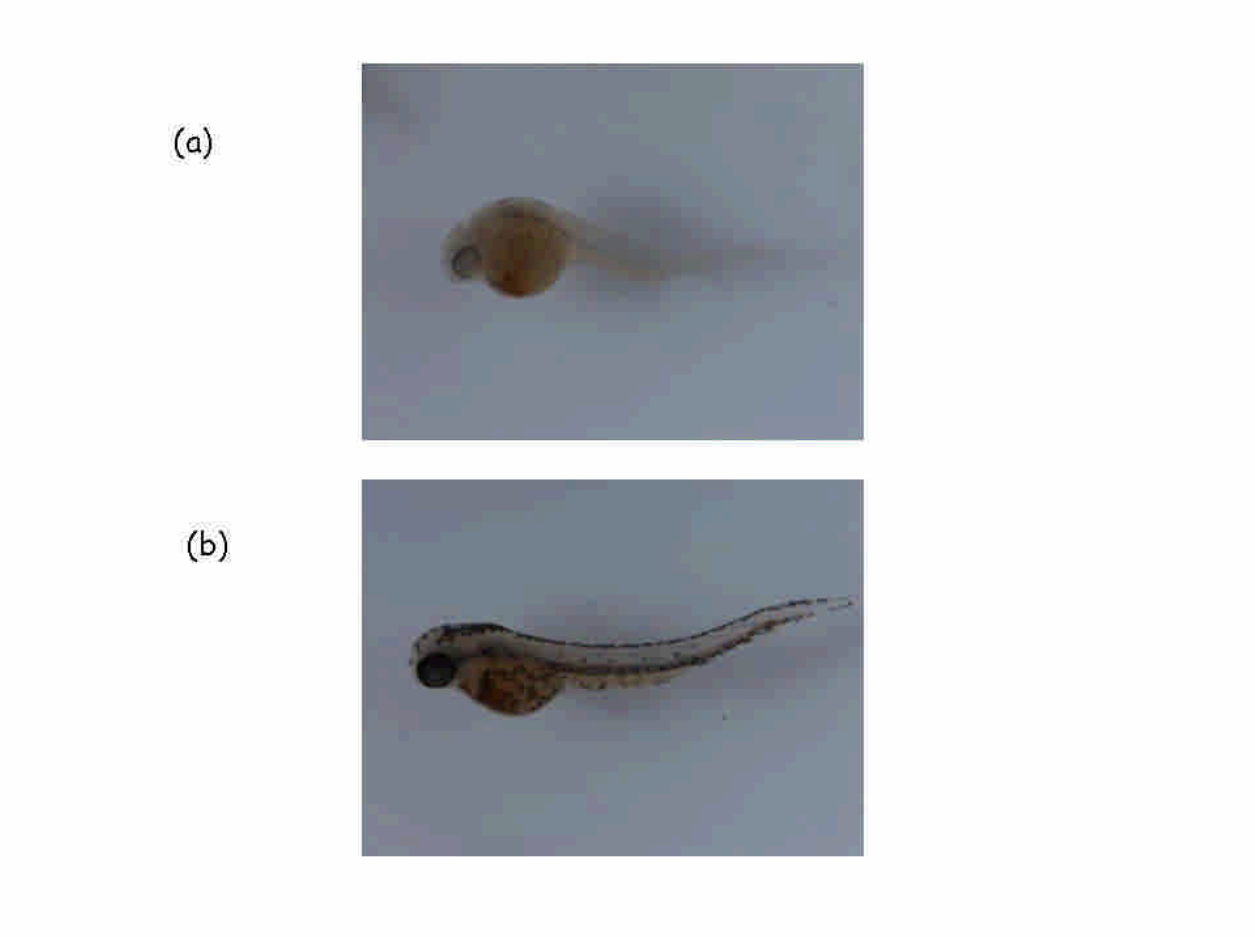

Fig. 4. O-dianisidine stain of morphants. Zebrafish embryos received 8 ng morpholino (a) and (b) wild type was subjectd to o-dianisidine stain.

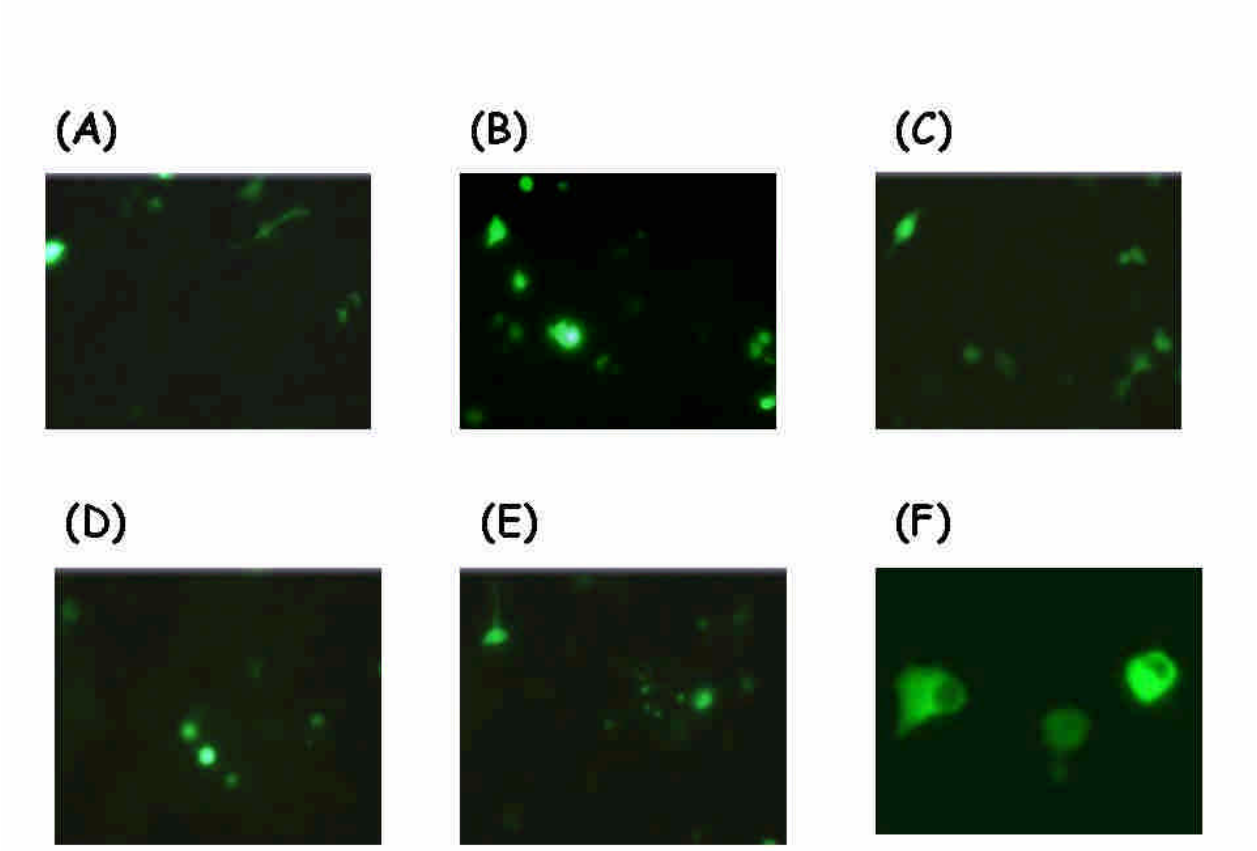

Fig. 5. Nuclear export of FOXO5 by AKT phosphorylation. H1355 cells were transfcted with (a) pEGFP/control AKT, (b) pEGFP/ dominant negative AKT , , (c) pEGFP/ constitutive activated AKT, (d) pEGFP-FOXO5/control AKT, (e) pEGFP-FOXO5/ dominant negative AKT,or (f) pEGFP-FOXO5/ constitutive activated AKT. After 48 hours, cells were pictured by fluorescent microscopy.