# III. Materials and Methods

## 1. Materials

**(1).** Adult zebrafish (*Danio rerio*)

**(2).** Embryo Medium (Westerfield M, 1994):

0.137 M NaCl, 5.4 mM KCl, 0.25 mM $Na_2H\ PO_4$, 0.44 mM $KH_2\ PO_4$, 1.3 mM $CaCl_2$, 1.0 mM $Mg\ SO_4$, 4.2 mM $NaH\ CO_3$

**(3).** Stock salts (Westerfield M, 1994): 40g Sea Salts in 1L distilled water.

**(4).** Egg water (Westerfield M, 1994): 1.5ml stock salts added to 1L distilled water= 60ug/ml final concentration.

**(5).** Suitable host strains: DH5 $\alpha$

**(6).** Luria-Bertani(LB) medium (per liter):

10g Bacto®-tryptone, 5g Bacto®-yeast extract, 5g NaCl. Adjust pH to 7.0 with NaOH.

**(7).** IPTG stock solution (0.1M):

1.2g IPTG (Cat.# V3951). Add water to 50ml final volume. Filtersterilize and store at 4°C.

**(8).** X-Gal (2ml):

100mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Cat.# V3941). Dissolve in 2ml N,N´-dimethylformamide. Cover with aluminum foil and store at –20°C.

**(9).** LB plates with ampicillin:

Add 15g agar to 1 liter of LB medium. Autoclave. Allow the medium to cool to 50°C before adding ampicillin to a final concentration of 100µg/ml. Pour 30–35ml of medium into 85mm petri dishes. Let the agar harden. Store at 4°C for up to 1 month or at room temperature for up to 1 week.

**(10).** LB plates with ampicillin/IPTG/X-Gal:

Make the LB plates with ampicillin as above; then supplement with 0.5mM IPTG and 80µg/ml X-Gal and pour the plates. Alternatively, 100µl of 100mM IPTG and 20µl of 50mg/ml X-Gal may be spread over the surface of an LB ampicillin plate and allowed to absorb for 30 minutes at 37°C prior to use.

**(11).** LB plates with kanamycin:

Add 15g agar to 1 liter of LB medium. Autoclave. Allow the medium to cool to 50°C before adding kanamycin to a final concentration of 50µg/ml. Pour 30–35ml of medium into 85mm petri dishes. Let the agar harden. Store at 4°C for up to 1 month or at room temperature for up to 1 week.

**(12).** 2M $Mg^{2+}$ stock:

20.33g MgCl2 • 6H2O, 24.65g MgSO4 • 7H2O. Add distilled water to 100ml. Filter sterilizes.

**(13).** SOC medium (100ml):

2.0g Bacto®-tryptone, 0.5g Bacto®-yeast extract, 1ml 1M NaCl, 0.25ml 1M KCl, 1ml 2M $Mg^{2+}$ stock, filtersterilized, 1ml 2M glucose, filtersterilized. Add Bacto®-tryptone, Bacto®-yeast extract, NaCl and KCl to 97ml distilled water. Stir to dissolve. Autoclave and cool to room temperature. Add 2M $Mg^{2+}$ stock and 2M glucose, each to a final concentration of 20mM. Bring to 100ml with sterile, distilled water. The final pH should be 7.0.

**(14).** Plasmid:

- pGEMT®-easy (Fig.3)(Promega, Madison, WI).
- pEGFP-1 (Fig.4) (ClonTech).
- pEGFP-C1 (Fig.5) (ClonTech).

**(15).** Restriction Enzyme: *Xho*I, *Eco*RI, *Sal*I, *Bam*HI, *Pst*I (Fermentas).

**(16).** pGEM-T Easy Vector System I (Promega, Madison, WI).

**(17).** PureLink HiPure Plasmid Filter Maxiprep Kit (Invitrogen).

**(18).** PureLink Micro-to-Midi Total RNA Purification System (Invitrogen).

**(19).** SuperScript™ III One-Step RT-PCR with Platinum® Taq (Invitrogen).

## 2. Methods

### (1). Zebrafish maintenance

Adult zebrafish (*Danio rerio*) were obtained from the Oregon State University A/B strain (Westerfield M, 1994) and maintained in our own fish facility with a controlled light cycle of 14 hours light / 10 hours dark at 28°C. They spawned soon after the onset of light, and the fertilized eggs were collected at the one-cell stage.

### (2). Transgenic vector preparation

### (2-1). Isolation of 5'-Flanking Region from Carp (*Cyprinus carpio*) M3-CK Genomic DNA

A Thermo PCR SPRINT PCR machine was used for PCR with the following program: 95°C 5 min.; 94°C 30 sec., 45°C 50 sec., 72°C 2 min., 5 cycles; 94°C 30 sec., 58°C 50 sec., 72°C 2 min., 25 cycles; 72°C 10 min. Primer sequences are: M3CKpro (Table. 1). The PCR product was ligated into the pGEM®-T Easy Vector System I (Promega, Madison, WI) for amplification and sequencing, and the resulting plasmid was named pM3CKPro as below.

Briefly centrifuge the pGEM®-T Easy Vector tubes to collect contents at the bottom of the tubes. Set up ligation reactions as described below. Vortex the 2X Rapid Ligation Buffer vigorously before each use. Mix the reactions by pipetting. Incubate the reactions 1 hour at room temperature.

Prepare two LB/ampicillin/IPTG/X-Gal plates each ligation reaction. Equilibrate the plates to room temperature prior to plating. Centrifuge the tubes containing the ligation reactions to collect contents at the bottom of the tube. Add 2µl of each ligation reaction to a sterile 1.5ml micro-centrifuge tube on ice. Set up another tube on ice with 0.1ng uncut plasmid for determination of the transformation efficiency of the competent cells. Remove tube of frozen DH5$\alpha$ Competent Cells from -70°C storage and place in an ice bath until just thawed (about 5 minutes). Mix the cells by gently flicking the tube. Carefully transfer 50µl of cells into each tube prepared (100µl cells for determination of transformation efficiency). Gently flick the tubes to mix and place them on ice for 35 minutes. Heat-shock the cells for 42 seconds in water bath at exactly 42°C. Immediately return the tubes to ice for 3 minutes. Add 950µl room temperature SOC medium to the tubes containing cells transformed with ligation reactions. Incubate for 1 hour at 37°C with shaking.

Plate 100µl of each transformation culture onto duplicate LB/ampicillin/IPTG/X-Gal plates. Incubate the plates overnight (16–24 hours) at 37°C. In our experience, approximately 100 colonies per plate are routinely seen when using competent cells that are $1 \times 10^8$cfu/µg DNA, if 100µl is plated. White colonies generally contain inserts; however, inserts may also be present in blue colonies.

**(2-2). Promoter expression analysis**

We analyze the potential transcriptional elements by paper searching on PubMed in NCBI and MatInspector database in Genomatix (http://www.genomatix.de/). For expression vector construction, the M3CK promoter from pM3CKpro was digested with *Xho*I / *Eco*RI and ligated into pEGFP-1 vector (ClonTech). The resulting plasmid was named pM3CKpro-EGFP.

Approximately 200 *p*l of 100 *n*g / *μ*l DNA solution in 5 mM Tris, 0.5 mM EDTA, 100 mM KCl, and 0.1% phenol red was injected into the blastomere of early one-cell-stage embryos using a glass micropipette.

At 96 h post injection, fish were examined using fluorescent microscopy and the GFP-expressing fish were saved for isolating different kind of tissues for RNA extraction (PureLink Micro-to-Midi Total RNA Purification System) and RT-PCR (SuperScript™ III One-Step RT-PCR with Platinum® Taq). The following RT-PCR primers were designed for the detection of EGFP gene: GFP. The zebrafish ß-actin primer set for internal control was pß-actin (Table. 2).

**(2-3). Plasmid construction**

A Thermo PCR SPRINT PCR machine was used for PCR with the following program: 95°C 5 min.; 94°C 30 sec., 43°C 50 sec., 72°C 2 min., 5 cycles; 94°C 30 sec., 57°C 50 sec., 72°C 2 min., 25 cycles; 72°C 10 min. Primer sequences are: CP1 and CP3 (Table. 2). The PCR product was ligated into the pGEM-T Easy Vector System I (Promega, Madison,

WI) as above for amplification and sequencing, and the resulting plasmids was named pGEMT-M1CK and pGEMT-M3CK.

The three promoters (CMV, CIP, M3CKpro) were digested with *Xho*I / *Eco*RI and the two genes (M1-CK and M3-CK) were digested with *Sal*I / *Bam*HI. We link the two genes (M1-CK and M3-CK) up the three promoters (CMV, CIP, M3CKpro) and ligating with pEGFP-C1 individually or only ligating promoter sequence with pEGFP-C1. We used transformation culture method as same as pGEM®-T Easy Vector system but plated 100 μl transformation culture onto duplicate LB/kanamycin plates. After acquiring the vector that we need, sequencing the nine plasmids used primers: EGFP-C, CIP1605, MP1321, BamHI-MCK (Table. 4).

**(3). Generation of transgenic zebrafish**

**(3-1). Microinjection**

Eggs were collected using breeding tanks containing nuptial chambers (Tidwell JH & Allan GL, 2001). Fertilized eggs were collected from the breeding tank by pouring water through a strainer. For transient expression, an intact circular form of plasmid DNA constructs was adjusted to 100 *n*g / *μ*l in 5 mM Tris, 0.5 mM EDTA, 100 mM KCl, and 0.1% phenol red.

Approximately 200 *p*l of DNA solution was injected into the blastomere of early one-cell stage embryos with a glass micropipette.

### (3-2). Green fluorescence protein (GFP) expression analysis

At 48 h post injection, fish were examined using fluorescent microscopy and the GFP-expressing fish were saved for further analysis and breeding.

### (3-3). mRNA expression analysis

We isolate different kind of tissues for RNA extraction (PureLink Micro-to-Midi Total RNA Purification System) and RT-PCR (SuperScript™ III One-Step RT-PCR with Platinum® Taq).

The following RT-PCR primers were designed for the detection of foreign Carp CK gene: CP1D and CP3D. The primers: ZF1D and ZF3D (Table. 4) set for the detection of original zebrafish CK gene. The zebrafish ß-actin primer set for internal control was A (Table. 2).

### (3-4). Immunoblot analysis

Zebrafish muscle total proteins were separated by 10% polyacrylamide gel. Separated proteins were electrotransferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 3% non-fat dried milk in Tris-buffered saline (20 mM Tris-HCl, pH 7.6, 137 mM NaCl) for 2 h at room temperature with agitation, followed by 1 h incubation in the presence of the primary antibody (antisera CKM1+2: 1:5000 dilution; antisera CKM3: 1:5000 dilution) as pervious study (Sun *et al.*, 1998). After washing in TBST (TBS buffer with 0.1% Tween-20), a secondary antibody, goat anti-rabbit

IgG conjugated to alkaline phosphatase (1:5000 dilution, Zymed) was added for 30 min at room temperature, followed by development with AP Development Buffer (Pierce).

**(3-5). Determination of swimming speeds**

At the end of thermal acclimation experiment, swimming ability was filmed at 28℃, 23℃, 18℃ and 13℃, respectively, for each fish. In an acute temperature change experiment, fish were first filmed at their acclimation temperature (28℃) and then transferred to low temperatures (23℃, 18℃ and 13℃) directly.

Swimming ability was filmed using a system that recorded 30 frames s-1. The camera was placed directly above the filming cylindrical tank (diameter: 20cm; height: 20cm), which was filled with water from the aquaria. For each filming distance, a known length was filmed, and its image was measured on the monitor. The relation between the known length and its monitor length was used to convert monitor speeds into real speeds.

**(4). Statistical analysis**

The effects of temperature change on all experimental variables for transgenic and wild type fish were analyzed using ANOVA test which $\alpha$=0.05. If the results have significant effect, using multiple comparisons to finding the significant differences in groups.