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Generation of a *mef2aa:EGFP* transgenic zebrafish line that expresses EGFP in muscle cells

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Abstract Transgenesis is an important tool for exploring gene expression and function. The *myocyte enhancer factor 2a* (*mef2a*) gene encodes a member of the Mef2 protein family that is involved in vertebrate skeletal, cardiac, and smooth muscle development and differentiation during myogenesis. According to studies on human and animal models, *mef2a* is highly expressed in the heart and somites. To explore the potential of *mef2a* as a tool for selective labeling of muscle cells in living zebrafish embryos, we constructed a transgene *mef2aa:EGFP* to induce the expression of green fluorescent protein (GFP) under the control of *mef2a* promoter. A ~2-kb DNA fragment, upstream of the translational start site of *mef2aa*, was identified to drive muscle-specific expression of EGFP in zebrafish embryos. Interestingly, the cranial muscles, abductor muscle, and adductor muscle were clearly labeled with EGFP in the established line *Tg(mef2aa:EGFP)*^{ntu803}. In addition, we showed that *mef2aa* mRNA was highly present in adult zebrafish heart, but not the skeleton muscle, whereas it was expressed in both embryonic heart and myotome, suggesting that *mef2a* is vital to the function of adult heart in vertebrates.

Keywords *mef2a* · Transgenic · Zebrafish · Heart · Muscle

Feng Lv and Chenwen Zhu have contributed equally to this work.

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Introduction

The myocyte enhancer factor 2 (Mef2) proteins belong to the ancient MADS family of transcription factors (Shore and Sharrocks 1995) and play an important role in morphogenesis and myogenesis of skeletal, cardiac, and smooth muscle cells (Olson et al. 1995). The N-terminal end of MEF2 factors contains a Mef2 domain and a highly conserved MADS-box, which together mediate DNA binding, dimerization, and co-factor interactions (Potthoff and Olson 2007; Black

and Olson 1998; McKinsey et al. 2002), while the C-terminal regions of Mef2 proteins function as transcriptional activation domains (Potthoff and Olson 2007; Martin et al. 1994). There are four vertebrate *Mef2* genes, *Mef2a*, *Mef2a-b*, *Mef2a-c*, and *Mef2a-d*, which are expressed in distinct, but overlapping patterns during embryogenesis through adulthood (McKinsey et al. 2002). Zebrafish is a popular model system for studying early embryo development and human disease (Lieschke and Currie 2007). It has also become an attractive model for studying regeneration of various tissues and organs, such as fins, heart, spinal cord, brain, and muscle (Gemberling et al. 2013). To fully exploit zebrafish in these studies, great emphasis has been placed on generation of fish lines that express desired transgenes, usually the fluorescence proteins, in either specific subsets of cells and tissues or a wide range of cells (Burket et al. 2008). These lines allow application of high-resolution imaging in vivo on zebrafish model. In addition, studies in zebrafish demonstrated that *mef2aa* is highly expressed in the heart (Wang et al. 2005) and somites (Wang et al. 2006) during zebrafish embryogenesis. Currently, we aimed to construct a transgene *mef2aa:EGFP* to induce the expression of EGFP in cardiomyocytes and muscle cells under the control of *mef2aa* promoter. The generation of *Tg(mef2aa:EGFP)* line will provide the new insights of understanding the *mef2a* expression in embryo and facilitate further analysis of the roles of *mef2a* in vivo during the development and tissue regeneration in adults.

Materials and methods

Zebrafish

Zebrafish (AB strain) were raised and maintained at 28.5 °C as previously described (Huang et al. 2013; Wang et al. 2015, 2016). Embryos were collected through natural mating. Embryonic stages were determined according to time post-fertilization and characteristics described by Kimmel et al. (1995). Embryos after 24 h post-fertilization (hpf) were treated with 0.2 mM 1-phenyl-2-thio-urea (PTU, Sigma) to prevent the pigmentation for further observation and imaging. Zebrafish embryos used for whole-mount in situ hybridization were collected at various stages, fixed with 4 % paraformaldehyde (PFA) in phosphate-buffered saline (PBS) overnight at 4 °C or 2 h at room temperature, washed with PBST, dehydrated in methanol, and stored at −20 °C until use. Embryos at stages earlier than 24 hpf were dechorionated after fixation, prior to storage.

Construction of *mef2a:EGFP* plasmid

Mef2aa promoter fragments (2044 bp) were amplified from the adult zebrafish genomic DNA by polymerase chain reaction (PCR) (Primers are listed in Table S1). Subsequently, the fragments were sub-cloned into pENTR™5′-TOPO® vector (Life Technologies) to produce 5′ Entry clone. pME-EGFP plasmid and p3E-polyA plasmid were obtained from Tol2Kit (Kwan et al. 2007). Three entry clones and the pDestTol2pA2 destination vector were used to generate the expression construct by LR recombination reaction as described in the Multisite Gateway Manual (Life Technologies).

RNA synthesis

Transposase RNA was generated using the pCS2-transposase plasmid as a template. Plasmid DNA was linearized with restriction endonuclease KpnI (NEB) and purified using the Zymoclean™ Gel DNA Recovery kit from agarose gels. Then, the mMESSAGE mMACHINE (Ambion, Life Technologies) was used to synthesize the capped RNA in vitro. The synthesized transposase RNA was purified by the RNeasy® MinElute® Cleanup kit (Qiagen).

RNA extraction, reverse transcription, and PCR

Tissue was homogenized and frozen in TRIzol Reagent (Life Technologies) and stored at −80 °C. The RNA was extracted following the manufacturer's instruction. 1 μg of RNA was reverse transcribed into cDNA by the use of Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's instructions. Synthesized cDNA was stored at −20 °C. All PCR amplifications were carried out in a total volume of 50 μl using specific primers and Advantage 2 Polymerase Kit (Clontech). The RT-PCR primers for zebrafish *mef2aa* and *ef1α* are listed in Table S1.

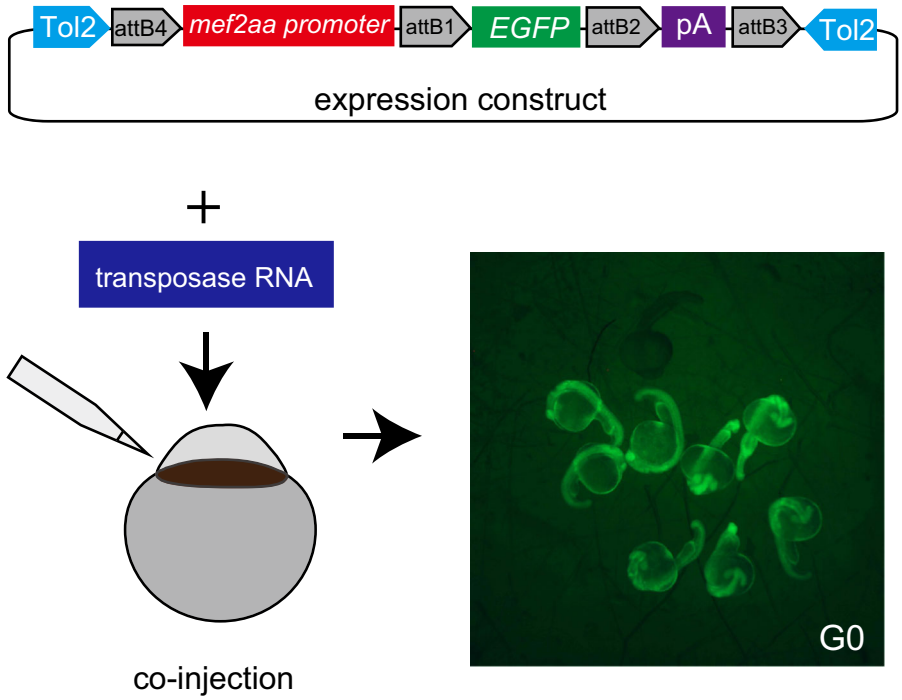


Fig. 1 Establishment of *Tg(mef2aa:EGFP)* zebrafish. The synthetic transposase mRNA and the expression construct containing a Tol2 construct with a *mef2aa* promoter, a ~2-kb DNA upstream of the translational start site of *mef2aa*, and EGFP are co-injected into zebrafish fertilized eggs

Microinjection of plasmid DNA and transposase RNA

The plasmid DNA and transposase RNA were mixed and microinjected into eggs at single-cell or two-cell stage. Approximately, 75 pg of plasmid DNA and 25 pg of transposase RNA were injected per embryo. Post-injection embryos were incubated at 28 °C in incubator.

Transgenic zebrafish screening

Injected embryos were screened through examining fluorescence expression using the stereo fluorescence microscopy (Olympus, MVX10; Zeiss, SteREO Discovery V20). Founders were raised until sexually mature and individually mated with wild-type (WT) fish, and the offspring were examined for fluorescence expression in muscle cells. F1 embryos were obtained from one of the positive founders and raised. Eight surviving positive male F1 fish were mated with wild-type females to generate F2 fish. F2 males were mated with F2 females to breed F3 generation.

Preparation of *mef2aa* RNA probe and whole-mount in situ hybridization (WISH)

The cDNA fragment (approximately 500 bp) of zebrafish *mef2a* was amplified by PCR. The primers used are listed in Table S1. Digoxigenin-labeled RNA probe was transcribed in vitro using T7 RNA polymerase, with the linearized pGEM®-T Easy vector (Promega) containing the DNA fragment of zebrafish *mef2aa*. The WISH procedure to detect *mef2aa* expression was carried out as described previously (Huang et al. 2013).

Imaging

Embryos subjected to whole-mount in situ hybridization were mounted in 75 % glycerol/PBST and photographed under a stereomicroscope (Olympus, MVX10) with DP71 camera. A Leica TCS SP5 was used for confocal imaging. Embryos were embedded in 0.8 % low melting point agarose dissolved in E3 medium with 0.03 % tricaine methanesulfonate (MS222, Sigma). All images acquired were compiled and edited using Adobe Photoshop and Illustrator software.

Results

Establishment of *Tg(mef2aa:EGFP)* zebrafish

There are two *mef2a* genes in zebrafish, *mef2aa* and *mef2ab*. It was reported that *mef2aa* was expressed in developing heart and somites (Wang et al. 2005, 2006), whereas *mef2ab* gene was not detected in zebrafish heart and somites at the corresponding stages (Yogev et al. 2013). Therefore, we choose to generate *Tg(mef2aa:EGFP)* transgenic zebrafish line that expresses EGFP in muscle cells. Firstly, we generated the *mef2aa:EGFP* transgenic construct (Fig. 1) containing a pair of Tol2 elements with *mef2aa* regulatory elements and the gene encoding EGFP using the multisite gateway system (Fig. 1; Figure S1). Then, the synthetic transposase mRNA and the expression construct were co-injected into zebrafish fertilized eggs at single-cell stage (Fig. 1). A fragment of 2044-bp DNA sequence was identified to drive muscle-specific expression of EGFP in zebrafish

Fig. 3 Confocal images showing expression of EGFP transgene in *Tg(mef2aa:EGFP)*^{ntu803} embryos at late stages. **a** 3 dpf, lateral view, GFP expression in somite and heart, **b** 4 dpf, lateral view, GFP expression in somite, heart and pectoral fins muscles, **c**, **c″** 3 dpf, Ventral view, GFP expression in heart co-localized with mCherry in *Tg(cmlc2:mCherry)*. *V* ventricle, *A* atrium, **d**, **d′** 3 dpf, Ventral view and lateral view, GFP expression in head muscles. *INTM-A and INTM-P* intermandibularis anterior and posterior, *INTE* interhyoideus, *HH-INF* hyoideus inferior, *SH* sternohyoideus, *OM* ocular muscles, *LEV-AP* levator arcus palatine, *LEV-OP* levator opercula, *DIL-OP* dilatator operculi, *AD-HYO* adductor hyomandibulae, **e**, **e″** 4 dpf, Ventral view and lateral view, GFP expression in pectoral fins muscles. *White arrowhead* indicates abductor muscle (ABM), *Red arrowhead* indicates adductor muscle (ADM). (Color figure online)

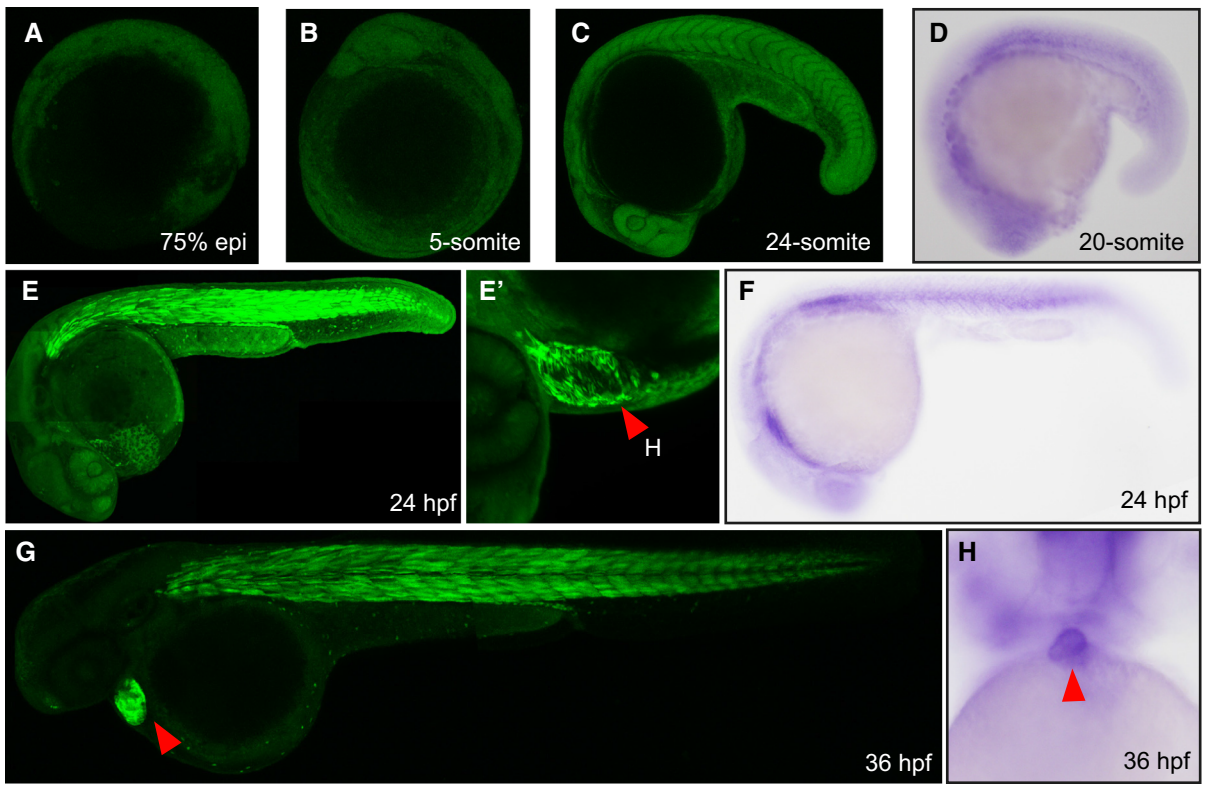
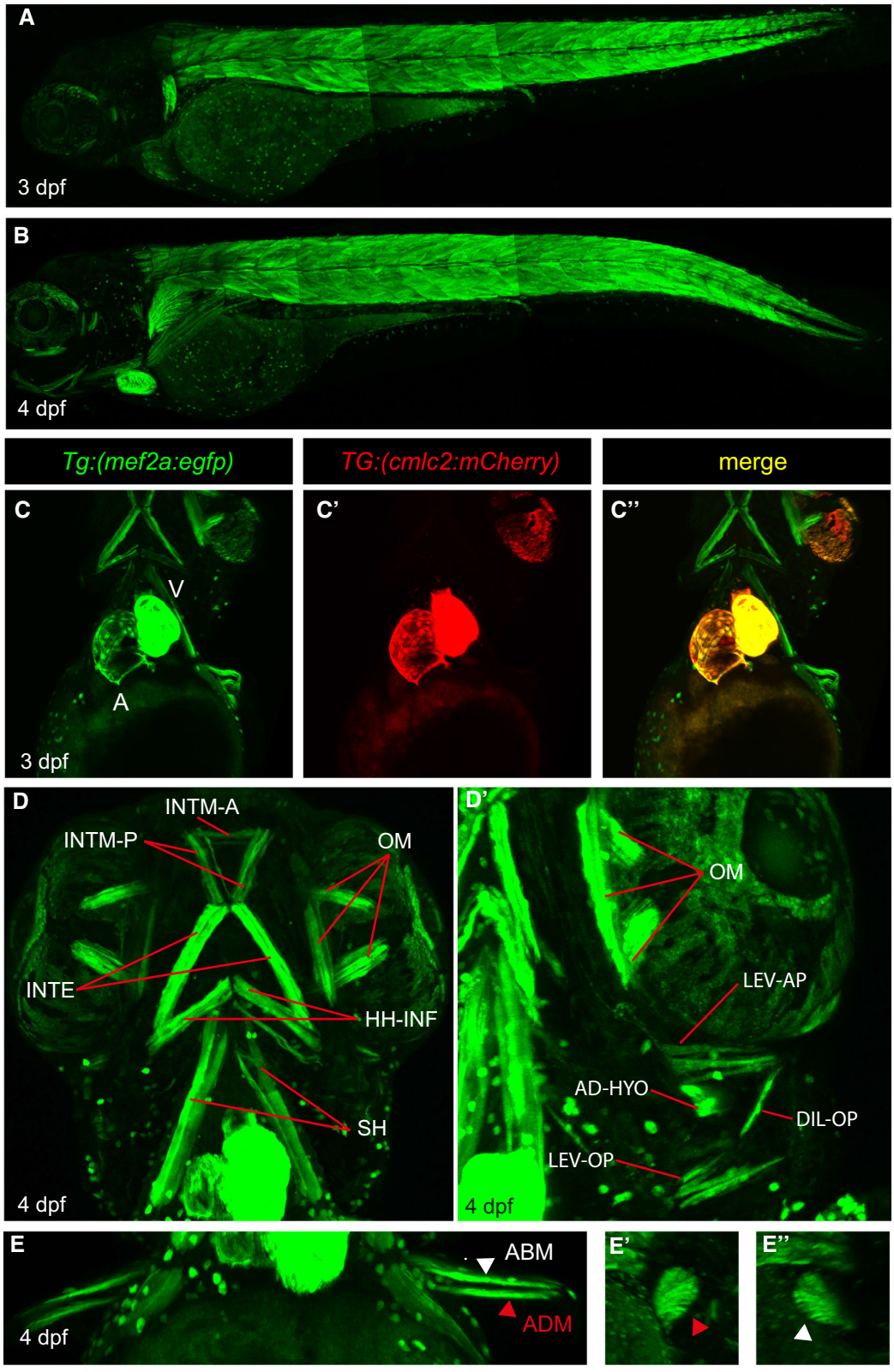


Fig. 2 Confocal images showing expression of EGFP transgene in *Tg(mef2aa:EGFP* ^{ntu803} embryos. **a** 75 % epiboly, lateral view, weak expression of GFP, **b** 5-somite stage, lateral view, weak expression of GFP in head, **c** 24-somite stage, lateral view, weak expression of GFP in somite and head, **d** 20-somite stage, lateral view, in situ hybridization analysis of *mef2aa* expression, weak expression in somite and head, **e** 24 hpf, lateral view, GFP expression in somite and heart, **e′** GFP expression in heart, *arrowhead* indicates heart, **f** In situ hybridization analysis of *mef2aa* expression at 24 hpf, lateral view, **g** 36 hpf, lateral view, GFP expression in somite and heart, *arrowhead* indicates heart, **h** in situ hybridization analysis of *mef2aa* expression at 36 hpf, *arrowhead* indicates heart


A
3 dpf
B
4 dpf
Tg:(mef2a:egfp)
TG:(cmlc2:mCherry)
merge
C
V
A
3 dpf
C'
C''
D
INTM-A
INTM-P
OM
INTE
HH-INF
SH
4 dpf
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AD-HYO
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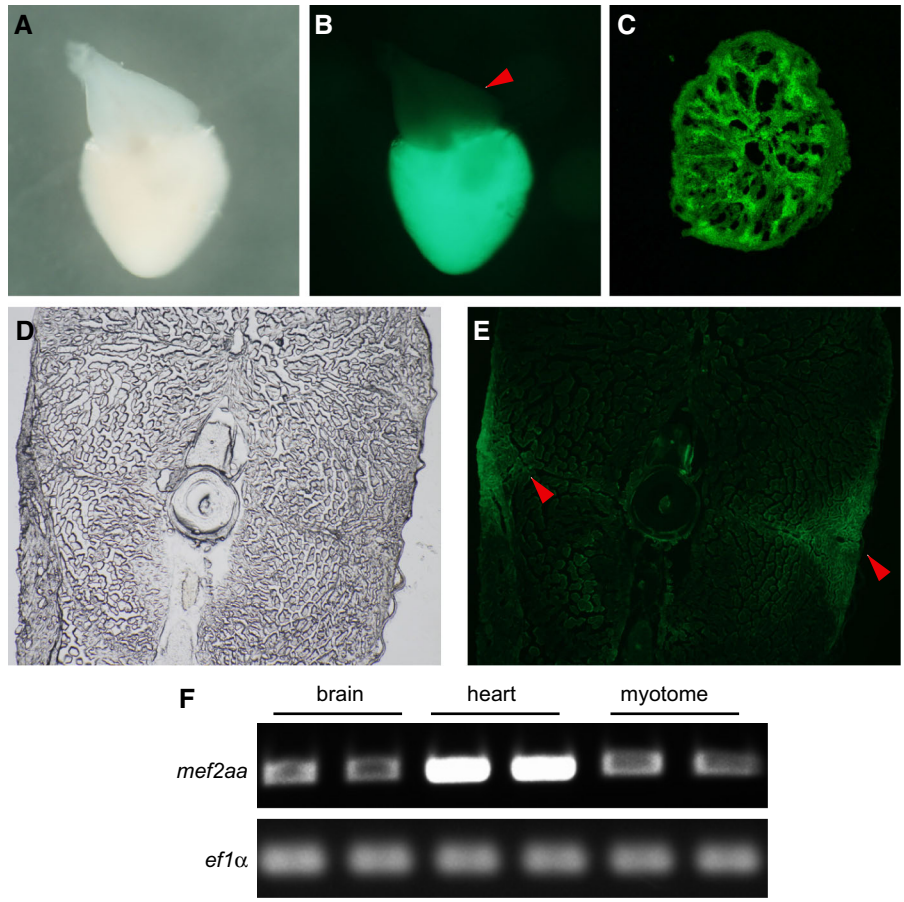

Fig. 4 *Mef2aa* expression in adult zebrafish heart and myotome. **a** Adult *Tg(mef2aa:EGFP)*^{ntu803} zebrafish heart in bright field, **b** morphology of adult *Tg(mef2aa:EGFP)*^{ntu803} zebrafish heart under fluorescence stereo microscope. *Red arrowhead* indicates bulbus arteriosus, **c** cross section of adult *Tg(mef2aa:EGFP)*^{ntu803} zebrafish heart under fluorescence microscope, **d** transverse section analysis of adult *Tg(mef2aa:EGFP)*^{ntu803} zebrafish myotome in bright field, **e** transverse section analysis of adult *Tg(mef2aa:EGFP)*^{ntu803} zebrafish myotome under fluorescence microscope. *Arrowheads* indicate GFP expression sites, **f** reverse transcription polymerase chain reaction analysis of *mef2aa* expression in brain, heart, and myotome. (Color figure online)

embryos. Founders were raised until sexually mature and individually mated with WT fish line. Of the 67 founders, 20 germ-line transmitted transgenic fish were identified. F1 embryos obtained from one of the positive founders, which were screened through examining the expression of fluorescence under a fluorescence stereomicroscope, and the positive F1 fish were raised. Eight surviving positive male F1 fish were mated with WT females to generate F2 fish. About 50 % of F2 offspring embryos carried EGFP expression, suggesting that the germline transmission rate of transgene complied with Mendelian inheritance law. F2 males were mated with F2 females to breed F3 generation. Finally, the *Tg:(mef2aa:EGFP)*^{ntu803} transgenic line was confirmed by around 75 % fluorescence induction in the F3 generation.

EGFP expression in *Tg(mef2aa:EGFP)*^{ntu803} zebrafish embryo in vivo

We examined the green fluorescence in *Tg(mef2aa:EGFP)*^{ntu803} zebrafish embryos through confocal imaging analysis. It was observed that GFP was weakly expressed at 75 % epiboly stage (Fig. 2a). In 5-somite and 20-somite stage embryos, we found GFP was present in telencephalon and myotomes (Fig. 2b, c). The transgene expression at these stages was similar to the endogenous expression of *mef2aa* shown

through whole-mount in situ hybridization analysis (Fig. 2d). At 24 hpf stage, GFP was strongly expressed in myotomes and heart (Fig. 2e, e′). The endogenous expression of *mef2aa* was also detected in myotomes and heart at the corresponding stage (Fig. 2f). At 36 hpf, 3 dpf, and 4 dpf stage, the GFP expression in heart and myotomes increasingly remained (Figs. 2g, h, 3a, b, c, c′, c″). In addition, GFP expression was detected in head muscle including intermandibularis anterior and posterior (INTM-A and INTM-P), interhyoideus (INTE), hyoideus inferior (HH-INF), sternohyoideus (SH), ocular muscles (OM), levator arcus palatini (LEV-AP), levator operculi (LEV-OP), dilatator operculi (DIL-OP), adductor hyomandibulae (AD-HYO) (Fig. 3d, d′). The pectoral fin muscles were also clearly labeled with EGFP including abductor muscle (ABM) and adductor muscle (ADM) (Fig. 3e, e′, e″).

EGFP expression in adult *Tg(mef2aa:EGFP)^{ntu803}* zebrafish heart and myotomes

Next, we examined GFP expression in adult *Tg(mef2aa:EGFP)^{ntu803}* zebrafish heart and myotomes. We dissected the heart by surgery and found that EGFP was highly present in adult zebrafish heart (Fig. 4a, b), but not in bulbus arteriosus (Fig. 4b). Cryostat sections of the *Tg(mef2aa:EGFP)^{ntu803}* zebrafish heart confirmed GFP expression in cardiomyocytes (Fig. 4c). In the transection analysis of zebrafish trunk, we demonstrated that GFP expression in myotome was weakly distributed in restricted region (Fig. 4d, e). RT-PCR analysis revealed that the endogenous expression of *mef2aa* in adult zebrafish heart is higher than that in brain and myotome (Fig. 4f), which supports that *mef2aa* is highly expressed in adult heart but not in skeleton muscle.

Discussion

Currently, we generated a *Tg(mef2aa:EGFP)^{ntu803}* transgenic zebrafish line that expresses EGFP in muscle cells. *Mef2a* transcripts are expressed in a wide array of tissues in mammals, while MEF2A protein appears to be considerably more restricted and is abundant in skeletal muscle, heart, and brain tissues (Suzuki et al. 1995) Accumulating evidence indicates that *Mef2a* is associated with cardiovascular and muscle development (Estrella et al. 2015). In addition, knockdown of *mef2a* in zebrafish embryos impairs the cardiac contractility (Wang et al. 2005) and posterior somite development (Wang et al. 2006). Our data are consistent with these findings that *mef2a* is highly expressed in developing heart and muscles. A loss-of-function mutation in the human *Mef2a* causes an autosomal dominant form of coronary artery disease, including its most serious complication myocardial infarction (Wang et al. 2003; Bhagavatula et al. 2004). Most mice lacking *Mef2a* die suddenly within the immediate perinatal period and exhibit pronounced dilation of the right ventricular, myofibrillar disarray and activation of a fetal cardiac gene program. The few *Mef2a* knock out mice that survives to adulthood is also susceptible to sudden cardiac death with mitochondrial deficiency (Naya et al. 2002). It was revealed that the level of *mef2aa* transcripts in adult zebrafish heart is higher than that in myotome. However, *mef2aa* was expressed in both heart and myotome in zebrafish embryos. Taken together, these observations suggest that *mef2a* is vital to the function of adult heart in vertebrates. Interestingly, the cranial muscles and pectoral fin muscles were clearly labeled with EGFP in *Tg(mef2aa:EGFP)^{ntu803}* line embryos, which allows high-resolution imaging of these muscles in vivo and promotes further studies of the role of *mef2a* in the development of these muscles. In addition, *Mef2a* was revealed to involve skeletal muscle regeneration in mice (Liu et al. 2014). It will be of interest to explore the expression and function of *mef2a* during skeletal muscle regeneration in the established *Tg(mef2aa:EGFP)^{ntu803}* line adults.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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