Front. Med. 2011, 5(3): 239–247
DOI 10.1007/s11684-011-0123-0

REVIEW

In vivo imaging of hematopoietic stem cell development in the zebrafish

Panpan Zhang, Feng Liu (✉)

State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China

Abstract ***In vivo*** **imaging is crucial for developmental biology and can further help to follow cell development/differentiation in normal and pathological conditions. Recent advances in optical imaging techniques has facilitated tracing of the developmental dynamics of a specific organ, tissue, or even a single cell. The zebrafish is an excellent model for imaging of hematopoiesis due to its transparent embryo at early stage; moreover, different zebrafish hematopoietic stem cells (HSCs) transgenic lines have been demonstrated as very useful tools for illustrating the details of the HSC developmental process. In this review, we summarize recent studies related to the non-invasive *in vivo* imaging of HSC transgenics, to show that zebrafish transgenic lines are powerful tools for developmental biology and disease. At the end of the review, the perspective and some open questions in this field will be discussed.**

Keywords hematopoietic stem cell; hematopoiesis; *in vivo* imaging; transgenics; zebrafish

Introduction

The zebrafish, *Danio rerio*, has been demonstrated to be an ideal model organism for vertebrate developmental biology studies in last decade due to some specific features. First, different from the traditionally-used mouse model, zebrafish eggs are fertilized externally, which is convenient for early stage developmental studies. Secondly, the zebrafish embryos are optically transparent, which makes it possible to study the formation of internal structures, such as vessel, liver, thymus etc *in vivo*. Thirdly, compared with mouse, zebrafish produces more offspring and the embryos develop more quickly. More importantly, the genetic programming, such as many transcriptional factors and signaling pathways in mammals have been demonstrated well conserved in zebrafish [1]. Therefore, zebrafish has gained more attention as human disease models for the mechanistic investigation of disease processes, including cancer, congenital heart disease and tissue regeneration, etc [2].

Given the above-mentioned properties, zebrafish emerged as an excellent model for studying hematopoiesis and cardiovascular development in early stage [3]. In zebrafish, the process of hematopoiesis is divided into two stages: the primitive hematopoiesis and the definitive hematopoiesis (Table 1) [1,4,5]. During the primitive hematopoiesis, mainly erythrocytes and some primitive myeloid lineages are generated [3], while during the definitive hematopoiesis, hematopoietic stem cells (HSCs) are generated, which then give rise to all kinds of blood cells such as erythrocytes, granulocytes, monocytes, lymphocytes, thrombocytes and others [6]. Newly published studies revealed that HSCs are derived directly from the ventral endothelial cells of dorsal aorta (DA) in zebrafish and mouse [7–9]. In zebrafish, HSCs enter into the posterior cardinal vein (PCV) afterwards and around 48 h postfertilization (hpf), migrate to the caudal hematopoietic tissue (CHT), the counterpart of the fetal liver in mammals [10]. In the CHT, HSCs proliferate and migrate to the thymus and pronephros, respectively. By 6 days postfertilization (dpf), the primary and lifelong site of definitive hematopoiesis is shifted to kidney marrow [11].

Imaging technologies in zebrafish

Zebrafish embryo has been widely used for optical imaging to address fundamental developmental biology questions due to its transparency. Optical imaging, which usually involves in ultraviolet, visible and infrared light, has been proven to be a fundamental tool in biologic research. However, it is well

Received December 29, 2010; accepted February 14, 2011
Correspondence: liuf@ioz.ac.cn

Table 1 A brief summary of primitive and definitive hematopoiesis in zebrafish

	Location	Time	Process	Cell types	Representative markers
Primitive hematopoiesis	ALM	18 to 24 hpf	Myelopoiesis	Myelocyte	*pu1*, *l-plastin, lyz, mpo*
	PLM	18 to 24 hpf	Erythropoiesis	Erythrocyte	*gata1*, *gata2*, *globin*
	ICM	24 hpf	Erythropoiesis	Erythrocyte	*gata1*, *gata2*, *globin*
Definitive hematopoiesis	AGM	24 to 60 hpf	HSC specification, EHT	HSC	*runx1*, *cmyb*, *cd41*
	CHT	2 to 4 dpf	HSC proliferation	HSC, EMP	*runx1*, *cmyb*, *cd41*, *cd45*
	Thymus	3 to 5 dpf	Thymopoiesis	T cell	*ikaros*, *rag1*, *rag2*
	Kidney	3 dpf to adulthood	Permanent HSC pool	HSC and all other blood cells	*cmyb*, *scl*

Abbreviations: ALM, anterior lateral plate mesoderm; PLM, posterior lateral plate mesoderm; ICM, intermediate cell mass; AGM, aorta-gonad-mesonephros; CHT, caudal hematopoietic tissue; EHT, endothelial-hematopoietic-transition; EMP, erythroid-myeloid progenitor; dpf, day postfertilization; hpf, hour postfertilization.

known that the application spectrum of the conventional imaging was limited. The thickness of the material must be no more than 10 μm, otherwise the image will get blur due to light scattering. In addition, to some extent the frequency of scattering events has a negative correlation with the wavelength of the light source [12]. The emergence of confocal microscopy brought about a revolution in this field. Different from conventional imaging techniques, confocal microscopy can eliminate out of focus light beyond the focal plane with the help of a pinhole device, and obtain reconstructed three dimensional structures from the captured images. Consequently, it is feasible to observe life phenomena and obtain high resolution images at depth as far as hundreds of micrometers in a noninvasive setting (Fig. 1). The achievable thickness of the specimen highly depends on the excitation light and the optical property of the specimen. Furthermore, the two photon excitation microscopy, which uses red shifted excitation light, makes it feasible to capture *in vivo* images from the living organism at a depth up to about one millimeter, since the near infrared (NIR) light can generate less scattering events and thus can penetrate a thicker depth compared with visible or ultraviolet light [13]. Moreover, the infrared light reduces the phototoxicity and confers less damage to the living tissue than visible or ultraviolet light. All of these have rendered the confocal and two-photon microscopy superior to the conventional imaging techniques. Recently, confocal microscopy combined with time lapse microscopy has been regarded as a very useful tool in developmental biology to reveal or uncover the dynamics or detailed processes of the organism at cellular or subcellular level *in vivo* over time. With the help of fluorescence correlation spectroscopy (FCS) [14], either from fluorescence protein in transgenic lines or from fluorophore injected into the cells, researchers can easily trace the origin, transition, and mitotic events of a single cell or detect the location or interactions of two or more fluorescent proteins in an unperturbed environment. For example, Herbomel and his colleagues discovered the emergence of HSCs from the ventral wall of the dorsal aorta in fluorescence labeled transgenic zebrafish *in vivo* by using confocal time-lapse microscopy [9]. Here, we briefly introduce the procedure of how to prepare and how to image zebrafish embryos using conventional and confocal microscopy.

Preparation of the embryo sample for imaging

Zebrafish embryos are collected and raised in fish water at 28°C. At the stage of 22 to 24 hpf, 1-phenyl-2-thiourea is added to prevent the pigmentation. For imaging, the chorion of the embryos at particular stage was removed. After anesthetized with tricaine, the embryo is mounted on the bottom in 35 mm glass dish with 1% low melting agarose covered with fish water. It should be noted that the trunk of the embryos needs to be close to the bottom of the dish as far as possible so as to get clearer images at high magnification.

In vivo imaging of the fluorescent zebrafish embryos

The conventional microscopy was performed on Nikon SMZ1500. The confocal microscopy was performed on Zeiss LSM 510 META, and the images were captured with 20 × and 40 × oil objectives. The parameters we used in our system are as follows. In case of 20 × objective, the power of 488 nm laser is at 30%, and gain at 597, with 0.1 offset, while at 40 × oil objective, the power of 488 nm laser is at 50%, and gain at 712, with 0.02 offset. The size of the images is all 1024 × 1024 pixels, and the space interval between two consecutive confocal planes is set at 2 μm. 3D projection from z-stack of each individual image was assembled. Time series combined with 3D projection were processed by Zeiss LSM software. As shown in Fig. 1, comparison between conventional and confocal imaging in zebrafish embryos clearly demonstrates that confocal imaging can provide much better three-dimensional structure of zebrafish blood vessels where HSCs were closely associated with.

Cell lineage tracing

It has long been developmental biologists' dream to thoroughly understand the detailed processes of cell

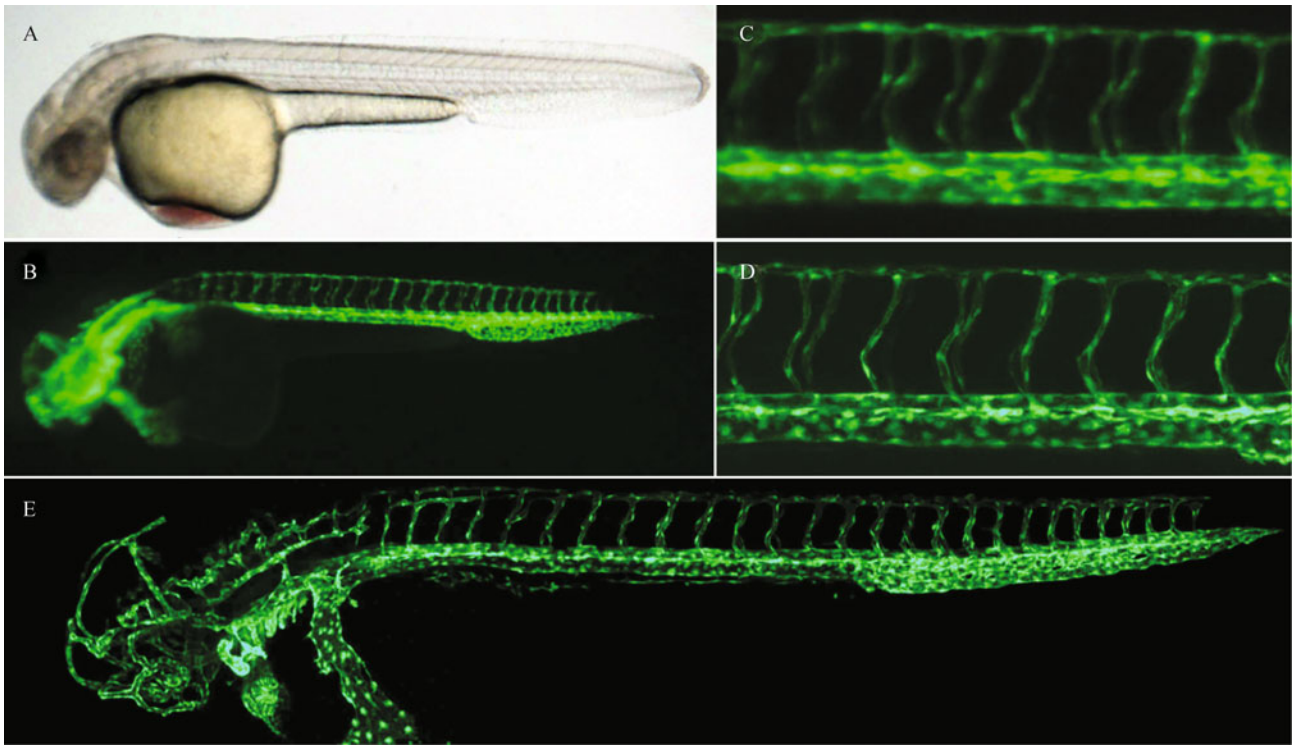

Fig. 1 Comparison of conventional and confocal microscopy used in zebrafish embryo imaging. (A) Bright-field imaging of a 36 hpf zebrafish embryo. Lateral view, anterior to the left. (B, C) Fluorescent imaging of the same embryos *(fli1:GFP)* showing green blood vessels. Lateral view, anterior to the left. (D, E) Confocal imaging of *flk1:GFP* embryo at day 2 using Zeiss LSM 510 showing 3D structures of blood vessels in green. Lateral view, anterior to the left.

migration, transition and cell fate determination during the early developmental stage of the organism. Zebrafish, owing to its special attributes, like maintaining optically transparent in early developmental stage, and being highly conserved in genetics, emerged as an ideal organism to trace the cell lineages or probe the fate of a particular cell in developmental biology when combined with various imaging techniques nowadays. The key point is how to label a specific single cell effectively with relatively ideal duration time. Up to now, there are mainly two ways to conduct cell lineage tracing in zebrafish, including fluorescence chromophore (or dye) injection, transgenic lines expressing various fluorescent proteins including photoconvertible fluorescent protein. Here, we will give a brief summary of each technique and exemplify their applications in recent published studies.

Fluorescence dyes and their derivatives

As early as the 1980s, Kimmel and his colleagues have developed the method of fluorescence dye injection in the early zebrafish embryo. By injecting tracer dye like rhodamine-dextran into single blastomeres between midblastula and late blastula stage, they can detect and trace the dynamics and location of the single cell over time [15,16]. They identified that the lineages of epithelial enveloping layer (EVL) labeled cells generate exclusively periderm, while the deep layer (DEL) labeled cells generate single deep embryonic tissues [15]. They further established a detailed fate map at the beginning of gastrulation stage for the zebrafish embryo [15]. However, it is laborious and relatively difficult to identify the exact site of a cell lineage before the gastrulation stage simply by observing the morphology of the embryo. Activation of caged fluorescein-dextran, as a new cell tracing approach with several advantages over single cell injection, emerged and gained wide popularity in developmental studies. For example, unlike conventional single cell injection, this technique can be applied at just one-cell stage, and can be used to trace target cells, which display different color after uncaging, at any developmental stages. Stainier and his colleagues injected the caged fluorescein dextran into embryos, and uncaged the ventral margin parts at shield stage. They found that a substantial number of cells displayed hemangioblast features and gave rise to both endothelial and hematopoietic lineages, as is evidenced by the observation that the uncaged cells with fluorescein isothiocyanate (FITC) colocalized with endothelial cells or with hematopoietic cells. Thus, they presented the *in vivo* evidence of the existence of hemangioblast for the first time [17]. Similarly, Murayama *et al*. used an *in vivo* photoactivatable cell tracer, caged fluorescein-dextran, and identified the CHT as an

intermediate HSC organ in zebrafish [10]. Photoconvertible fluorescent protein, like Kaede and Eos, which can be photoconverted irreversibly from green to red by UV light, are newly emerging tools to study the cell migration, and fate in noninvasive conditions [18]. These proteins can be transiently expressed by injecting mRNA or plasmid DNA. After photoconvertion, researchers can detect and trace the target cell or cells by time lapse imaging. Compared with other methods of cell labeling, this approach could be more convenient and effective, which will warrant a promising application in zebrafish developmental studies.

Transgenics and modifiers

Utilization of transgenic lines has been proven to be another effective way to trace the cell fate. As we will discuss below, different transgenic lines like *scl:GFP*, *cd41:GFP*, *flk1:GFP*, *runx1:GFP*, and so on, can be powerful tools to probe and trace the emergence and transition of HSCs *in vivo* with the help of modern imaging techniques. Similar to regular fluorescent proteins, photoconvertible fluorescent protein can be permanently expressed in specific tissues in zebrafish by constructing transgenic line under the control of a tissue specific promoter. In addition, the GAL4-UAS system can promote the specificity for transient mosaic expression [18]. Recently, Lewis and his colleagues created a new transgenic line called mosaic analysis in zebrafish (MAZe), which may provide new insight into the cell lineage studies and the clonal analysis of gene function in zebrafish. This line consists of a self-excising *hsp70*:Cre cassette. Once heat shocked, the Cre recombinase mediated recombination leads to the expression of Gal4:VP16 under *ef1a* promoter, and then the fluorescent protein controlled by UAS sequence is activated. By adjusting the time and duration of the heat-shock, the amount of cells induced and the time of origination can be controlled. Besides, after altering the UAS conjugated protein, they can make it possible to conditionally coexpress a specific protein, like some ligands or receptors in some vital signaling pathways, with fluorescent signals. Furthermore, they pointed out that the tissue specific induction of fluorescent signals can be generated by injecting the DNA plasmid with Cre gene under the control of a tissue-specific promoter [19].

Hematopoiesis in zebrafish

In zebrafish primitive hematopoiesis, the anterior lateral mesoderm (ALM) mainly gives rise to myelocytes and head vasculature, while the posterior lateral plate mesoderm (PLM) mainly gives rise to erythrocytes which assemble and form intermediate cell mass (ICM), and trunk vessels [5,20–23]. It has been proven that these blood cells and vascular endothelial cells share a common progenitor called hemangioblast [17], which emerges at about 10 hpf. During this stage, *scl* and *lmo2* begin to express and function as important regulators of hemangioblasts [24]. Then at about 14 hpf, the hemangioblast generates angioblasts that will later migrate medially under the notochord and start the process of vasculargenesis and angiogenesis [25]. By about 22 hpf, the dorsal aorta and caudal vein have been generated. Nearly simultaneously, the putative HSCs or progenitor of HSCs in the ventral wall of the dorsal aorta begin detectable, as is indicated by the *runx1*'s expression in the aorta-gonad-mesonephros (AGM) region [22]. At about 30–36 hpf, the process of endothelial-hematopoietic-transition (EHT) occurs [9], and the primitive hematopoiesis ceases and is followed by the definitive hematopoiesis (Table 1; Fig. 2). The emergence of HSC is quite a complicated process which is governed by several signaling pathways. Previous studies have demonstrated that bone morphogenetic protein (BMP), Hedgehog, vascular endothelial growth factor (VEGF), as well as Notch signaling pathways all play important roles in this process. For instance, it is suggested that Hedgehog signaling is essential for angioblast migration, artery specification and sprouting [26], and also functions in maintaining dorsal artery programming [27]. BMP signaling can affect posterior hemangioblast formation through *Fli1* and thus influence on the definitive hematopoiesis [28], and besides this, it is also reported that BMP can induce ventral HSC program [27]. Epistasis experiments revealed that VEGF signaling which

Fig. 2 Hematopoiesis is closely associated with vessel development in zebrafish. (A) Whole mount *in situ* hybridization of *fli1*, a hemangioblast marker, in lateral plate mesoderm at 5 somite stage. Dorsal view, anterior to the top. (B) *flk1*-positive cells mark angioblasts migrating to the midline at 18 somite stage. Dorsal view, anterior to the top. (C) *flk1* staining demarcates artery and vein at 24 hs postfertilization (hpf). Lateral view, anterior to the left. (D) At 36 hpf, *cmyb* positive cells represent hematopoietic stem cells (arrow) which were derived from the dorsal aorta. Lateral view, anterior to the left.

lies downstream of Hedgehog signaling, functions upstream of Notch signaling on the regulation of artery differentiation and HSC formation [26,29]. However, there arises an inexplicable question on whether the process of AGM HSC specification and the artery specification are uncoupled or not, as some studies suggested that overexpressed notch can expand *runx1*$^{+}$ and *cmyb*$^{+}$ cells but failed to expand artery markers [30,31].

Zebrafish transgenic lines for HSC studies

The transgenic lines with the vascular endothelial cells and ongoing efforts of producing more putative HSCs transgenic lines have offered us great favor to reveal the emergence and transition of HSCs. To distinguish HSC from other cells, researchers have identified many HSC or (hematopoietic progenitor cell) HPC specific markers, many of which belong to the cluster of differentiation factors, such as CD34, CD41, CD45, CD90, CD133, C-kit. In addition, there are some specifically expressed transcriptional factors with key regulatory roles in different stages of HSC development. For example, *runx1* is a master regulator in HSC development [9,32,33]. It has been widely used to identify HSCs in transgenic imaging, *in situ* hybridization and flow cytometry (i.e. fluorescence-activated cell sorting, FACS), together with *cmyb* and other markers. *In vivo* imaging, owing to its immediacy and accuracy, is indispensable in the study of developmental biology, especially in the field of HSC emergence and transition, and combined with other techniques, a lot of achievements have been made during recent years. For example, the time-lapse confocal microscopy, especially combined with the 3D techniques for 4D imaging, allows us to detect and even trace a single cell's process of formation, transition, mitosis and differentiation along time. Here, we introduce the construction of transgenic lines in zebrafish and summarize the application of some of HSC- and endothelium-specific transgenic lines which will facilitate *in vivo* imaging of such cell types of interest in hematopoietic stem cell development (Table 2).

Construction of zebrafish transgenic line

There are commonly three types of methods to construct

Table 2 Molecular markers and available transgenic lines in zebrafish hematopoiesis

Molecular marker	Hemangioblast	Angioblast	Artery/Vein	HSC	Transgenic line	References
CD41				√	Y	[34]
CD45				√	Y	[35]
c-kit				√		
cmyb				√	Y	[36]
deltaC			√			
dll4			√			
ephB4			√			
ephrinB2			√			
etsrp	√	√				
fli1		√			Y	[37]
flk1		√			Y	[38]
flt4			√			
gata1	√				Y	[39]
gata2	√				Y	[40]
gfi1a				√		
gridlock		√	√			
ikaros				√	Y	[41]
lmo2	√	√			Y	[42]
msr			√			
notch1b			√			
notch5			√			
pu1	√				Y	[43]
runx1				√	Y	[44]
scl	√				Y	[45]
tbx20			√			

transgenic lines in zebrafish, such as DNA fragment or plasmid injection, I-SceI mediated DNA fragments injection, and transposon mediated DNA fragments integration. We will give a brief description of these methods below.

The first method often relates to injecting constructed DNA fragment or plasmid containing particular promoter of the target gene and the fluorescence protein, like green fluorescent protein (GFP) or red fluorescent protein (RFP), into the one-cell stage embryos. The injected embryos are raised and their fluorescence protein expression can be detected in the following days. The embryos with specific fluorescence whose expression pattern is analogous to the target gene are chosen as F0 and are raised. About three months later, F1 generation can be generated. We screen the embryos for the most intensive and specific fluorescent protein expression. When these embryos grow into adulthood, they can generate hereditarily stable F2 transgenic line.

I-SceI is a meganuclease characterized by its large recognition site [46]. When constructed plasmid with I-SceI recognition site flanking the target sequence is injected into the one-cell stage embryo combined with I-SceI mRNA, the translated I-SceI will recognize its binding site and cause double-strand breaks in plasmid and genomic DNA. Then the DNA repair system is triggered and results in increased homologous recombination. Accordingly, this method can generate higher percentage of ideal F0 embryos, compared with the first one.

Transposon mediated DNA fragment integration relates to injecting transposase mRNA and constructed plasmid with transposase recognition site sandwiching the target sequence into the one-cell stage embryos [47]. With the help of transposase, the external DNA fragment can transpose and integrate into the host chromosome more effectively. Due to its high chromosome integration efficiency, transposon mediated transgenics has been widely used in zebrafish for promoter trapping, gene trapping and enhancer trapping [48].

Available HSC- and endothelium-related transgenic lines

Friend leukemia integration 1 (fli1)

In zebrafish, *fli1* expression can be detected in the blood and endothelial common progenitors (i.e. hemangioblast) as early as at one somite stage but is restricted in blood vessels later on [28,49]. Therefore, *fli1:GFP* transgenic line is widely used as a tool to study vascular development in zebrafish [37]. Although some controversy remains, most researchers in this field believed that HSCs are derived from the floor of the dorsal aorta in the AGM region. Wen and his colleagues took use of this *fli1:GFP* transgenic line to study the HSCs development, because some of the GFP^+ cells in the ventral wall of DA express *cmyb*, a well known marker of HSC [50]. The presence of an intermediate functioning organ was noticed as temporal hematopoiesis site before the definitive hematopoiesis in pronephros is launched. They referred this putative transient hematopoietic organ as posterior blood island (PBI) [50], which is also known as CHT [10].

Stem cell leukemia (scl)

Scl, also known as Tal1, is one of the earliest markers and regulators of primitive and definitive hematopoiesis, as well as vasculargenesis. Zhang and Rodaway cloned the whole zebrafish *scl* locus with GFP and inserted it into the P1-derived artificial chromosome (PAC) to generate transgenic fish, and found that in the germline transgenic fish, GFP expression properly recapitulated endogenous expression of *scl* [45]. Using *in vivo* time-lapse imaging as early as at tail bud stage, it was confirmed that the ICM which generates erythroblasts and trunk vessel angioblasts, is indeed formed by cells from PLM [20,45]. GFP expression between the DA and PCV is likely the origin of definitive hematopoiesis, since GFP expression in this region decreased when treated with runt-related transcription factor 1-MO (runx1-MO) knock-down or the drug TCDD [20]. Our recent work also suggested that *scl:GFP* positive cells in the ventral wall of the dorsal aorta represent putative hematopoietic stem cells (Fig. 3) [5].

Flk1

flk1, also known as *kdrl*, is specifically expressed in endothelial cells in zebrafish as well as in other species. Although the HSCs are widely believed to derive from the dorsal aorta floor, this hypothesis still lacks experimental evidence. In this regard, *flk1* transgenic line may be a useful tool to validate the hypothesis (Fig. 1). In early 2010, with the help of high-resolution imaging techniques, three different groups confirmed one same thing that HSCs derive directly

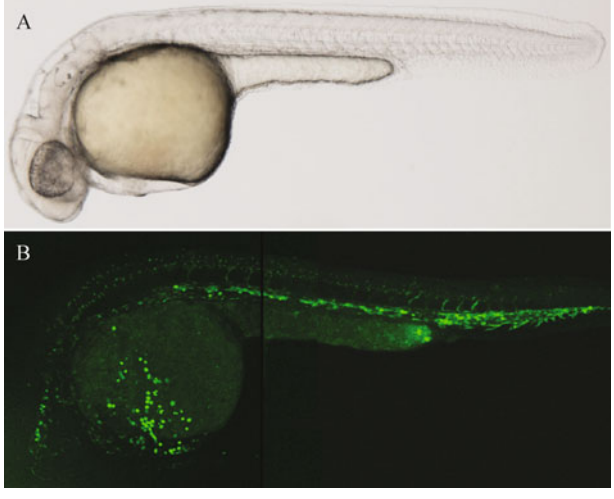

Fig. 3 Bright-field (A) and 3D confocal (B) images of *scl:GFP* transgenic zebrafish embryo show GFP expression in blood and blood vessels within the whole embryo at 30 h postfertilization (hpf).

from the ventral wall of dorsal aorta. *flk1:GFP* transgenic zebrafish was employed in two of these studies. It was revealed that the HSCs emergence relates to the endothelium bending and egressing of single cell from the ventral wall of dorsal aorta into the subaortic mesenchyme [9]. In the process of cell transition, the endothelial cells begin to possess hematopoietic potential concomitantly, as confirmed by the finding that in the *lmo2:dsRed* and *cd41:GFP* double transgenic fish the egressed dsRed$^+$ cells begin to express GFP after entering into the subaortic mesenchyme [9]. This process of cell transition was termed as EHT [9], which is highly dependent on Runx1, as the egressed cells bursted into pieces when embryos were treated with *runx1*-MO [9].

CD41

cd41 is a surface marker of early platelet progenitor and mature platelet. Its expression has been noticed on the potential HSCs in zebrafish, as indicated by the *cd41:GFP* transgenic fish. The non-mobile pool of GFP$^+$ cells between the DA and PCV decreased when treated with *scl*-MO, but not with *cmpl*-MO knockdown [34], implying that *cd41:GFP* transgenic line may be a useful tool to study the origin and transition routes of HSCs. Using this transgenic line, Herbomel's laboratory reported the living imaging of putative HSCs, and found that without intra-aorta cell clusters in AGM, the *cd41:GFP^{low}* HSCs emerge in the sub-aorta of the mesenchyme, and enter the circulation through the axial vein [10,32]. *cd41:GFP$^+$* cells are first detected by 33 to 35 hpf between the dorsal aorta and the posterior cardinal vein, and migrate to the CHT by 48 to 56 hpf. Around 54 to 56 hpf, the GFP$^+$ cells were found to colonize the nascent thymus through the mesenchyme, not by circulation [32]. A novel migration route was further identified from the *cd41:GFP$^+$* cells in AGM to the developing kidney by the pronephric tubules at 48 to 96 hpf [35].

Runx1

Runt-related transcription factor 1 (runx1) is well known for its critical role in HSCs emergence, both in mouse [33] and in zebrafish [7,9]. Flores and her colleague found that in zebrafish, *runx1* is driven by two promoters P1 and P2, resulting in two *runx1* isoforms, and they cloned and generated *runx1-P1:GFP* and *runx1-P2:GFP* transgenic lines, respectively [44]. They found that *runx1*-P1 transgenic fish first marks the erythromyeloid progenitors (EMPs) at around 18 hpf which populate the posterior blood island (PBI). However, neither primitive nor definitive hematopoiesis requires *runx1*-P1 expression, since knockdown of *runx1*-P1 by MO does not interfere with either primitive hematopoietic markers, such as *gata1*, *hhex*, and *scl* in ICM, or definitive hematopoietic markers *runx1* and *cmyb* expression [44]. On the other hand, GFP driven by *runx1*-P2 was able to label definitive HSCs in transgenic line and the GFP expression was first observed in the ventral wall of DA at around 22 hpf. Injecting *runx1* or *scl*-MO dramatically decreased the GFP expression in the AGM region [44]. Osato's group has recently identified an intronic *runx1* enhancer, which was active specifically in hemogenic endothelial cells in the AGM and targeted only precursors of HSCs as evidenced by the *runx1* enhancer transgenic line [51].

Besides the transgenic lines mentioned above, there are also some other useful lines available. For instance, *cmyb*, which is analogous to *runx1*, is widely used as a definitive HSCs marker [35]. *lmo2* is expressed in blood and endothelial progenitor cells, and *lmo2:GFP* transgenic line is a useful tool to study hematopoietic and vascular specification and may shed light on the process of HSC formation and homeostasis [52]. CD45 is known as a relatively late marker of HSCs in murine AGM [53]. In zebrafish, *cd45* expression maintains at a low level in AGM cells, and becomes detectable at a later stage than *cmyb* [35]. The *cd45*:dsRed transgenics label a subset of *cd41$^+$* cells and differentiated leukocytes, and the immigrant cells in the developing kidney show a lower expression level of *cd45* [35].

Perspectives

Although great progress in the study of HSCs development by the use of the proper HSC transgenics and *in vivo* imaging techniques has been achieved, there are also some open questions remained. For example, it is known that several signaling pathways take part in the process of HSC emergence and transition, but it is still a mystery how these pathways interact with each other and/or affect the structure alteration of cytoskeleton accordingly to facilitate cell movements. With the advances of multi-color fluorescent imaging [54], we could generate signaling-specific transgenic lines, combined with HSC imaging, to visualize HSC development in more details under normal and perturbed conditions. Besides this, how do the mesenchymal cells contribute to the process of EHT and HSCs migration? What genes are specifically expressed to distinguish the HSCs prone to migrate from their non-mobile counterparts? To address these questions, we need to identify more molecular markers at different stages of HSC development and to improve imaging techniques at more detailed subcellular-resolution level [55]. Moreover, we don't appreciate why and how some particular cells in the ventral endothelium are determined to be HSCs while others not. In addition, the WISH staining of *runx1* and *cmyb* indicate the distribution of HSCs on dispersive pattern in ventral wall of dorsal aorta. Do the putative HSCs in the ventral endothelium of the dorsal aorta have some correlation with the somite or intersomitic segmental vessel (ISV)? Some particular transgenic lines, such as specific ventral endothelium in dorsal aorta marked by fluorescence protein, will certainly help to address these questions. Thus, new transgenic lines with these features

together with newly developed imaging technologies will open a new window for us to better understanding hematopoietic stem cell development.

Acknowledgements

This work was supported by grants from the National Basic Research Program of China (2010CB945300, 2011CB943900) and the National Science Foundation of China grant (30971678).

References

1. Zon LI. Developmental biology of hematopoiesis. Blood 1995; 86 (8): 2876–2891
2. Ingham PW. The power of the zebrafish for disease analysis. Hum Mol Genet 2009; 18(R1): R107–R112
3. de Jong JL, Zon LI. Use of the zebrafish system to study primitive and definitive hematopoiesis. Annu Rev Genet 2005; 39(1): 481–501
4. Amatruda JF, Zon LI. Dissecting hematopoiesis and disease using the zebrafish. Dev Biol 1999; 216(1): 1–15
5. Ciau-Uitz A, Liu F, Patient R. Genetic control of hematopoietic development in Xenopus and zebrafish. Int J Dev Biol 2010; 54(6–7): 1139–1149
6. Chen AT, Zon LI. Zebrafish blood stem cells. J Cell Biochem 2009; 108(1): 35–42
7. Bertrand JY, Chi NC, Santoso B, Teng S, Stainier DY, Traver D. Haematopoietic stem cells derive directly from aortic endothelium during development. Nature 2010; 464(7285): 108–111
8. Boisset JC, van Cappellen W, Andrieu-Soler C, Galjart N, Dzierzak E, Robin C. *In vivo* imaging of haematopoietic cells emerging from the mouse aortic endothelium. Nature 2010; 464(7285): 116–120
9. Kissa K, Herbomel P. Blood stem cells emerge from aortic endothelium by a novel type of cell transition. Nature 2010; 464 (7285): 112–115
10. Murayama E, Kissa K, Zapata A, Mordelet E, Briolat V, Lin HF, Handin RI, Herbomel P. Tracing hematopoietic precursor migration to successive hematopoietic organs during zebrafish development. Immunity 2006; 25(6): 963–975
11. Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. Cell 2008; 132(4): 631–644
12. Ntziachristos V. Going deeper than microscopy: the optical imaging frontier in biology. Nat Methods 2010; 7(8): 603–614
13. Denk W, Strickler JH, Webb WW. Two-photon laser scanning fluorescence microscopy. Science 1990; 248(4951): 73–76
14. Shi X, Teo LS, Pan X, Chong SW, Kraut R, Korzh V, Wohland T. Probing events with single molecule sensitivity in zebrafish and Drosophila embryos by fluorescence correlation spectroscopy. Dev Dyn 2009; 238(12): 3156–3167
15. Kimmel CB, Warga RM, Schilling TF. Origin and organization of the zebrafish fate map. Development 1990; 108(4): 581–594
16. Warga RM, Kimmel CB. Cell movements during epiboly and gastrulation in zebrafish. Development 1990; 108(4): 569–580
17. Vogeli KM, Jin SW, Martin GR, Stainier DY. A common progenitor for haematopoietic and endothelial lineages in the zebrafish gastrula. Nature 2006; 443(7109): 337–339
18. Hatta K, Tsujii H, Omura T. Cell tracking using a photoconvertible fluorescent protein. Nat Protoc 2006; 1(2): 960–967
19. Collins RT, Linker C, Lewis J. MAZe: a tool for mosaic analysis of gene function in zebrafish. Nat Methods 2010; 7(3): 219–223
20. Detrich HW 3rd, Kieran MW, Chan FY, Barone LM, Yee K, Rundstadler JA, Pratt S, Ransom D, Zon LI. Intraembryonic hematopoietic cell migration during vertebrate development. Proc Natl Acad Sci USA 1995; 92(23): 10713–10717
21. Davidson AJ, Ernst P, Wang Y, Dekens MP, Kingsley PD, Palis J, Korsmeyer SJ, Daley GQ, Zon LI. cdx4 mutants fail to specify blood progenitors and can be rescued by multiple hox genes. Nature 2003; 425(6955): 300–306
22. Kalev-Zylinska ML, Horsfield JA, Flores MV, Postlethwait JH, Vitas MR, Baas AM, Crosier PS, Crosier KE. Runx1 is required for zebrafish blood and vessel development and expression of a human RUNX1-CBF2T1 transgene advances a model for studies of leukemogenesis. Development 2002; 129(8): 2015–2030
23. Liu F, Wen Z. Cloning and expression pattern of the lysozyme C gene in zebrafish. Mech Dev 2002; 113(1): 69–72
24. Patterson LJ, Gering M, Eckfeldt CE, Green AR, Verfaillie CM, Ekker SC, Patient R. The transcription factors Scl and Lmo2 act together during development of the hemangioblast in zebrafish. Blood 2007; 109(6): 2389–2398
25. Fouquet B, Weinstein BM, Serluca FC, Fishman MC. Vessel patterning in the embryo of the zebrafish: guidance by notochord. Dev Biol 1997; 183(1): 37–48
26. Gering M, Patient R. Hedgehog signaling is required for adult blood stem cell formation in zebrafish embryos. Dev Cell 2005; 8(3): 389–400
27. Wilkinson RN, Pouget C, Gering M, Russell AJ, Davies SG, Kimelman D, Patient R. Hedgehog and Bmp polarize hematopoietic stem cell emergence in the zebrafish dorsal aorta. Dev Cell 2009; 16 (6): 909–916
28. Liu F, Walmsley M, Rodaway A, Patient R. Fli1 acts at the top of the transcriptional network driving blood and endothelial development. Curr Biol 2008; 18(16): 1234–1240
29. Lawson ND, Vogel AM, Weinstein BM. sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation. Dev Cell 2002; 3 (1): 127–136
30. Burns CE, Galloway JL, Smith AC, Keefe MD, Cashman TJ, Paik EJ, Mayhall EA, Amsterdam AH, Zon LI. A genetic screen in zebrafish defines a hierarchical network of pathways required for hematopoietic stem cell emergence. Blood 2009; 113(23): 5776–5782
31. Burns CE, Traver D, Mayhall E, Shepard JL, Zon LI. Hematopoietic stem cell fate is established by the Notch-Runx pathway. Genes Dev 2005; 19(19): 2331–2342
32. Kissa K, Murayama E, Zapata A, Cortés A, Perret E, Machu C, Herbomel P. Live imaging of emerging hematopoietic stem cells and early thymus colonization. Blood 2008; 111(3): 1147–1156
33. Chen MJ, Yokomizo T, Zeigler BM, Dzierzak E, Speck NA. Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. Nature 2009; 457(7231): 887–891
34. Lin HF, Traver D, Zhu H, Dooley K, Paw BH, Zon LI, Handin RI. Analysis of thrombocyte development in CD41-GFP transgenic zebrafish. Blood 2005; 106(12): 3803–3810

35. Bertrand JY, Kim AD, Teng S, Traver D. CD41+ cmyb+ precursors colonize the zebrafish pronephros by a novel migration route to initiate adult hematopoiesis. Development 2008; 135(10): 1853–1862
36. North TE, Goessling W, Walkley CR, Lengerke C, Kopani KR, Lord AM, Weber GJ, Bowman TV, Jang IH, Grosser T, Fitzgerald GA, Daley GQ, Orkin SH, Zon LI. Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. Nature 2007; 447 (7147): 1007–1011
37. Lawson ND, Weinstein BM. *In vivo* imaging of embryonic vascular development using transgenic zebrafish. Dev Biol 2002; 248(2): 307–318
38. Jin SW, Beis D, Mitchell T, Chen JN, Stainier DY. Cellular and molecular analyses of vascular tube and lumen formation in zebrafish. Development 2005; 132(23): 5199–5209
39. Long Q, Meng A, Wang H, Jessen JR, Farrell MJ, Lin S. GATA-1 expression pattern can be recapitulated in living transgenic zebrafish using GFP reporter gene. Development 1997; 124(20): 4105–4111
40. Jessen JR, Meng A, McFarlane RJ, Paw BH, Zon LI, Smith GR, Lin S. Modification of bacterial artificial chromosomes through chi-stimulated homologous recombination and its application in zebrafish transgenesis. Proc Natl Acad Sci USA 1998; 95(9): 5121–5126
41. Bajoghli B, Ramialison M, Aghaallaei N, Czerny T, Wittbrodt J. Identification of starmaker-like in medaka as a putative target gene of Pax2 in the otic vesicle. Dev Dyn 2009; 238(11): 2860–2866
42. Bertrand JY, Kim AD, Violette EP, Stachura DL, Cisson JL, Traver D. Definitive hematopoiesis initiates through a committed erythro-myeloid progenitor in the zebrafish embryo. Development 2007; 134(23): 4147–4156
43. Hsu K, Traver D, Kutok JL, Hagen A, Liu TX, Paw BH, Rhodes J, Berman JN, Zon LI, Kanki JP, Look AT. The pu.1 promoter drives myeloid gene expression in zebrafish. Blood 2004; 104(5): 1291–1297
44. Lam EY, Chau JY, Kalev-Zylinska ML, Fountaine TM, Mead RS, Hall CJ, Crosier PS, Crosier KE, Flores MV. Zebrafish runx1 promoter-EGFP transgenics mark discrete sites of definitive blood progenitors. Blood 2009; 113(6): 1241–1249
45. Zhang XY, Rodaway AR. SCL-GFP transgenic zebrafish: *in vivo* imaging of blood and endothelial development and identification of the initial site of definitive hematopoiesis. Dev Biol 2007; 307(2): 179–194
46. Thermes V, Grabher C, Ristoratore F, Bourrat F, Choulika A, Wittbrodt J, Joly JS. I-SceI meganuclease mediates highly efficient transgenesis in fish. Mech Dev 2002; 118(1–2): 91–98
47. Kawakami K, Shima A, Kawakami N. Identification of a functional transposase of the Tol2 element, an Ac-like element from the Japanese medaka fish, and its transposition in the zebrafish germ lineage. Proc Natl Acad Sci USA 2000; 97(21): 11403–11408
48. Kawakami K. Transposon tools and methods in zebrafish. Dev Dyn 2005; 234(2): 244–254
49. Liu F, Patient R. Genome-wide analysis of the zebrafish ETS family identifies three genes required for hemangioblast differentiation or angiogenesis. Circ Res 2008; 103(10): 1147–1154
50. Jin H, Xu J, Wen Z. Migratory path of definitive hematopoietic stem/progenitor cells during zebrafish development. Blood 2007; 109 (12): 5208–5214
51. Ng CE, Yokomizo T, Yamashita N, Cirovic B, Jin H, Wen Z, Ito Y, Osato M. A Runx1 intronic enhancer marks hemogenic endothelial cells and hematopoietic stem cells. Stem Cells 2010; 28(10): 1869–1881
52. Zhu H, Traver D, Davidson AJ, Dibiase A, Thisse C, Thisse B, Nimer S, Zon LI. Regulation of the lmo2 promoter during hematopoietic and vascular development in zebrafish. Dev Biol 2005; 281(2): 256–269
53. Bertrand JY, Giroux S, Golub R, Klaine M, Jalil A, Boucontet L, Godin I, Cumano A. Characterization of purified intraembryonic hematopoietic stem cells as a tool to define their site of origin. Proc Natl Acad Sci USA 2005; 102(1): 134–139
54. Finley KR, Davidson AE, Ekker SC. Three-color imaging using fluorescent proteins in living zebrafish embryos. Biotechniques 2001; 31(1): 66–70
55. Beis D, Stainier DY. *In vivo* cell biology: following the zebrafish trend. Trends Cell Biol 2006; 16(2): 105–112