Migration of cardiomyocytes is essential for heart regeneration in zebrafish

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SUMMARY
Adult zebrafish possess a significant ability to regenerate injured heart tissue through proliferation of pre-existing cardiomyocytes, which contrasts with the inability of mammals to do so after the immediate postnatal period. Zebrafish therefore provide a model system in which to study how an injured heart can be repaired. However, it remains unknown what important processes cardiomyocytes are involved in other than partial de-differentiation and proliferation. Here we show that migration of cardiomyocytes to the injury site is essential for heart regeneration. Ventricular amputation induced expression of cxcl12a and cxcr4b, genes encoding a chemokine ligand and its receptor. We found that cxcl12a was expressed in the epicardial tissue and that Cxcr4 was expressed in cardiomyocytes. We show that pharmacological blocking of Cxcr4 function as well as genetic loss of cxcr4b function causes failure to regenerate the heart after ventricular resection. Cardiomyocyte proliferation was not affected but a large portion of proliferating cardiomyocytes remained localized outside the injury site. A photoconvertible fluorescent reporter-based cardiomyocyte-tracing assay demonstrates that cardiomyocytes migrated into the injury site in control hearts but that migration was inhibited in the Cxcr4-blocked hearts. By contrast, the epicardial cells and vascular endothelial cells were not affected by blocking Cxcr4 function. Our data show that the migration of cardiomyocytes into the injury site is regulated independently of proliferation, and that coordination of both processes is necessary for heart regeneration.

KEY WORDS: Heart regeneration, Cardiomyocytes, Zebrafish, Directed migration, CXCL12-CXCR4

INTRODUCTION
Regeneration is a complex biological process by which animals restore the shape, structure and function of body parts lost to injury or experiment (Brockes and Kumar, 2005; Poss, 2010). One of the most remarkable examples is heart regeneration (Ausoni and Sartore, 2009; Laflamme and Murry, 2011). Several non-mammalian vertebrates, including zebrafish, possess the significant ability to restore injured heart. After resectioning of the ventricular apex, zebrafish restore lost heart tissue within 1-2 months (Poss et al., 2002; Raya et al., 2003), which contrasts to the inability of mammals to do so after the immediate postnatal period (Bergmann et al., 2009; Porrello et al., 2011). The regenerative ability is also observed in response to a variety of injuries, including cryoprobe-induced injury (Chablais et al., 2011; Gonzalez-Rosa et al., 2011; Schnabel et al., 2011) and genetic ablation of cardiomyocytes (CMs) by transgenic induction of toxin expression (Wang et al., 2011). It therefore provides a model system to study how an injured heart can be repaired (Poss, 2007; Raya et al., 2004). Recent reports showed that the major source of regenerated CMs was pre-existing CMs, rather than stem/progenitor cells, that undergo partial de-differentiation, re-enter the cell cycle and proliferate to restore the injured heart (Jopling et al., 2010; Kikuchi et al., 2010).

Simultaneously, vascularization of the regenerating area takes place, which requires the activities of fibroblast growth factor (FGF) signaling and vascular endothelial growth factor (VEGF) signaling (Kim et al., 2010; Lepilina et al., 2006). However, it is still unknown whether CMs are involved in other processes, in addition to proliferation, to restore the injured heart. More specifically, cell migration is known to be involved in the development of a variety of organs (Friedl and Gilmour, 2009; Raz and Mahabaleeshwar, 2009), and organ regeneration involves reactivation of developmental processes and genes (Iovine, 2007).

The recent advent of photoconvertible fluorescent reporter proteins provided a new approach for cell migration analysis (Stark and Kulesa, 2007). Natural and engineered green fluorescent proteins, which can be photoconverted into red fluorescent proteins, have been developed (Lukyanov et al., 2005). Such proteins include Kaede (Ando et al., 2002), PA-GFP (Patterson and Lippincott-Schwartz, 2002), KikGR (Tsutsui et al., 2005), EosEP (Nienhaus et al., 2006), PA-mRFP (Verkhusha and Sorkin, 2005) and Dendra (Gurskaya et al., 2006). Kaede is one of the founding members of photoconvertible fluorescent proteins, serendipitously found from a stony coral, *Trachyphyllia geoffroyi* (Ando et al., 2002). The unique photoconvertible characters of Kaede represent several advantages for optic marking. First, the red state is comparable to the green in terms of brightness and stability. Second, photoconversion is shown to result in a more than 2000-fold increase in the red/green fluorescent ratio. Third, completely separate wavelengths of light can be used for observation and photoconversion. By taking advantage of these unique properties, several reports have demonstrated cell tracing in vivo, such as monitoring cell migration during zebrafish embryogenesis (Hatta et al., 2006), cell migratory behaviors in cortical slices of mice (Mutoh et al., 2006) and T-cell migration during cutaneous immune response in mice (Tomura et al., 2010; Tomura et al., 2008). The
use of cell-type-specific promoters/enhancers has also facilitated monitoring specific types of cells in combination with photoconversion technology, such as differentiation of cardiomyocytes (de Pater et al., 2009), beta cells of the pancreas (Pisharath et al., 2007) and postmitotic neurons (Pan et al., 2012).

Here we show that migration of CMs to the injury site is essential for heart regeneration. We found that the chemokine ligand, cxcl12a and its receptor, cxcr4b, were induced after heart injury in zebrafish. Blocking Cxcr4 function caused mis-localization of proliferating CMs outside of the injury site without affecting their proliferation. By localized photoconversion in hearts of the CM-specific Kaede transgenic zebrafish, we show that CMs migrate toward the injury site only after heart damage, and this migration requires Cxcr4 function. Our data show that the migration of CMs into the injury site is regulated independently of proliferation, and that coordination of both processes is essential for heart regeneration.

MATERIALS AND METHODS
Zebrafish maintenance and surgery
Zebrafish were maintained under standard conditions at around 28°C, and adult zebrafish (6 to 18 months old) were used for experiments. Ventricular amputation was performed as previously published (Raya et al., 2003). Care and experimentation were done in accordance with the Institutional Animal Care and Use Committee of the University of Minnesota and the National Institute of Advanced Industrial Science and Technology, Japan.

In situ hybridization, immunostaining and TUNEL assay
In situ hybridization on sections 14 μm thick was performed as previously described (Kawakami et al., 2011). Immunostaining on sections was performed according to a standard procedure (Kawakami et al., 2006; Raya et al., 2003). The primary antibodies used were anti-myosin heavy chain (MHC; Developmental Studies Hybridoma Bank, MF20, 5.14 μg/ml), anti-CXCR4 (Santa Cruz Biotechnology, sc-6190, 1:100), anti-PCNA (Santa Cruz Biotechnology; sc-56, 1:100), anti-MEF2 (Santa Cruz Biotechnology; sc-313, 1:50), anti-GFP (Molecular Probes, A1122, 1:500) and anti-DsRed2 (Clontech, 632496, 1:500). Secondary antibodies used were Alexa fluorophore-labeled anti-mouse or rabbit or goat IgG (Invitrogen, 1:1000) or biotinylated anti-mouse IgG (Vector Laboratories, BA-9200, 1:500). An In Situ Cell Death Detection Kit (Roche Diagnostics) was used for TUNEL assay according to the manufacturer’s instruction. Counterstaining was done with DAPI or hematoxylin. The number of proliferating CMs was counted manually with the sections that represent the largest injury area at the center of the injury site of each heart.

EdU-labeling experiment
To label proliferating cells, 20 μl of 0.5 mg/ml EdU solution was intraperitoneally injected into zebrafish at 7 days post amputation (dpa) and 10 dpa. Hearts were collected at 13 dpa and subjected to standard analysis according to the manufacturer’s instructions (Invitrogen, Click-IT EdU cell proliferation assays).

Pharmacological blocking of Cxcr4 function and genetic loss of cxcr4b function
For pharmacological blocking of Cxcr4 function, each fish was maintained in 50 ml system water with or without 40 nM FC131 after surgery (Narumi et al., 2010) (Wako Chemical, Osaka, Japan), and the water was refreshed daily. Because the carrier is H2O, nothing was added into the containers of control fish. To assist with the penetration of FC131, the pericardiac cavity was surgically opened weekly in both control and treated fish. For genetic loss of cxcr4b function, we used the odysseus mutant fish line, which possesses a null mutation in the cxcr4b gene (Knaut et al., 2003). Wild-type siblings were used as controls for the odysseus mutants (hereafter odysseus mutants are referred to as cxcr4b−/−).

Generation of the cmlc2a-Kaede line
The cmlc2a-Kaede line was established with the zebrafish cmlc2a promoter (Huang et al., 2003) and Kaede (Medical & Biological Laboratories, Nagoya, Japan) using the Tol2 transposon system (Kawakami et al., 2004; Urasaki et al., 2006). The P0 fish were outcrossed with AB fish, and stable transgenic lines were established after evaluating cardiac-specific expression of Kaede.

Photoconversion of Kaede
For photoconversion of cmlc2a-Kaede transgenic fish hearts, we made a small window by manually dissecting the pericardiac cavity after anesthetizing fish. A compound microscope (Nikon LABPHOT) equipped with a 100 watt mercury lamp, diachronic mirror of 380 nm and a barrier filter of 420 nm were used to irrigate the heart for 90 seconds with approximately 2 mm distance between the 20× objective lens and the heart. To injure the heart, we enlarged the pericardic window and amputated the apex of the ventricle under fluorescent monitoring with a Zeiss V12 stereomicroscope.

Imaging
Fluorescent confocal images were obtained by using a Zeiss LSM 710 laser scanning microscope system, and analyzed by ZEN2009 software. For Kaede imaging, non-fixed hearts were embedded in optimal cutting temperature compound and frozen. Sections at 20 μm thickness were dried, washed with PBS, rinsed with water and mounted for confocal imaging. Kaede green fluorescence was excited by 514 nm light and 519–556 nm fluorescence was detected. Wavelengths for excitation and detection of Kaede red fluorescence were 562 nm and 566–674 nm, respectively.

Statistical analysis
Statistical significance was analyzed by the Student’s t-test, and shown as average ± standard deviation. P-values are indicated within each panel.

RESULTS
Induction of cxcl12a and cxcr4b expression after heart injury
During expression screening of genes expressed in developing and regenerating zebrafish hearts, we found that cxcl12a, a gene encoding a chemokine ligand (also known as sdf1a), and its receptor cxcr4b were expressed during heart regeneration (Fig. 1). The transcripts of cxcl12a were detected between 3 dpa and 7 dpa at the surface and inside of the regenerating area (Fig. 1). The signals of cxcr4b became evident at 5 dpa, and persisted until 10 dpa (Fig. 1). cxcr4b expression was detected at the surface layer of the regenerating area and around the injury site. These expression patterns were not detected in sham-operated hearts at 3 dpa (cxcl12a) and 7 dpa (cxcr4b), the stages at which they were detected at high levels (n=5 each, data not shown). By contrast, we did not detect transcripts of the related ligand-receptor pair cxcl12b and cxcr4a during heart regeneration by in situ hybridization (supplementary material Fig. S1). The CXCL12-CXCR4 system is known to regulate directed cell migration during embryonic development (Friedl and Gilmour, 2009; Raz and Mahabaleshwar, 2009; Schier, 2003). Thus, induction of the expression of cxcl12a and cxcr4b after heart injury suggests that directed cell migration plays a role during heart regeneration.

CMs express cxcr4b
As a first step to investigate the possible involvement of directed cell migration during heart regeneration, we sought to identify cell types that express cxcr4b and cxcl12a. Immunofluorescence analysis showed that the CM-specific nuclear signal of Me2 (supplementary material Fig. S2) is associated with membrane-localized Cxcr4 signal on the same confocal plane (Fig. 2A,B), indicating that CMs express Cxcr4b. Further analysis of the Cxcr4 signal and CM-specific cmlc2a-EGFP signal also showed that 287 Cxcr4-positive signals from six sections were associated with the cmlc2a-EGFP signal (Fig. 2C-E). Similarly, the Cxcr4 signal was
These data indicate that epicardial cells express EGFP-positive endothelial cells (supplementary material Fig. S3G-H) or in fli1-cxcl12a-DsRed2 reporter (Glass et al., 2011) in MHC-epicardial cells (Perner et al., 2007). We did not detect expression (supplementary material Fig. S3A-C/H11032) of the cxcl12a-DsRed2 reporter (Fig. 1A-H) in the regenerating area (arrowheads in B). B shows a higher magnification image of the boxed area in A. The dotted line indicates the amputation plane. (C-E) Confocal images of Cxcr4 (C) and cmlc2a-EGFP signal, detected by anti-GFP antibody (D), and a merged image (E). The arrowheads point to cells positive for both Cxcr4 and cmlc2a-EGFP signals. (F-H) Confocal images of Cxcr4 (F) and fli1-EGFP signal, detected by anti-GFP antibody (G), and a merged image (H). Scale bars: 50 μm.

Also associated with MHC and Mef2 signals (Fig. 3). Moreover, optic sectioning of Cxcr4-expressing CMs showed Cxcr4 signal in the cytoplasm, in addition to the membrane (Fig. 3B,C), suggesting Cxcl12a-dependent internalization of Cxcr4b (Orsini et al., 1999). These results demonstrate that CMs express Cxcr4. In the mouse, endothelial cells also express Cxcr4 (Gupta et al., 1998; Volin et al., 1998); however, we did not detect Cxcr4 in endothelial cells (Fig. 2F-H). Two hundred and sixty Cxcr4 signals from five sections of fli1-EGFP transgenic fish heart were not associated with the endothelial-cell-specific fli1-EGFP signal.

Next, we investigated which cells express cxcl12a. Consistent with the expression of cxcl12a at the surface of the regenerating area (Fig. 1), the cxcl12a signals overlapped with wrt signals (supplementary material Fig. S3A-C′), which is expressed in the epicardial cells (Perner et al., 2007). We did not detect expression of the cxcl12a-DsRed2 reporter (Glass et al., 2011) in MHC-positive CMs (supplementary material Fig. S3D-F) or in fli1-EGFP-positive endothelial cells (supplementary material Fig. S3G-I). These data indicate that epicardial cells express cxcl12a after heart injury.

**Cxcr4 function is required for heart regeneration**

To determine the functional significance of the Cxcl12-Cxcr4 system during heart regeneration, we blocked Cxcr4 function by treating fish with an antagonist after ventricular amputation. We first tested whether FC131, a recently developed selective CXCR4 antagonist (Narumi et al., 2010), can block zebrafish Cxcr4 function. AMD3100, a widely used CXCR4 antagonist, was not used because our previous studies indicated that it did not efficiently block Cxcr4 function, either in the adult zebrafish hematopoietic system in vivo (Glass et al., 2011) or in the migration of primordial germ cells in zebrafish embryos (data not shown). Treating zebrafish embryos with FC131 significantly impaired directed migration of primordial germ cells into the gonad-forming region (supplementary material Fig. S4), a process that has been shown to depend on cxcr4b function (Doitsidou et al., 2002; Knaut et al., 2003), demonstrating that FC131 can effectively block Cxcr4 function in zebrafish. We then treated zebrafish with FC131 after amputation of the ventricle and found that this resulted in failure to regenerate the heart (Fig. 4A-F′). In control regenerating hearts, MHC signals (CM marker) were strongly detected in the injury site, where the heart tissue was removed, at 14 dpa, and the regenerating tissue was filled with CMs at 30 dpa (Fig. 4A, A′, C,C′). At 60 dpa, the regenerated myocardium exhibited a strong MHC signal, comparable to the non-injured area (Fig. 4E, E′). However, significantly lower levels of MHC signals were detected in the injury site of CXCR4-antagonist-treated hearts at 14 dpa (Fig. 4B′, n=4/5 hearts). After 30 days and 60 days the CXCR4-antagonist-treated heart lacked a sealed wall (Fig. 4D, D′, red arrows, n=5/5 hearts; 4F′, n=3/4 hearts), which was fully formed in control hearts (Fig. 4C, C′; black arrows, n=5/5 hearts; 4E, E′, n=5/5 hearts). These results indicate that the CXCR4-antagonist-treated heart did not regenerate. The significant reduction of MHC signals in the injury site was also observed in cxcr4b−/− fish at 14 dpa (Fig. 4G, G′, n=3/3 hearts). Similar to the CXCR4-antagonist-treated heart, cxcr4b−/− fish hearts failed to form the sealed wall at 30 dpa (Fig. 4J, J′, n=2/2 hearts) and 60 dpa (Fig. 4L, L′, n=2/2 hearts), compared with control hearts (Fig. 4I, I′,
Fig. 4. Cxcr4 function is required for heart regeneration.


n=3/3 hearts; 4K,K’, n=3/3 hearts). Fibrin clearance at 14 dpa was similar in control and cxcr4b–/– fish (supplementary material Fig. S5, n=2/2 for both control and cxcr4b–/– hearts), and thus, the failure to regenerate the heart is unlikely to be caused by abnormal fibrin deposition. These data demonstrate that cxcr4b function is required for heart regeneration in zebrafish. These results strongly support our hypothesis that cxcr4b functions in CMs that contribute to heart regeneration. Although cxcr4b expression was specific to CMs in regenerating zebrafish hearts (Fig. 2), Cxcr4 is also known to be involved in heart development in mice (Ma et al., 1998; Tachibana et al., 1998; Zou et al., 1998). In order to avoid complications caused by an unidentified role of cxcr4b in zebrafish heart development, if any, we focused our study of cxcr4b function by pharmacological antagonism using FC131.

Mis-localization of proliferating CMs by blocking Cxcr4 function

Given that the proliferation of CMs is a major factor for heart regeneration (Jopling et al., 2010; Kikuchi et al., 2010), we asked whether blocking Cxcr4 function affected CM proliferation and/or survival. The number of proliferating CMs, visualized by proliferating cell nuclear antigen (Pcna, an S-phase marker) and Mef2 (CM marker), did not change significantly in Cxcr4-blocked hearts compared with control hearts at 7, 14, 21 and 30 dpa (Fig. 5A–C, n=5 hearts at each time point). This result also indicates that the failure to regenerate the heart by blocking Cxcr4 function is not due to delayed CM proliferation. The number of Pcna-positive cells, which includes CMs and non-CMs, was also not changed at these time points (supplementary material Fig. S6), indicating that the proliferation of other cell types was also unaffected by CxCR4 antagonist treatment. TUNEL analysis did not show an increase in the number of cell deaths at 7 dpa and 14 dpa (supplementary material Fig. S7). These data indicate that the failure to regenerate the amputated heart by blocking Cxcr4 function is not caused by defects in proliferation or survival of CMs and other cells. A recent study showed that the number of genetically labeled CMs located at the sub-epicardial area increases in the injury site, whereas their number reduces at the edge of the injury site during heart regeneration (Kikuchi et al., 2010). Thus, we compared the number of proliferating CMs in the injury site, similar to the study by Kikuchi and colleagues (Kikuchi et al., 2010). We found a change in the localization of proliferating CMs in Cxcr4-blocked hearts. In control hearts we found 74.0% of proliferating CMs around the center of the regenerating area, and the majority of the other proliferating CMs at the edge of the regenerating area at 14 dpa. By contrast, only 23.5% of the proliferating CMs were located in the center of the regenerating area, similar to the study by Kikuchi and colleagues (Kikuchi et al., 2010). We found a change in the localization of proliferating CMs in Cxcr4-blocked hearts. In control hearts we found 74.0% of proliferating CMs around the center of the regenerating area, and the majority of the other proliferating CMs at the edge of the regenerating area at 14 dpa. By contrast, only 23.5% of the proliferating CMs were located around the center of the injury site in Cxcr4-blocked hearts (Fig. 5D). Because the total number of proliferating CMs was not altered (Fig. 5C), this observation suggests that Cxcr4 function is necessary for localization of proliferating CMs in the regenerating area.

Detection of Pena visualizes cells in the S phase of the cell cycle at the time of sample fixation. In order to further examine CM
proliferation, we labeled cells by EdU. Injection of EdU at 7 dpa and 10 dpa, followed by detection at 13 dpa, allows for labeling cells that underwent cell division during 7 dpa to 13 dpa (Fig. 5E-F'). Similar to the analysis of Pcna detection, the number of proliferating CMs, visualized as EdU and cmlc2a-mCherry double-positive cells, were at similar levels in control and CXCR4-antagonist-treated hearts (Fig. 5G). We found that 72.4% of EdU-labeled CMs were located around the center of the regenerating area. By contrast, only 12.4% of EdU-labeled CMs were located around the center of the injury site in Cxcr4-blocked hearts (Fig. 5H), similar to the case with Pcna analysis. This EdU-labeling experiment further confirmed a role for Cxcr4 function in the localization of proliferating CMs in the regenerating area.

**Localized labeling of CMs by photoconversion of the Kaede fluorescent protein**

Given that the Cxcl12-Cxcr4 system is known to regulate directed cell migration in a variety of processes (Friedel and Gilmour, 2009; Raz and Mahabaleshwar, 2009; Schier, 2003), the abnormal localization of proliferating CMs suggests that the migration of proliferating CMs, rather than local activation of CMs in the injury site, is necessary for heart regeneration. To address this hypothesis, we developed a fluorescent reporter-based CM-tracing assay. We established a transgenic zebrafish line that expresses Kaede (Ando et al., 2002), driven by the CM-specific cmlc2a promoter (supplementary material Fig. S8A,B, cmlc2a-Kaede). The green fluorescence of Kaede can be irreversibly converted into stable red fluorescence by ultraviolet irradiation for cell tracing in vivo (supplementary material Fig. S8C-H) (Ando et al., 2002; Tomura et al., 2008). Thus, we were able to label CMs in a restricted area of the ventricle with red fluorescence in order to trace labeled CMs during heart regeneration (Fig. 6A-F; Fig. 7A). Maintaining cmlc2a-Kaede fish under normal breeding conditions (Fig. 6G-J, n=8), as well as ventricular amputation under a fluorescent stereomicroscope (Fig. 6K-N, n=8), did not induce green to red photoconversion. For localized photoconversion of the heart, we exposed a small part of the ventricle and performed irradiation (see Materials and methods) (Fig. 6A-F). Photoconverted red fluorescence was observed immediately after irradiation and only in the irradiated area (Fig. 6O-R, n=8). Seven days after irradiation, the red fluorescence was stable and remained as a cluster (Fig. 6S-V, n=8), indicating that CMs labeled with red fluorescence did not migrate under normal conditions. We also observed newly synthesized green Kaede 7 days after irradiation such that the irradiated CMs fluoresced both red and green (compare Fig. 6O and 6S). This indicates that the irradiation was not toxic to CMs. Also, we did not detect cell death at 1 day and 3 days after irradiation in the irradiated area (data not shown).

**Cxcr4-dependent CM migration after injury**

To analyze the migration of CMs, we amputated the apex in the non-irradiated portions of the ventricle under a fluorescent stereomicroscope (Fig. 7A-C, n=6). Three days after amputation, we did not detect CMs in the regenerating area (data not shown, n=7), consistent with the lack of ccrx4b signal in the regenerating area at this time point (Fig. 1F). However, seven days after amputation, CMs with both green and red fluorescence, and CMs with only green fluorescence, were detected in the regenerating area (Fig. 7D,E, n=6). We detected 5.5% of photoconverted CMs in the regenerating area (Fig. 7P). This result demonstrates that CMs migrated into the regenerating area by 7 dpa. The ratio of photoconverted CMs in the regenerating area was 10.4% and 12.1% at 10 dpa (Fig. 7H,I,P, n=7 hearts) and 14 dpa (Fig. 7L,M,P, n=7 hearts), respectively. At 10 dpa and 14 dpa, a portion of photoconverted CMs exhibited strong green signals due to continued de novo production of Kaede. Blocking Cxcr4 function caused CMs to be excluded from the injury site at 7 dpa (Fig. 7F,G,P, n=6 hearts), 10 dpa (Fig. 7J,K,P, n=6 hearts) and 14 dpa (Fig. 7N,O,P, n=6 hearts). Our data revealed that the cxxcl12a-ccrx4b-dependent system regulates directed migration of CMs into the injury site, and that this process is essential for heart regeneration.
Epicardial cells are unlikely to be affected by blocking Cxcr4
Cells other than CMs, such as epicardial cells and vascular endothelial cells, are also known to participate in heart regeneration in zebrafish (Kikuchi et al., 2011b; Kim et al., 2010; Lepilina et al., 2006). To further understand how Cxcr4 regulates heart regeneration, we examined whether these cells are also affected by blocking Cxcr4 function during heart regeneration. Expression of aldehyde dehydrogenase 1a2 (aldh1a2, also known as retinaldehyde dehydrogenase 2, raldh2), a gene encoding a rate-limiting enzyme for retinoic acid synthesis, is upregulated in the epicardial tissue after cardiac damage (Lepilina et al., 2006), and retinoic acid signaling is required for heart regeneration (Kikuchi et al., 2011b). We observed aldhl1a2 expression in a wide region of the epicardium at 3 dpa, and at the surface of the injury site at 7 dpa, similar to the control heart (supplementary material Fig. S9A-F). aldhl1a2 expression is also detected in endocardial cells after heart injury (Kikuchi et al., 2011b), which can be visualized by flil-EGFP reporter at 3 dpa (supplementary material Fig. S9C,D). Thus, aldhl1a2 expression in both epicardial and endocardial tissue appeared to be unaffected by CXCR4 antagonist treatment. Expression of wtlb marks the epicardial tissue in regenerating hearts (González-Rosa et al., 2011; Kikuchi et al., 2011a; Schnabel et al., 2011). We detected comparable wtlb mRNA expression in the epicardial tissue of both control and Cxcr4-blocked hearts at 3 dpa (supplementary material Fig. S9G-H') and 7 dpa (supplementary material Fig. S9J-'). To further evaluate whether epicardial responses are affected by CXCR4 antagonist treatment, we measured the length of the wtlb-expressing domain (supplementary material Fig. S9G,H,I,J, blue lines). The ratio of the lengths of the wtlb-expressing domain to the length of the surface of the regenerating area were comparable in control and CXCR4-antagonist-treated hearts at both time points (supplementary material Fig. S9K). These results indicate that gene expression in the epicardial tissue was not affected by blocking Cxcr4 function during heart regeneration.

Blocking of CXCR4 function is unlikely to affect neo-vascularization in regenerating heart
Neo-vascularization of the regenerating area is crucial for heart regeneration (Kim et al., 2010; Lepilina et al., 2006). Although Cxcr4 was not detected in endothelial cells after ventricular amputation (Fig. 2), we sought to clarify whether vascularization was affected by blocking Cxcr4 function. The flil-EGFP signal was detected similarly in control and CXCR4-antagonist-treated hearts at 7 dpa (Fig. 8A,B, n=3) and 14 dpa (Fig. 8C,D, n=3). Analysis of the flil-EGFP-positive region in the regenerating area at 14 dpa by ImageJ software showed a similar level of vascularization (Fig. 8E). Thus, CXCR4-antagonist treatment is unlikely to affect neo-vascularization during heart regeneration in zebrafish.

It has been shown that FGF signaling is required for neo-vascularization of the regenerating area during heart regeneration (Lepilina et al., 2006); thus, we also examined activation of FGF signaling. Phosphorylation of ERK, a hallmark of the activation of FGF signaling, was detected both in control and Cxcr4-blocked hearts (Fig. 8F,G). Expression of mkp3/dusp6, a target of FGF signaling (Kawakami et al., 2003), was also detected similarly in the control and CXCR4-antagonist-treated heart (Fig. 8H,I). These results indicate that activation of FGF signaling occurred similarly in both control and Cxcr4-blocked hearts. Comparable activation of gene expression in the epicardial tissue (supplementary material Fig. S9) and vascularization (Fig. 8) further support the idea that the failure to regenerate the injured heart by blocking Cxcr4 function is caused by a defect in the CMs themselves.

DISCUSSION
Two cxcl12-cxcr4 systems in zebrafish
In this report, we identified cxcl12a-cxcr4b-dependent CM migration as an essential mechanism for heart regeneration in zebrafish. The
CXCL12-CXCR4 system is a major chemokine-receptor system that regulates directed migration of a variety of cells (Raz and Mahabaleshwar, 2009). Zebrafish have two cxcl12 genes and two cxcr4 genes as a result of the teleost genome duplication during evolution (Amores et al., 1998). This gene duplication appears to contribute to functional segregation of the cxcl12-cxcr4 system. For instance, during embryonic development, the cxcl12b-cxcr4a system functions for blood vessel development, endothelial cell migration (Bussmann et al., 2011; Siekmann et al., 2009), and endoderm migration during gastrulation (Mizoguchi et al., 2008; Nair and Schilling, 2008). The cxcl12a-cxcr4b system is known to function for primordial germ cell migration (Knaut et al., 2003; Raz, 2003), sensory ganglia assembly (Knaut et al., 2005) and lateral line migration (Haas and Gilmour, 2006), and is also expressed in regenerating fins (Bouzaffour et al., 2009). This functional segregation might have contributed to the viability of the cxcr4b mutant line, because mouse mutants that lack either Cxcl12 or Cxcr4 die before birth owing to a ventricular septal defect, defective formation of the large vessels in the gastrointestinal tract and impaired hematopoietic development (Ma et al., 1998; Tachibana et al., 1998; Zou et al., 1998). Our data demonstrated that the cxcl12a-cxcr4b system functions to regulate CM migration, which is essential for heart regeneration, and that the cxcl12b-cxcr4a system might be involved in the regeneration of other organs.
Cxcr4 function is necessary in CMs during heart regeneration in zebrafish

Our analysis showed that Cxcr4 functions for directed migration of CMs toward the injury site in zebrafish. This is in contrast to mammalian myocardial infarction models (Takahashi, 2010), in which Cxcr4 functions in CMs (Hu et al., 2007) and bone-marrow-derived mesenchymal stromal cells (Honzarenko et al., 2006). In mammals, Cxcr4 in CMs is shown to act for enhanced cell survival and reduction of infarction size (Hu et al., 2007). The Cxcl12-Cxcr4 system in the mesenchymal stromal cells functions for cardioprotection (Saxena et al., 2008), and a fraction of bone-marrow-derived cells can differentiate into CMs (Wojakowski et al., 2010). In amputated zebrafish hearts, CM proliferation and survival were not affected by blocking Cxcr4 function (Fig. 5; supplementary material Fig. S7). Moreover, it is yet to be determined whether mesenchymal stromal cells in zebrafish (Lund et al., 2012) can contribute to CMs. Nonetheless, our data highlight the requirement of Cxcr4 function in CMs during heart regeneration in zebrafish.

The Cxcl12-Cxcr4 system and neo-vascularization

Our analysis showed a lack of Cxcr4 signal in endothelial cells in regenerating hearts (Fig. 2). This contrasts to mammalian myocardial infarction models (Takahashi, 2010), according to which Cxcr4 is expressed in cell types involved in neo-vascularization, such as bone-marrow-derived mesenchymal stromal cells (Honzarenko et al., 2006; Yamaguchi et al., 2003) and endothelial progenitor cells (Yamaguchi et al., 2003). Although zebrafish stromal cells can exhibit endothelial-like properties (Lund et al., 2012), neo-vascularization seems to be unaffected in Cxcr4-antagonist treated fish (Fig. 8). Studies have demonstrated that neo-vascularization in regenerating zebrafish hearts involves the contribution of epicardial cells (Kim et al., 2010; Lepilina et al., 2006), which are unlikely to be affected by Cxcr4 antagonist treatment (supplementary material Fig. S9). The present study does not rule out the possibility that other unidentified cell types migrate to the injured heart in a Cxcr4-dependent manner and contribute to heart regeneration in zebrafish. However, data obtained in our analyses suggest that neo-vascularization occurs independently from Cxcr4 function during heart regeneration in zebrafish (Fig. 9).

Kaede-photoconversion system and cell tracing

Cell lineage analysis is an important issue to understand complex processes of regeneration, in which multiple cell types are involved (Tanaka and Reddien, 2011). A genetic recombination approach using CreER transgenic lines is a powerful method, especially for long-term lineage analysis (Jopling et al., 2010; Kikuchi et al., 2010). The advent of photoconvertible fluorescent proteins, such as Kaede, has led to the development of an effective approach for tracing migration of specific cell types in vivo (Ando et al., 2002). Our data show that CMs labeled by Kaede photoconversion migrate toward the injury site during heart regeneration (Fig. 7). Recent studies also showed neutrophil mobilization in zebrafish larvae (Deng et al., 2011) and developmental timing assays during zebrafish heart development (de Pater et al., 2009) with similar approaches. Thus, localized photoconversion in combination with the use of cell-type-specific promoters/enhancers would be a valuable approach for cell migration and lineage analysis in a variety of biological processes.

A role for CM migration during heart regeneration

The present study demonstrates that CM migration is an essential mechanism for heart regeneration, in addition to CM proliferation and vasculature formation. Considering that clearing fibrin scarring would also be important for heart regeneration, remodeling of the extracellular matrix should also be coupled to these processes. Such a process might involve unidentified molecular systems, and to be studied in the future. Our data also indicate that the proliferation of CMs and neo-vascularization are regulated independently from migration (Fig. 9). As both proliferation of CMs (Jopling et al., 2010; Kikuchi et al., 2010) and their migration (this study) are necessary, these two events need to be coordinated for the regeneration of the injured heart. Previous studies show that genes involved both in cell cycle regulation and cell movement are upregulated in regenerating zebrafish hearts (Lien et al., 2006; Sleep et al., 2010), which supports the conclusions of our research. Given that the neonatal mammalian heart also possesses the ability to regenerate after resectioning through the proliferation of pre-existing CMs (Porrello et al., 2011), the correct coordination of migration and proliferation may prove to be crucial for heart regeneration not only in zebrafish but also in mammalian species.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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