Retention of Stem Cell Plasticity in Avian Primitive Streak Cells and the Effects of Local Microenvironment

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ABSTRACT

Primitive streak (PS) is the first structure occurring in embryonic gastrulation, in which the epiblast cells undergo the epithelial-mesenchymal transition to become the loose mesoderm cells subsequently. Because the mesoderm cells departing from different portions of PS are blessed with disparate migration trajectory and differentiation fate, one question is when the cell fate is determinated. To understand whether the cell fate and cell migration pattern will be alternated along with the microenvironment transformation, the traditional transplantation technology was used to replace the anterior PS cells in HH4 host embryo using posterior PS tissue labeled by green fluorescent protein (GFP) in the same stage donor embryo, and then, we tracked the migration trajectory of the GFP-positive cells with fluorescence stereomicroscope after incubation, and eventually verified the cell contribution from the transplants with *in situ* hybridization and immunocytochemistry. The same experimental strategy applied for posterior PS site replacement in host embryo. We found that the transplanted posterior PS cells to anterior part of streak followed the anterior PS cell migration pattern rather than kept its posterior streak cell migration trajectory, and so did vice versa. In addition, the transplants were involved in the contribution to the subsequent organogenesis as the local PS tissues affirmed by specific expression of myocardial or hematopoietic markers. Therefore, our data strongly suggest that the PS cells still keep stem cell plasticity during gastrulation and the eventual cell fate will depend on the spatial gene expression within local microenvironment along with development. Anat Rec, 296:533–543, 2013.

Key words: gastrulation; cell migration; local microenvironment; primitive streak; graft

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The primitive streak (PS) is a long rod-like structure first emerging in the embryonic gastrula stage. It originates from the medial posterior margin of the embryo and extends rostrally over 80% of the chick embryo before regressing. The PS not only present in chick but also in mouse and other vertebrate embryos (Tam and Behringer, 1997); the PS can be considered the symbol for the commencement of gastrulation (Chuai et al., 2006). During gastrulation, epiblast cells undergo an epithelial-mesenchymal transition (EMT) in the PS and shape the mesoderm and endoderm by migrating anterolaterally and posterolaterally (Fig. 1A–D) to form three germ layers, ectoderm, mesoderm, and endoderm (Fig. 1A,B; Chuai and Weijer, 2008; Downs, 2009). These in turn give rise to all types of adult tissues (Mikawa et al., 2004).

When studying autonomous cell movement during early embryo development, although the effect of tissue movement on cell migration cannot be ignored, especially extra-cellular matrix-induced passive epithelial-like sheet movement (Zamir et al., 2008), cell migration patterns, and fates in the PS seem to depend on their location in the streak. Thus, one open question is when is cell fate determined and what is the crucial factor that commits the cell fates? In this study, we pay close attention to the plasticity of the progenitor cells in different regions of the chick PS.

The exact timing of the specification of the fate of the mesoderm and endoderm in the gastrulating embryo has bedeviled developmental biologists since long ago. Chapman et al. reported that in the chick embryo the cell fate of

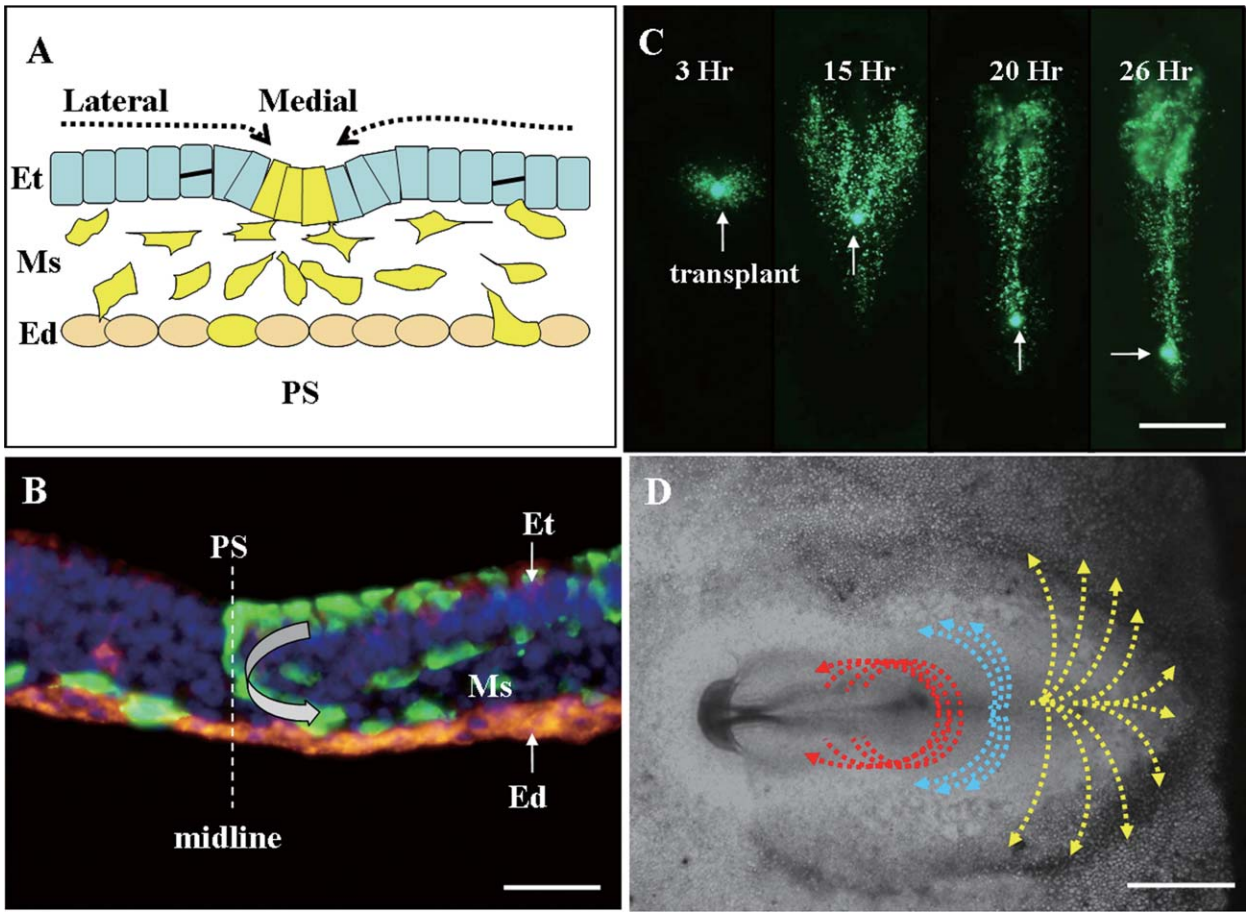

Fig. 1. The migration pattern of mesoderm cell derived from primitive streak during chick gastrulation. **A**: Schematically drawing of cross section shows that epiblast cell move sheet-likely to medial primitive streak (PS), and then ingress there to generate mesoderm and endoderm cells later on. **B**: Like schematically drawing indicated, the GFP-transfected cells in epiblast (ectoderm) underwent EMT in primitive streak to give rise to mesoderm and endoderm indicated by line arrowhead. The endoderm was labeled by the expression of HNK1 (red). **C**: The mesoderm cells derived from anterior primitive streak transplant migrate anterolaterally while the Hensen's node and primitive streak regress, which is overtly exhibited in the series photographs at 3, 15, 20, and 26 hr after orthotropic transplantation between donor and host $HH3^{+}$ chick embryo. **D**: Using the same orthotropic transplantation approach, the mesoderm cells migration pattern from primitive streak is richly illustrated as in the three color dotted lines, red ones stand for the cells from anterior primitive streak cell [as in (D)], blue ones stand for the cells from middle primitive streak, migrating more laterally before back to midline, and moreover, the yellow lines stand for the cells from posterior primitive streak, migrating in extra-embryo region. Scale bar = 30 μm in (B) and 1 mm in (C) and (D). Abbreviations used: Ed = endoderm; Et = ectoderm; Ms = mesoderm; PS = primitive streak.

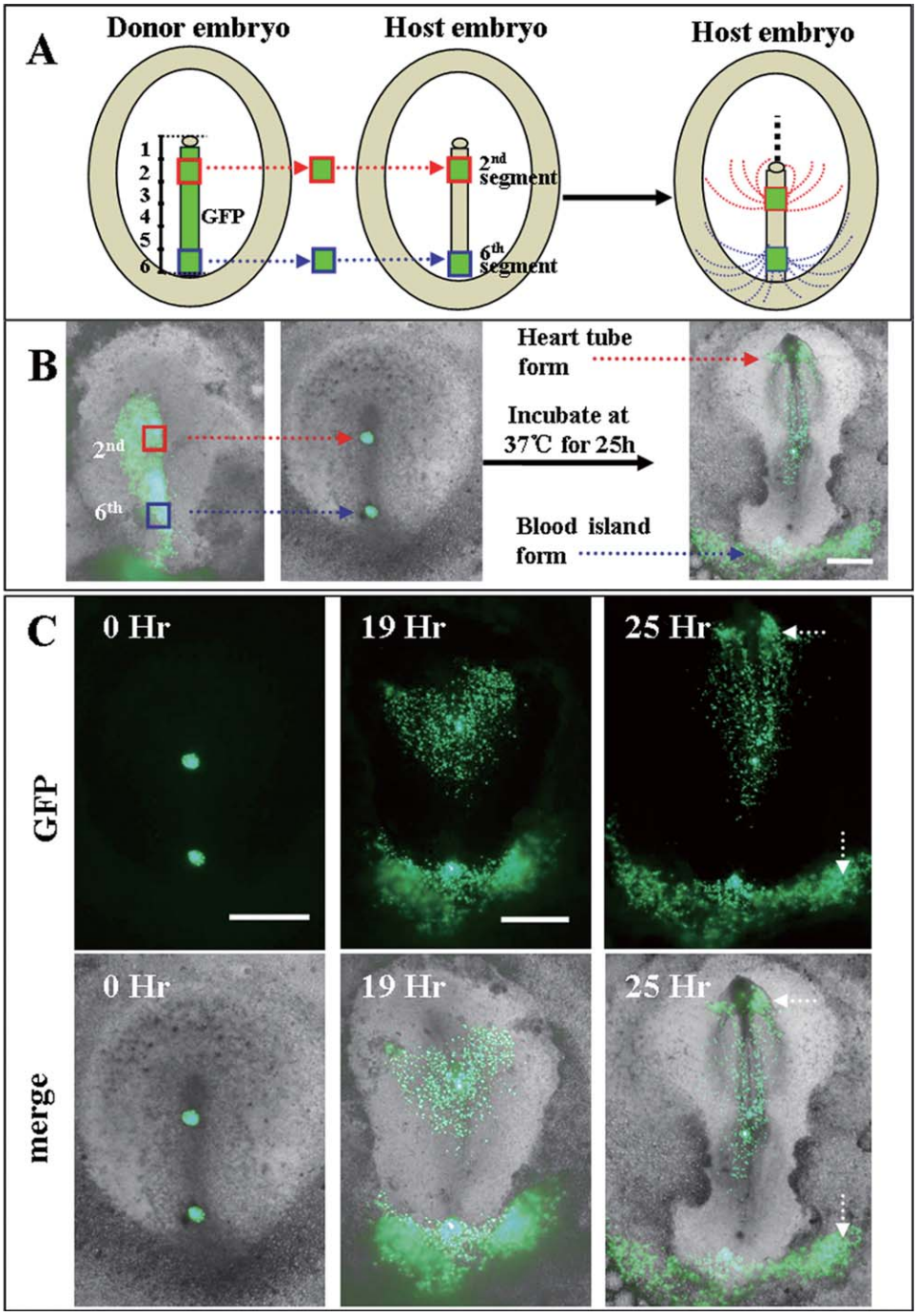

Fig. 2. The migration pattern and derivation for the anterior and posterior primitive streak cells during chick gastrulation. **A**: To compare the cell migration and derivation of different region of primitive streak, first, we divided the whole streak into six equal size segments for the $HH3^{+}$ chick embryo primitive streak. Schematically drawing of orthotropic transplantation strategy shows that the transfected anterior (2nd segment) and posterior (6th segment) primitive streak tissue were grafted to the same position in untransfected same stage ($HH3^{+}$) host chick embryo, respectively, and then assess the transplanted GFP-labeled cell migration pattern and contribution after overnight incubation. **B**: The transplantation was performed according to the schematic photograph (A) and the 2nd segment and 6th segment primitive streak GFP cells gave rise to the heart tube (including somites and mesoderm structure) and blood islands in area opaca, respectively, after 25-hr incubation. **C** and **D**: The transplanted embryo was photographed at incubating hour 0, 19, and 25 with fluorescence images in upper panel and merge images in lower panel, in which transplant GFP cells were in original points at 0 hr, and migrating on the way at 19 hr, overtly nearly arrived in the heart tube forming and blood island forming regions, respectively, at 25 hr as indicated by white dotted line arrowheads. Scale bars = 1 mm in (B) and (C).

prospective mesoderm and endoderm is committed at Hamburger–Hamilton stage 3 (HH3) (Hamburger and Hamilton, 1951), but that the cell fate depends on the microenvironment. That is, the cell fate is altered if the population of cells is transplanted to a new site (Chapman et al., 2007). Fate-mapping studies demonstrate that the order and the site of progenitor cell ingression through the streak determines the fate of mesodermal cells (Kinder et al., 1999), although some details of cell fate determination especially the relationship to the local microenvironment during early embryo development remain obscure.

Here, using heterotopic grafting of PS tissue, we reveal that anterior PS cells are subjected to site-specific influences from the posterior PS region when transplanted to the caudal PS. Transplanted cells migrate like native cells, moving toward extra-embryonic areas to form blood islands. Similarly, when posterior PS tissue is transplanted into the anterior PS region, the transplanted posterior cells also undergo the native anterior migration trajectory and eventually contribute to heart tube formation.

MATERIALS AND METHODS

Chick Embryo Incubation and Explant Culture

Fertilized leghorn eggs were obtained from the Avian Farm of South China Agriculture University and incubated in a humidified incubator (Yiheng Instruments, Shanghai, China) at 38°C with humidity until the required HH stage (Hamburger and Hamilton, 1951).

We divided the HH3 chick embryo streak into six equal size portions, and the explants from the anterior (second segment) and posterior (sixth segment) (Fig. 2A) were cultured *in vitro* in culture medium (DMEM-F12 GIBCO) at 37°C and 5% CO_2 for 48 hr (James and Schultheiss, 2003).

Chick Embryo Electroporation and Transplantation

HH3 chick embryos in early chick (EC) (Chapman et al., 2001) culture were transfected by electroporation (BTX-ECM399) using two-wire parallel electrodes made of platinum, and twice pulsed at 10 V as described before (Yang et al., 2002). The transfected embryos were then continuously incubated at 37°C with humidity until the required stage. Transplantation was performed as previously described (Yang et al., 2002). In brief, the PS from a transfected donor embryo was orthotopically transplanted into an untransfected host embryo of the same stage (HH3) at the same position (Fig. 2A). Alternatively, heterotopic transplantation was performed as outlined in the schematic drawing in Figs. 4 and 6A. After transplantation, embryos were incubated for various times to assess green fluorescent protein (GFP) positive cell migration patterns and spatial contributions.

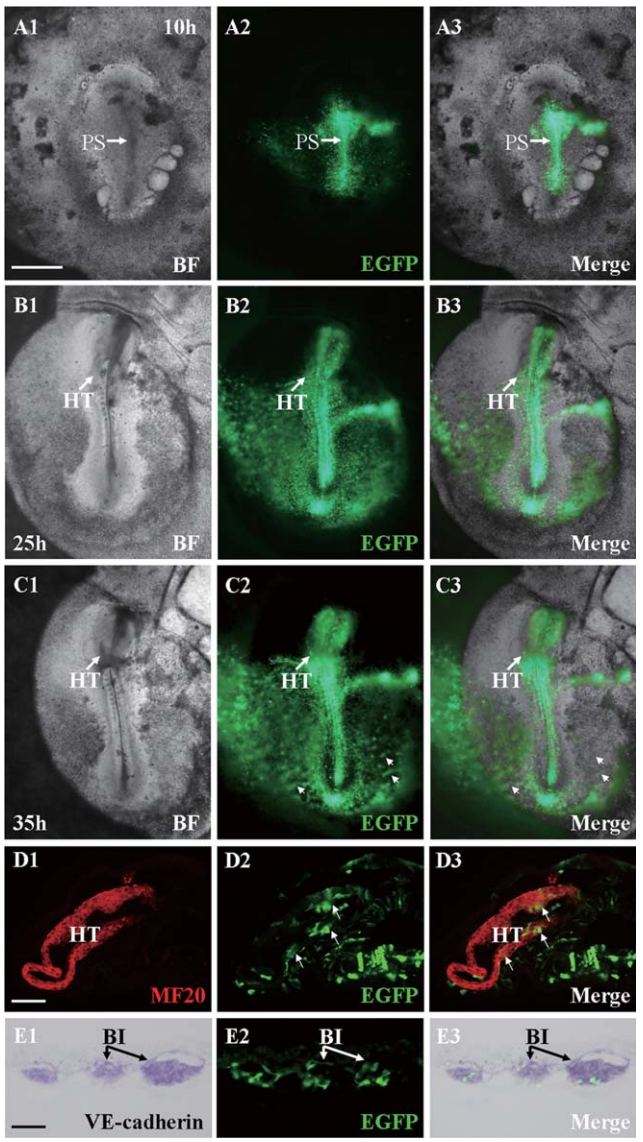

Fig. 3. **A1–A3**: Bright-field (A1), fluorescence (A2), and merge images (A3), respectively, at 10-hr incubation following transfection. **B1–B3**: Bright-field (B1), fluorescence (B2), and merge images (B3), respectively, at 25-hr incubation following transfection. **C1–C3**: Bright-field (C1), fluorescence (C2), and merge images (C3), respectively, at 35-hr incubation following transfection. **D1–D3**: Transverse section for MF20 immunohistochemistry (D1), EGFP transfected (D2), and merge of D1 and D2, respectively, at heart tube level of 35-hr incubation embryo following transfection. **E1–E3**: Transverse section for VE-cadherin *in situ* hybridization (E1), EGFP transfected (E2), and merge of E1 and E2, respectively, at blood island level of 35-hr incubation embryo following transfection. Abbreviation used: BI = blood island; HT = heart tube; PS = primitive streak. Scale bar = 1 mm in (A–C), 50 μm in (D), and 100 μm in (E). Abbreviations used: BI = blood island; HT = heart tube; PS = primitive streak.

Immunohistochemistry and *In Situ* Hybridization

Immunohistochemistry of whole-mount chicken embryos was performed to detect sarcomeric myosin (MF20) expression as previously described (Yang et al., 2008). Briefly, embryos were fixed with 4% paraformaldehyde at 4°C overnight. Nonspecific interactions were blocked with 2% bovine serum albumin, 1% Triton-X, and 1% Tween 20 in phosphate-buffered saline (PBS) for 2 hr at room temperature, followed in a brief wash with PBS before the embryos were incubated with a MF20 mouse monoclonal antibody (Developmental Studies Hybridoma Bank) (Bader et al., 1982) overnight at 4°C on rocker. After extensive washing, the embryos were incubated with goat anti-mouse antibody

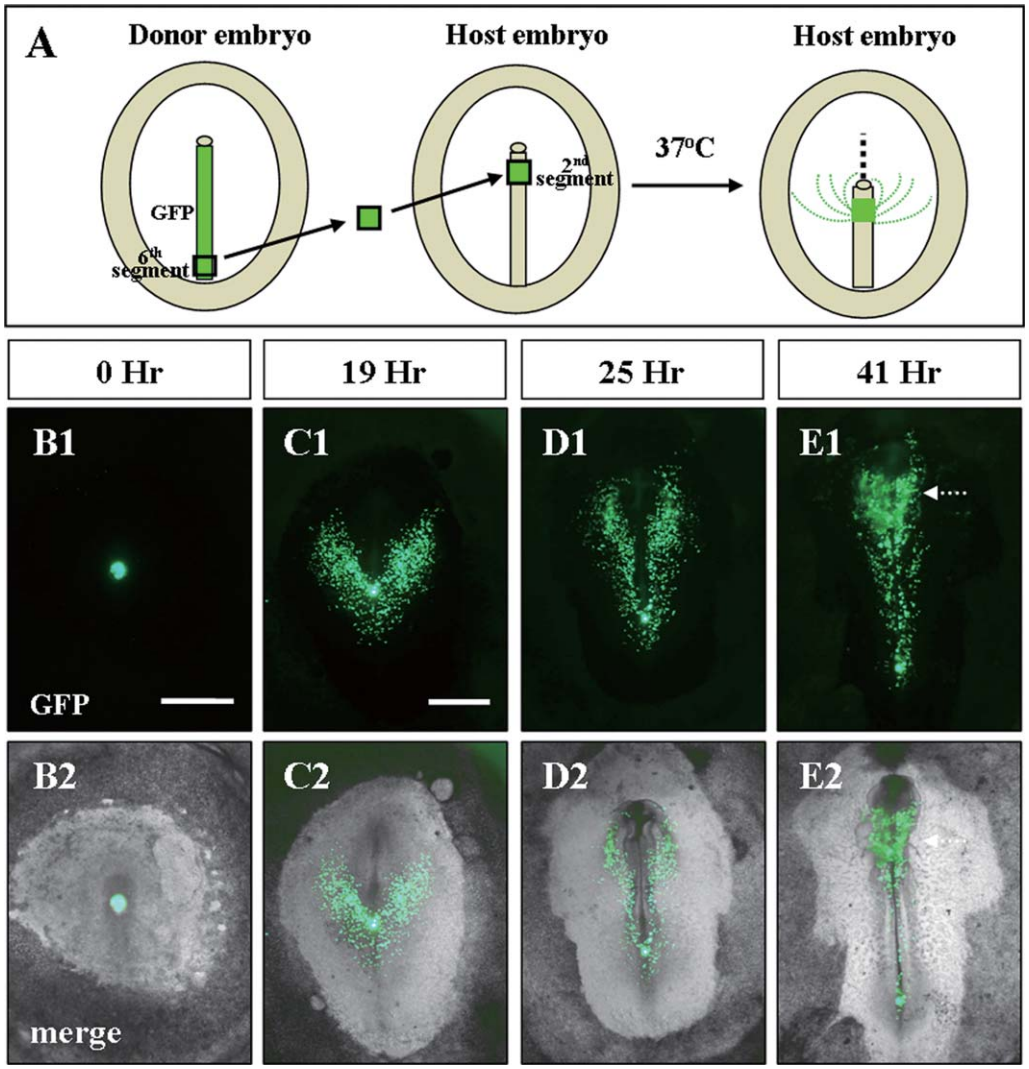

Fig. 4. The migration trajectory of transplanted posterior primitive streak (6th segment) cells follows the instinct one of anterior primitive streak (2nd segment) after heterotopic transplantation. **A**: Schematically drawing of heterotopic transplantation shows that the transplant from 6th segment was grafted to 2nd segment position in an untransfected same stage host embryo. **B–E**: The transplanted embryo was photographed at incubating hour 0, 19, 25, and 41 with fluorescence images in upper panel (B1–E1) and merge images in lower panel (B2–E2), in which transplanted GFP cells were in original points at 0 hr, and migrating on the way at 19 and 25 hr, overtly arrived in the region of heart tube forming at 41 hr as indicated by dotted line arrowheads. Obviously, the posterior primitive steak cells transformed their migration trajectory in keeping with anterior primitive streak one after transplantation. The number of samples in this analysis is 56. Scale bars = 1 mm in (B–E).

coupled with Alexa Fluor 555 (Invitrogen) overnight at 4°C on rocker to visualize the antigen.

Whole mount *in situ* hybridization was performed as previously described (Henrique et al., 1995). For *in situ* hybridization on explants, pieces of sheet-like tissues were dissected from the culture dish and then processed as for whole-mount *in situ* hybridization.

Photography

Whole-mount embryos were photographed using a stereoscopic fluorescence microscope (Olympus MVX10) using the Olympus software package Image-Pro Plus 7.0 after immunohistochemistry or *in situ* hybridization. Subsequently, the embryos were sectioned at 15 μm with a cryostat (Leica CM1900) and photographed with an epifluorescent microscope (Olympus IX51, Leica DM 4000B) at 200 × or 400 × magnification using the Olympus software package Leica CW4000 FISH.

RESULTS

Normal Cell Migration Patterns of Primitive Streak Cells During Chick Gastrulation

We modified our previous transplantation approach (Yang et al., 2002) and used orthotropic double-site grafting of the PS to assess the cell migration patterns

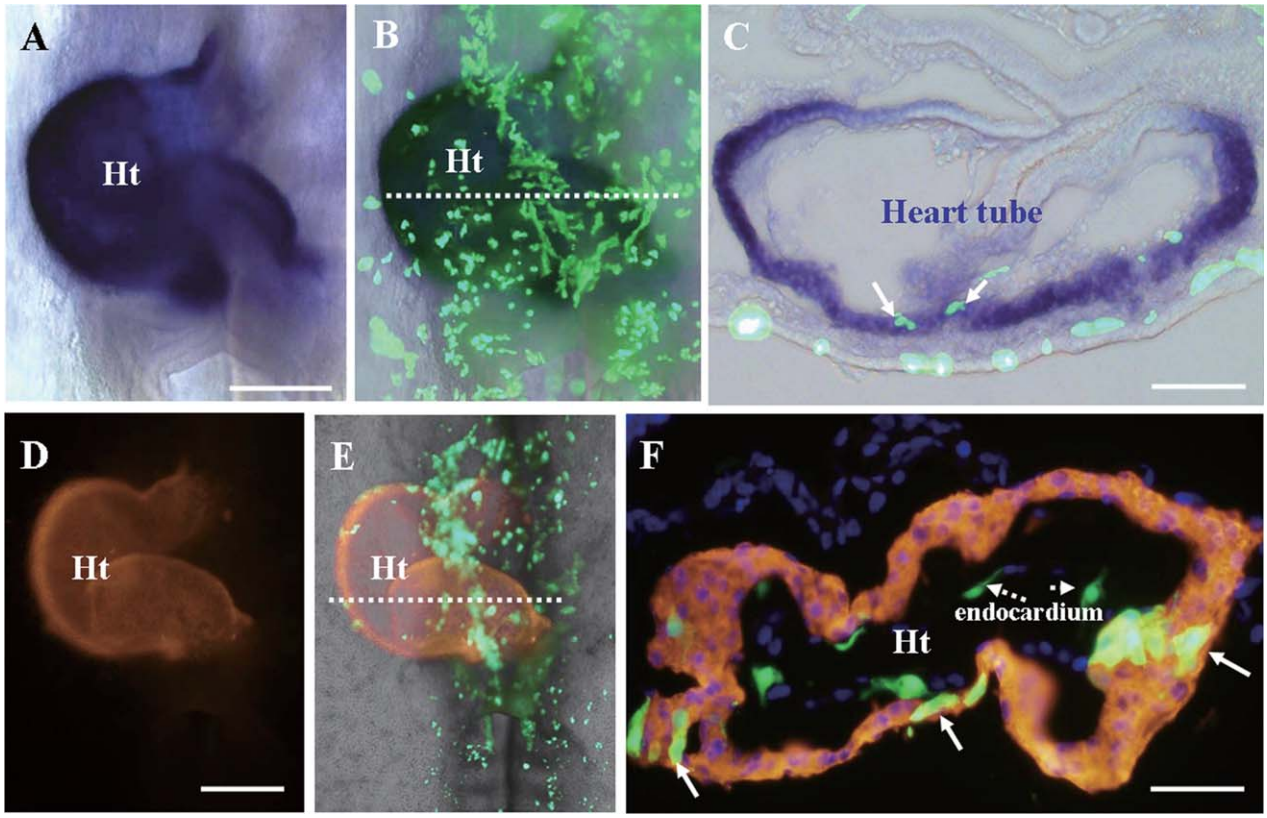

Fig. 5. The convertibility verification of the transplanted posterior primitive streak cells after heterotopic grafting to the site of anterior primitive streak. **A**: *In situ* hybridization shows that vMHC gene specifically express in the looping heart tube of the host embryo. **B**: The merge image of *in situ* and GFP transfection shows that GFP-positive cells derived from posterior primitive streak gave rise to looping heart tube, which should have been contributed from anterior primitive streak cells. **C**: The transverse section at the level indicated with dotted line in (B) shows that GFP positive cells were involved in the heart tube formation indicated by white solid line arrowheads. **D**: Immunocytochemistry against MF20 shows that MF20 specific expression in the host embryo looping heart tube. **E**: The merge image of MF20 immunocytochemistry and GFP transfection shows that GFP-positive cells derived from posterior primitive streak gave rise to looping heart tube regardless of scattering, which should have been contributed from anterior primitive streak cells. **F**: The transverse section at the level indicated with dotted line in (E) shows that GFP positive cells were involved in the heart tube formation indicated by solid line arrowheads (myocardium) and dotted line arrowheads (endocardium). Scale bar = 200 μm in (A and B), 50 μm in (C), 300 μm in (D and E), and 30 μm in (F). Abbreviation used: Ht = heart tube.

of cells leaving the anterior and posterior PS, respectively (Fig. 2A).

No migrating GFP positive single cells could be seen at the initial time point. At 19 hr, GFP cells of the second segment transplant had migrated anterolaterally, whereas the cells of sixth segment had migrated to the extra-embryonic area opaca. At 25 hr, the anterolaterally migrating cells were situated toward the midline, where the somites and heart tube form, whereas the migrating cells in the area opaca were situated more laterally and reached the region of blood island formation (Fig. 2B,C).

We used electroporated cells in the PS, along with the native cells, as a control to show whether electroporation itself has an effect on cell differentiation. We transfected the whole streak with enhanced green fluorescent protein (EGFP) plasmid at HH3. GFP positive cells in the PS were observed after 10-hr of incubation following transfection (Fig. 3A1–A3). At 25 hr of incubation (Fig. 3B1–B3), part of the GFP positive cells migrated to the area opaca (Fig. 3B2), and some GFP positive cells participated in the formation of mesoderm tissues such as somites (Fig. 3B2) and the left/right heart tube (Fig. 3B3) as indicated by white arrows (Fig. 3B1–B3). At 35-hr of incubation (Fig. 3C1–C3), GFP positive cells located in the heart tube (Fig. 3C1–C3), and in the area opaca, some GFP positive cells participated in the formation of blood islands as indicated by white arrow heads (Fig. 3C1–C3). To verify whether the GFP positive cells participated in the heart tube and blood islands, we performed MF20 immunohistochemistry and VE-cadherin *in situ* hybridization, respectively. As shown in Fig. 3D1–D3, the co-localization of GFP positive cells and MF20 positive cells in the heart tube was obvious as indicated by white arrows (Fig. 3D2,D3). In blood islands (indicated by arrows in Fig. 3E1–E3), the co-localization of GFP positive cells and VE-cadherin positive cells were observed. These findings demonstrate that electroporation has no effect on the cell differentiation.

Migration and Differentiation of Posterior Primitive Streak Cells Transplanted to the Rostral Primitive Streak Site

To assess the plasticity of posterior PS cells, we performed heterotopic transplantation, in which the sixth segment PS tissue from an EGFP transfected donor

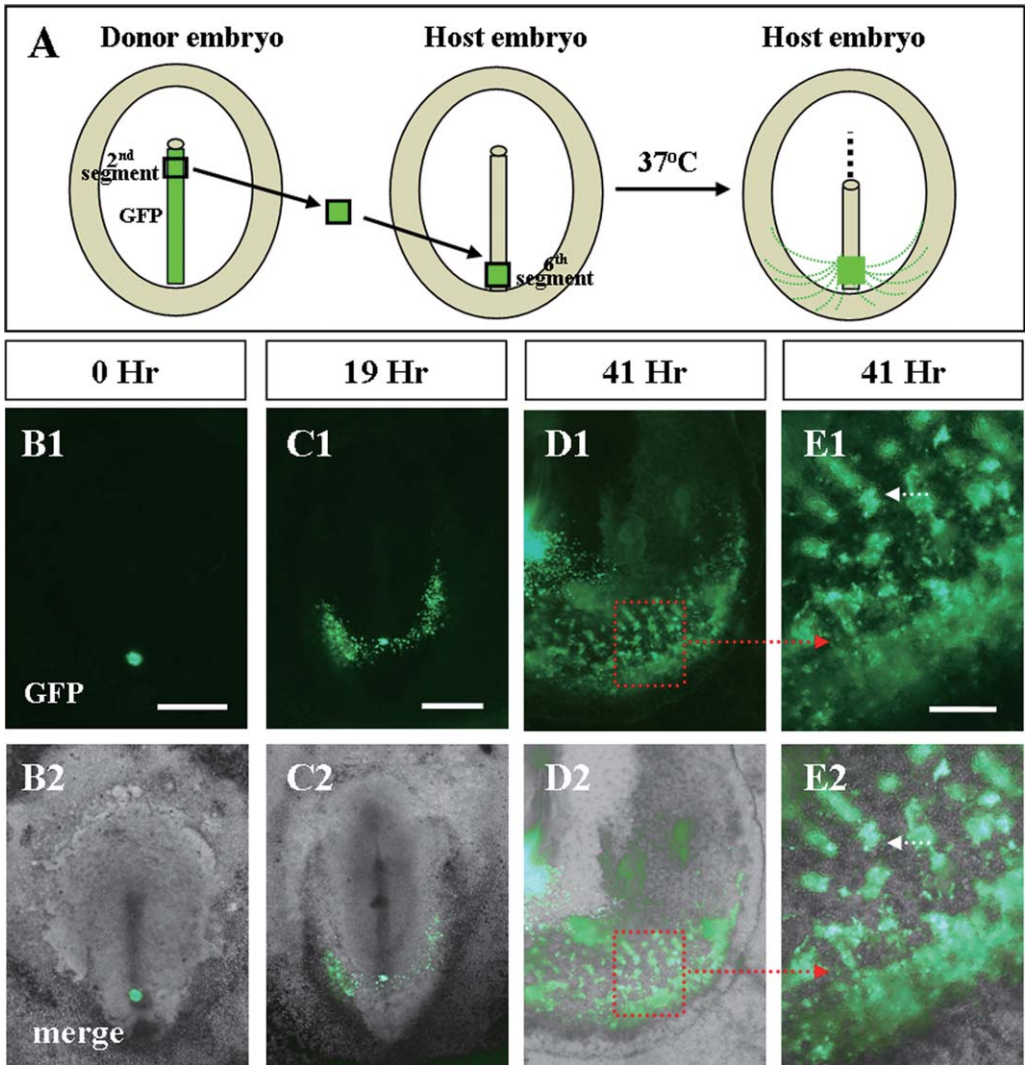

Fig. 6. The migration trajectory of transplanted anterior primitive streak (2nd segment) cells follows the instinct one of posterior primitive streak (6th segment) after heterotopic transplantation. **A**: Schematically drawing of heterotopic transplantation shows that the transplant from the 2nd segment was grafted to the 6th segment position in an untransfected host embryo with the same stage. **B–E**: The transplanted embryo was photographed at incubating hour 0, 19, and 41 with fluorescence images in upper panel (B1–D1) and merge images in lower panel (B2–D2); moreover, (E1) and (E2) are the magnified images indicated with red dotted line squares in (D1) and (D2), respectively, in which transplanted GFP cells were in original points at 0 hr, and migrating on the way at 19 hr, overtly arrived in the region of blood island forming in area opaca at 41 hr as indicated by white dotted line arrowheads. Obviously, the anterior primitive steak cells transformed their migration trajectory in keeping with posterior primitive streak one after transplantation. The number of samples in this analysis is 46. Scale bars = 1 mm in (B–D) and 300 μm in (E).

embryo was grafted to a second segment PS site (Fig. 4A). Whereas cells from the sixth segment would normally migrate caudal-laterally toward the area opaca, heterotopically transplanted cells followed the migration trajectory of the second segment cells, as evidenced by the GFP positive cells in the transplanted embryo at 19, 25, and 41 hr post-transplantation (Fig. 4B–E). GFP positive cells particularly accumulated in the region of straight heart tube formation (arrows in Fig. 4E).

Although cells derived from the transplanted sixth segment followed the second segment cell migration pattern, it remained to be determined if they could differentiate into myocardial precursor cells. Therefore, mRNA expression of ventricular myosin heavy chain (vMHC), a specific myocardial marker (Somi et al., 2006), was determined by *in situ* hybridization in the transplanted host embryo (Fig. 5A–C). As seen here, vMHC is expressed specifically in looping heart tube (Fig. 5A), an area where many GFP positive cells derived from the sixth segment transplant (Fig. 5B) are found. In high-power merged images of transverse sections, co-localized GFP positive cells (indicated by arrowheads in Fig. 5C) are observed.

To obtain more information about the cellular phenotype in the transplanted host embryo, we examined expression of sarcomeric myosin (MF20), a specific marker for myocardium (Bader et al., 1982). Sarcomeric myosin was

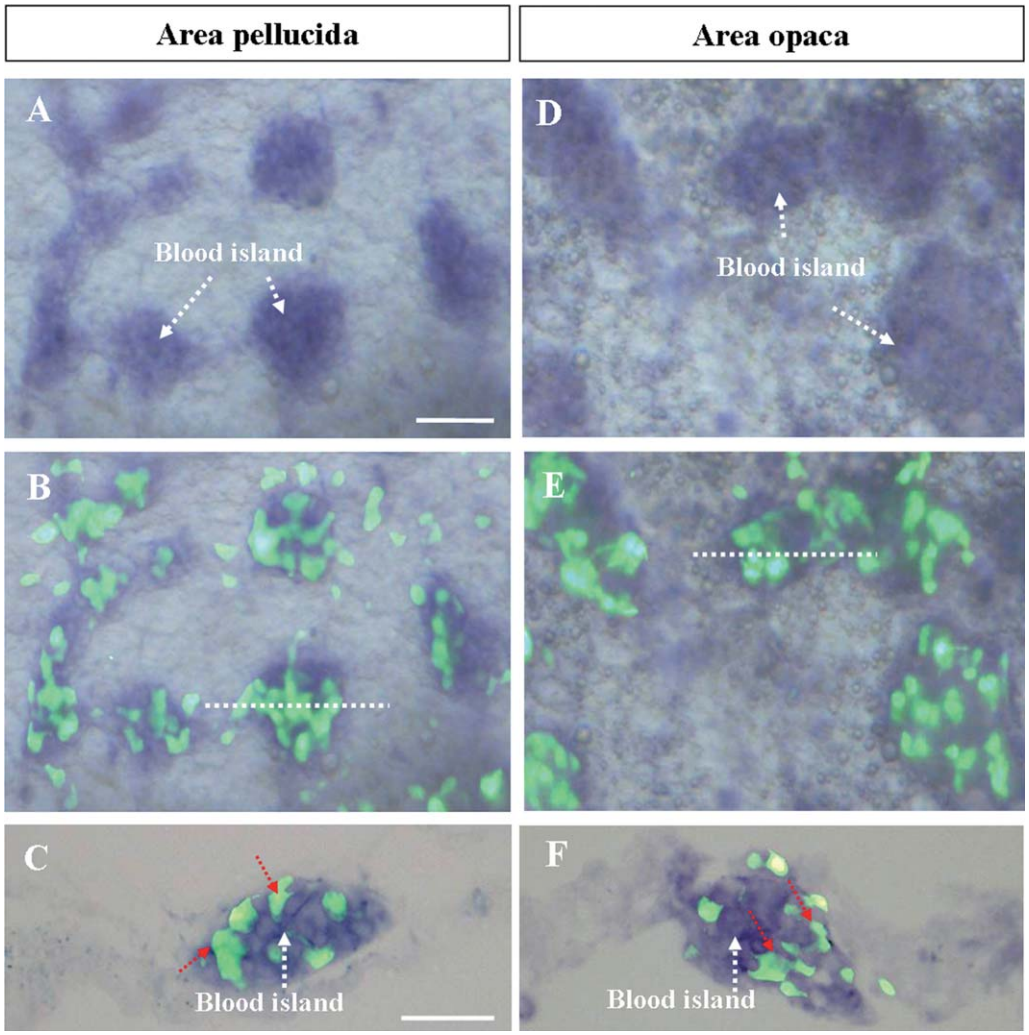

Fig. 7. The convertibility verification of the transplanted anterior primitive streak cells after heterotopic grafting to the site of posterior primitive streak. **A–E**: *In situ* hybridization shows that VE-Cadherin gene specifically express in the blood island in area pellucida (A–C) and area opaca (B–E). (A) and (D): VE-Cadherin mRNA expression in area pellucida and area opaca, respectively. (B) and (E): The merge image of *in situ* and GFP transfection shows that GFP-positive cells derived from anterior primitive streak gave rise to blood islands in the both regions, which should have been contributed from posterior primitive streak cells. (C) and (F): The transverse section at the level indicated by dotted line in (B) and (E) shows that GFP positive cells were involved in the blood island formation indicated by red dotted line arrowheads. Scale bar = 50 μm in (A, B, D, and E) and 10 μm in (C and F).

expressed uniformly in the entire looping heart tube (Fig. 5D), and GFP cells were scattered throughout the region. Moreover, GFP positive cells from posterior streak of donor embryo appeared in transverse sections of endocardium and myocardium (as indicated by arrowheads in Fig. 5F).

Migration and Differentiation of Anterior Primitive Streak Cells Transplanted to the Caudal Primitive Streak Site

Next, to assess the plasticity of anterior PS cells, we grafted the second segment PS tissue from transfected donor embryo to the sixth segment PS site (Fig. 6A). In control experiments, cells from the second segment migrated anterolaterally to give rise to somites and heart tube and other embryonic tissues. However, at 19, 25, and 41 hr, transfected GFP positive cells followed the migration trajectory of the sixth segment cells (Fig. 6B–E). In particular, GFP positive cells reached the region of blood island formation in the area opaca (Fig. 6E).

We next examined whether the second segment cells that migrated as sixth segment cells could differentiate into hematopoietic precursor cells, as native posterior streak cells do. Hence, expression of VE-Cadherin mRNA, a specific marker for hematopoietic and endothelial cells (Risau and Flamme, 1995; Larson et al., 2004), was determinated by *in situ* hybridization in the transplanted host embryo (Fig. 7A–F). *In situ* hybridization data demonstrated that VE-Cadherin is expressed specifically in blood islands in the area opaca and area pellucida (Fig. 7A,D) and is co-localized with GFP

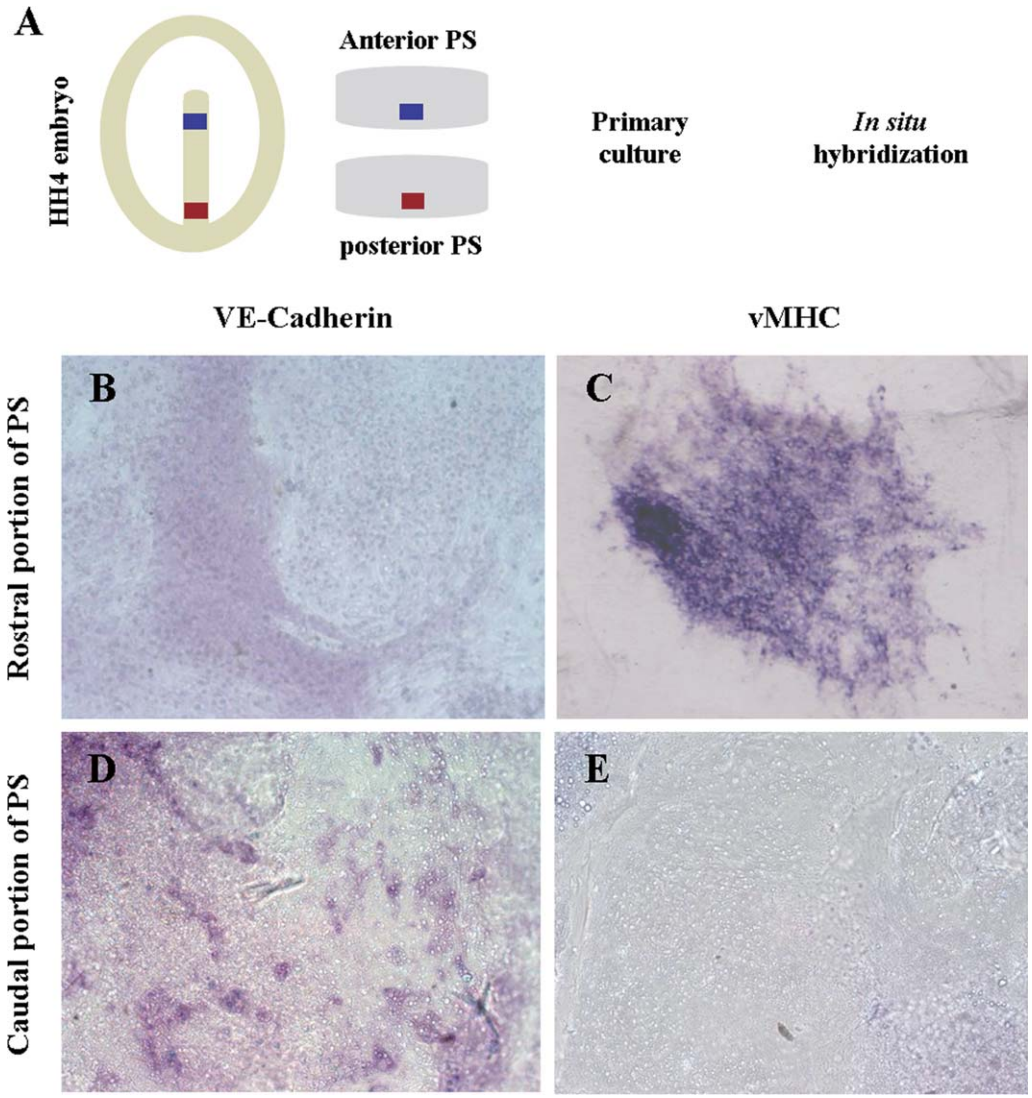

Fig. 8. VE-Cadherin and vMHC expression of primitive streak explants derived either from rostral or caudal portion after primary culture *in vitro*. **A**: Schematically drawing of primary culture shows that the explants from either anterior or posterior primitive streak was incubated *in vitro* for 41 hr, and then the mRNA expression of vMHC and VE-Cadherin were assessed by *in situ* hybridization. **B–C**: vMHC express much stronger (C) than VE-Cadherin (B) in anterior primitive streak explants, although there is dull sheet as well on VE-Cadherin expression. **D** and **E**: VE-Cadherin expresses dramatically with scattered spots (D) meanwhile no expression on vMHC (E) in posterior primitive streak explants. Scale bars = 100 μm in (B–E). Abbreviation used: PS = primitive streak.

positive cells from the second segment transplant as indicated by arrowheads (Fig. 7B,C,E,F).

Molecular Phenotype of Cultured Primitive Streak Cells

To preclude the potential of experimental artifact on cell differentiation due to transplantation, and more importantly, to verify the effect of local microenvironment on cell fate, we cultured PS segments for 41 hr (Fig. 8A). *In situ* hybridization on the explants from either anterior or posterior PS showed that vMHC rather than VE-Cadherin is expressed in anterior PS cells (Fig. 8B,C), whereas VE-Cadherin rather than vMHC is expressed in posterior PS cells (Fig. 8D,E).

DISCUSSION

The PS is a temporally and spatially temporary structure in avian gastrulation that leaves no residual structure as it elongates or regresses during formation of the three germ layers. As epiblast cells adjacent to PS are ingressing to form the streak, other existing streak cells are departing to migrate laterally and rostrocaudally to become mesoderm and endoderm (Rosenquist, 1972; Lawson and Schoenwolf, 2003). The three germ layers that ultimately evolve in turn form the basis of

perspective tissues and organs (Yasuo and Lemaire, 2001). Thus, one question that has long been raised is when and where cell fate is committed? That is, is the cell fate determined before or after cell ingression into the PS?

To answer the above question, the fate map with fluorescence DiI labeling could not only be direct and simple experimental approach but also has been successfully used by many laboratories (Selleck and Stern, 1991; Beddington, 1994; Olivera-Martinez et al., 2000; Cui et al., 2005; Chuai et al., 2006; Chuai and Weijer, 2008). However, DiI label can be diluted during subsequent growth. In this study, we adopted another frequently used approach for cell fate investigation, namely, grafting GFP-labeled cells (Knezevic et al., 1998; Yang et al., 2002, 2008; Yue et al., 2008). GFP has the advantage that the dye is not lost over time and detection can be easily combined with *in situ* hybridization and/or immunocytochemistry.

Our current double-site PS transplantation protocol (Fig. 2) confirmed previous cell migration trajectory data, which indicated that at HH4, chick embryo anterior PS cells migrate anterolaterally, whereas posterior PS cells migrate caudally and laterally toward to extraembryonic area opaca (Yang et al., 2002; Yue et al., 2008; Lopez-Sanchez et al., 2009).

When the cell fates of myocardial and hematopoietic precursor cells are committed? Is it before epiblast cells ingress into, while they reside within, or after they depart from the PS? Despite significant efforts to investigate endoderm plasticity in the EC embryo (Kimura et al., 2006), the timing of cell fate determination of PS cells remains obscure. To assess it, here we first evaluated the migration pattern of posterior PS cells transplanted into the anterior PS (Fig. 4) and found that the cell migration pattern appeared to be the same as native anterior PS cells. More importantly, the transplanted cells derived from posterior PS were localized in the myocardium. This was verified by GFP cell co-localization with myocardial marker expression (Fig. 5), suggesting that posterior streak cells can differentiate into myocardium after being in a rostral PS site environment.

In the reverse, transplant of GFP-labeled anterior PS cells into the posterior PS region, transplanted anterior cells followed the native posterior PS cell migration pattern (Fig. 6), and were found to contribute to blood islands in the area opaca with time (Fig. 7), suggesting that they transformed completely to match the original posterior PS cells.

Hence, at a minimum, we can say the cells in the PS have not become committed to their final fate. However, they have probably acquired the tendency toward one direction of cell fate, as was reflected by our primary culture experiments (Fig. 8). When anterior and posterior PS explants were cultured without the influence of the *in vivo* local environment, they developed into the cell types predicted by their former *in vivo* locations; either vMHC-expressing myocardial or VE-Cadherin-expressing hematopoietic cells, respectively. Our observations above suggest that cells are not committed when they depart from PS; they still possess stem cell plasticity at that stage. The subsequent cell fate or differentiation will depend on local gene expression and/or the environment.

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