Endoderm and mesoderm reciprocal signaling mediated by CXCL12 and CXCR4 regulates the migration of angioblasts and establishes the pancreatic fate

Keiichi Katsumoto\textsuperscript{1,2} and Shoen Kume\textsuperscript{1,2,§}

Department of \textsuperscript{1}Stem Cell Biology, Institute of Molecular Embryology and Genetics (IMEG), Kumamoto University Honjo 2-2-1 Kumamoto, Japan; \textsuperscript{2}The Global COE “Cell Fate Regulation Research and Education Unit”, Kumamoto University;

Key words: Pdx1, blood and vessel precursor, pancreas

\textsuperscript{§}Corresponding author: Shoen Kume, Ph.D. Fax: +81-96-373-6807 Email: skume@kumamoto-u.ac.jp

Divisions of \textsuperscript{1}Stem Cell Biology, Institute of Molecular Embryology and Genetics, Kumamoto University, Honjo 2-2-1, Kumamoto 860-0811, Japan

ABSTRACT

We discovered that angioblasts trigger an early inductive event of pancreas differentiation. This event occurs soon after gastrulation before the formation of blood vessels. Morphological studies revealed that Lmo2-expressing angioblasts reside in proximity to the somitic mesoderm and the gut endoderm from which pancreatic progenitors arise. The chemokine ligand Cxcl12 expressed in the gut endoderm functions to attract the angioblasts that express its receptor Cxcr4. Angioblasts then signal back to the gut endoderm to induce Pdx1 expression. Gain-of-function and loss-of-function experiments for CXCL12 and CXCR4 were performed to test their function on blood vessel formation and pancreatic differentiation. The ectopic expression of Cxcl12 in the endoderm attracted the angioblasts and induced ectopic Pdx1-expression. This resulted in an expanded pancreatic bud and an increased area of Insulin-expressing cells. In contrast, in embryos treated with beads soaked in AMD3100, an inhibitor of CXCR4, the migration of angioblasts towards the Cxcl12-expressing gut endoderm was arrested, causing a malformation of blood vessels. This led to the generation of a smaller pancreatic bud and a reduced area of Insulin-expressing cells. Taken together, these results indicate that the gut endoderm and angioblasts attract each other through reciprocal CXCL12 and CXCR4 signaling. This has a pivotal role in the fate establishment of the pancreatic progenitor cells and the potentiation of further differentiation into endocrine $\beta$-cells.

INTRODUCTION

The earliest pancreatic marker gene, Pancreatic and duodenal homeobox1 (Pdx1) (Jonsson et al., 1994; Offield et al., 1996), is expressed in the dorsal and ventral pancreatic bud and a portion of the stomach and duodenal endoderm. Pdx1-expressing precursor cells were shown to give rise to all three pancreatic lineages: the endocrine, acinar and duct cells (Gu et al., 2003; Gu et al., 2002). The analysis of mice with homozygous null mutations in Pdx1 demonstrates that Pdx1 plays a key role in pancreatic differentiation. In Pdx1-deficient mice the pancreatic buds are formed, but further development is arrested (Jonsson et al., 1994; Katsumoto et al., 2010; Kume, 2005; Offield et al., 1996). The dorsal and ventral pancreas are marked by Pdx1 expression and arise independently from
two distinct regions of the gut epithelium at an early somite stage (embryonic day 8.5, E8.5) both in the mouse and in the chick (Katsumoto et al., 2009; Kume, 2005; Matsuura et al., 2009). Fate map studies of the endoderm of chick embryos revealed that the definitive endoderm is derived from the extreme anterior end of the primitive streak, known as Hensen’s node (Grapin-Botton, 2005; Kirby et al., 2003; Lawson and Schoenwolf, 2003). Recently, using the lipophilic carbocyanine dye Dil (1, 10-dioctadecyl-3,3,30,30-tetramethyl indocarbocyanine perchlorate), we performed fate mapping of the endodermal progenitor cells, the pancreatic, stomach and intestinal progenitor cells (Katsumoto et al., 2009). The endodermal progenitor cells first appear near Hensen’s node immediately after the completion of gastrulation. Later, the pancreatic progenitor cells are segregated from the stomach and intestinal progenitor cells immediately after the completion of gastrulation, and their cellular fates are determined during their migration (Katsumoto et al., 2009). At 8 somite stage (ss), the dorsal pancreatic progenitor cells reside in the endoderm at the level of somites 3-7, whereas the progenitor cells of the stomach are located at the level of somites 1-2 and the intestinal progenitors are at the level of somite 8. At the 17 ss, the ventral pancreatic progenitor cells segregate lateral to somites at the level of somite 4 near the vitelline vein (Katsumoto et al., 2009; Matsuura et al., 2009).

Inductive interactions of the endoderm with mesodermal cells have been shown to play a role in the development of the endoderm germ layer into tissue specific gastrointestinal organs (Wells and Melton, 2000). We previously showed by transplantation experiments that pancreatic fate specification occurs between the 6 ss and 8 ss. During gastrulation, the dorsal pancreatic progenitor cells change their positions over time and receive signals from the mesoderm layer as they migrate posteriorly to their final destination where the pancreatic bud will form (Katsumoto et al., 2009). We showed that the mesodermal cells adjacent to the stomach and pancreatic endoderm, but not those adjacent to the intestinal endoderm have pancreas-inducing activities during this process (Katsumoto et al., 2009). Later on, the notochord comes in contact with the pancreatic endoderm and sends signals that permit pancreatic differentiation (Hebrok et al., 1998; Kim et al., 1997a). Notochord signals are considered to be permissive rather than instructive, since they cannot induce the posterior non-pancreatic endoderm to express pancreatic marker genes (Kim et al., 1997a). Activin and bFGF are candidate molecules for the notochord signal that can direct dorsal pancreatic morphogenesis and maintain the expression of early pancreatic genes through the repression of sonic hedgehog (Shh) (Hebrok et al., 1998). The notochord is subsequently separated from the gut endoderm, and the dorsal aorta, merging medially, comes into close contact with the gut endoderm. The aorta sends signals that promote pancreatic differentiation (Lammert et al., 2001; Yoshitomi and Zaret, 2004). The nature of these signals is unknown. The induced β cells which produce VEGF-A can signal back to attract blood vessels (Brissova et al., 2006; Lammert et al., 2001; Vasir et al., 1998).

Here, we aim to determine the nature of the earliest pancreatic inducing signal, which is emitted from the mesoderm layer at early somite stages (Katsumoto et al., 2009). By examining the tissue structure adjacent to the Pdx1-expressing cells, we revealed that prior to blood vessel formation and to Pdx1 expression, there are angioblasts that express the transcription factor LIM domain only 2 (Lmo2) between the lateral plate mesoderm and the endoderm layers at specific locations. Later on, the pancreatic progenitor cells give rise in the endoderm, close to the location where these angioblasts appear. The temporal and spatial emergence of the angioblasts corresponds to the timing of pancreatic progenitor cell fate specification and to their location, thereby strongly suggesting that the angioblasts have a role in pancreatic cell differentiation. We then examined the nature of the signaling molecules and their role in pancreatic differentiation.

**MATERIALS AND METHODS**

**Embryo culture and treatment with beads adsorbed with AMD3100 or CXCL12**

Fertilized white leghorn chicken eggs were incubated at 38°C in a humidified incubator and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). For short-term *in vitro* cultures, embryos were explanted in Pannett-Compton saline and cultured with ventral side up on 35-mm dish coated with agar-albumin (0.3% agar, 62.5 mM NaCl, 0.75% glucose, 50% albumin) supported by a filter paper ring (Sundin and Eichele, 1992). Alternatively, for long term experiments, beads soaked in 1 mM AMD3100 (A5602; Sigma), a CXCR4 inhibitor, or 1 μg/ml CXCL12 (50-NS-010; R&D systems) were...
deposited in ovo either above or underneath the embryo. At stage 6 to 2 ss, a window was opened on the side of the air chamber using forceps. For CXCR4 inhibition, 10 μl of a 1 mM AMD3100 solution containing the beads (AG® 1-X2 Resin, 143-1255, BIO-RAD) was applied to the pre-pancreatic region from the upper side of the embryo (ectoderm side). For the application of CXCL12, 10 μl of a 1 μg/ml CXCL12 solution containing the beads was added to the pre-intestine region from under the embryo (endoderm side). For control experiments, a PBS solution containing the beads was used. After applying the solution, the eggs were sealed with tape and parafilm, and incubated for 4 days until the embryos reached stage 26. Then, the embryos were dissected and processed for double whole mount in situ hybridization.

**Paraffin sections**

The embryos were fixed overnight with 4% paraformaldehyde at 4°C and dehydrated using an ethanol series finally replaced by xylene. The embryos were embedded in paraffin, serially sectioned at 6 μm, mounted on slides and dewaxed in xylene. The sections were stained with Hematoxylin and Eosin (HE).

**In situ hybridization**

Embryos were fixed overnight with 4% paraformaldehyde at 4°C, washed by PBS three times and stored in cold methanol. Whole mount in situ hybridization was done as described (Shimamura et al., 1995; Uchikawa et al., 1999). Probes used were as follows. Pdx1 (Grapin-Botton et al., 2001), Insulin (Matsuura et al., 2009), CD34 antigen (Cd34) (an EST clone; ChEST326d6), platelet /endothelial cell adhesion molecule 1 (Pecam1, also known as Cd31) 860bp (a gift from Dr. Ken Matsumoto), NK6 homeobox 2 (Nkx6-2) (Vallstedt et al., 2001), SRY-box containing gene 9 (Sox9) (Sakai et al., 2006) and aldehyde dehydrogenase family 1 sub family A2 (Aldh1a2, also known as Raldh2) (Bayha et al., 2009), cDNAs for chemokine (C-X-C motif) ligand 12 (Cxcl12, also known as stromal cell-derived factor-1, Sdf1), chemokine receptor 4 (Cxcr4), Lmo2, T-cell acute lymphocytic leukemia 1 (Tal1, also known as Sc1) and kinase insert domain protein receptor (Kdr, also known as Flk1) were cloned by RT-PCR. The primers used were as follows. Cxcl12 558bp, Fwd: 5′-GTCGCCAGATGGACACTC-3′; Rev: 5′-CTTGGGAGAACGGCTCTTTA-3′; Cxcr4 1180bp, Fwd: 5′-ACTCGGTGCTCGGAATG-3′; Rev: 5′-GGTCAGTCTTGTAGT TCTGA GAT-3′; Lmo2 876bp, Fwd: 5′-ACAGGCAAGC TACAAAGGAG-3′; Rev: 5′-GCACAATCTTACAG TCTGCAAGT-3′; Tal1 1035bp, Fwd: 5′-GGGCC TCTTTCCGAT CAAAAAGA-3′; Rev: 5′-GTGCTG CACAG-CCC TATGT T-3′; Kdr 1000bp, Fwd: 5′-GGA CTTGGCTGCTCGTAATA-3′; Rev: 5′-ACTGTTTTCGCGTAGATG T-3′. Double whole mount in situ hybridization was done as follows: digoxigenin labeled probe and fluorescence-labeled probe were added at the same time as the hybridization step. After first color development (NBT/BCIP (Roche) or Fast Red (Roche) staining), embryos were washed by PBS and treated at 70°C for 1 hour to inactivate the first antibody, followed by blocking and second color development.

**Measurement of Insulin expressing area and the size of pancreas**

After double whole mount in situ hybridization, embryos were embedded in OCT compound and cut into 10 μm frozen sections. PBS treated (n=13), 1 mM AMD3100 treated (n=12) or 1 μg/ml CXCL12 treated (n=9) embryos were analyzed. For measurements of the Pdx1-expressing area, 275 sections (PBS treated), 233 sections (AMD3100 treated) or 226 sections (CXCL12 treated) were used. For measurements of the Insulin-expressing area, 210 sections (PBS treated), 210 sections (AMD3100 treated) or 192 sections (CXCL12 treated) were used. Insulin- or Pdx1-positive areas were quantified using an Olympus IX2-ZDC microscope, Meta-IMAGE (Olympus) and MetaMorph software (Molecular Devices).

**Electroporation**

For Cxcl12 overexpression experiments, we constructed pCIG-Cxcl12 plasmid (Megacon and McMahon, 2002). First we cloned chick Cxcl12 by RT-PCR, using the following primers: Cxcl12 Fwd: 5′-GTGCCCA AATGGACACTC-3′; Rev: 5′-CTTGGGAGAACGGCTCTTTA-3′. We then inserted the Cxcl12 cDNA under the control of chicken β-actin promoter. The plasmid was electroporated in ovo into the chick embryos at stage 6 or 4 ss. A window was opened on the side of the air chamber in the eggs by a pair of forceps. A solution of 5-10 μg/μl DNA with 0.01% fast green was injected in the yolk under the embryo in ovo. A negative tungsten electrode was inserted under the embryo and a positive cover square platinum plate electrode (CUY701P2L, NEPA GENE) was held by a micromanipulator above
the embryo. Ten square pulses of 6.5 V for 99 msecond were applied 999 msecond apart using a CUY21 (NEPA GENE). After the electroporation, eggs were sealed with tape and parafilm and incubated until the desired stage. The embryos were then dissected, photographed and processed for double whole mount in situ hybridization.

RESULTS

Some angioblasts are located between the lateral plate mesoderm and the Pdx1-expressing endoderm layer.

At 8 ss, just after the pre-pancreatic endoderm fate decision occurs, Pdx1 expression becomes detectable by in situ hybridization. Pdx1 shows a periodic pattern, exhibiting strong expression in the endoderm cells located between the somites (Fig. 1A) (Katsumoto et al., 2009). Transversal sections revealed that although vasculature has not yet been formed at this stage, angioblasts are detected between the lateral plate mesoderm and the Pdx1-expressing endoderm cells at 8 ss (Fig. 1B, B’, arrow). Sagittal sections at 8 ss revealed that the angioblasts lied adjacent to the stomach and pancreatic endoderm (1-7 somite level), but not to the intestinal endoderm (8 somite level) (Fig. 1E, E’). Angioblasts were also observed earlier at 4 ss but not at 2 ss (Fig. 1C-D’). Since Pdx1 is detectable by RT-PCR in the pre-pancreatic region at 4 ss, but not at 2 ss (KK unpublished results) (Katsumoto et al., 2009), the emergence of angioblasts corresponds temporally and spatially to Pdx1 expression.

The effect of the blood vessels on pancreatic development was previously reported at a later stage (Lammert et al., 2001), but such an early role of angioblasts prior to blood vessel assembly has not been reported. This prompted us to further investigate the role of angioblasts in the pancreatic fate specification.

Cxcl12-expressing endoderm cells lie adjacent to the Lmo2-expressing angioblasts in the mesoderm.

In situ hybridization analysis confirmed that the angioblasts expressed Lmo2 (Fig. 2A-D’). These cells also expressed Tal1, which is known to act with Lmo2 in specifying haemangioblasts (Fig. S1A-B’ in the supplementary material). These cells were also positive for Cd34 and Kdr (Fig. S1C-F’ in the supplementary material) and negative for Pecam1 (data not shown), thereby indicating that these cells have the identity of angioblasts (Gering et al., 2003; Minasi et al., 2002; Royer-Pokora et al., 1995; Warren et al., 1994; Yamada et al., 1998) (Fig. S1 in the supplementary material). Interestingly, the Pdx1-expressing cells reside close to the angioblasts (Fig. 2A-B’).

We hypothesized that a cue released by the endoderm attracts the angioblasts towards this region. It is well known that chemokine signals are important for the mobilization of hematopoietic stem cells and the regulation of angiogenesis at a later stage (Kim and Broxmeyer, 1999). Therefore, we examined by in situ hybridization whether Cxcl12 and its receptor, Cxcr4, were expressed in these tissues.

Cxcl12 expression was detected in the endodermal cells in the pre-pancreatic endoderm, in a periodic pattern at 6 ss (Fig. 2C, D), prior to pancreas specification and before Pdx1 detection by in situ hybridization (Katsumoto et al., 2009). Lmo2-positive angioblasts were observed in close association with the Pdx1- or Cxcl12-positive endodermal cells (Fig. 2B’, D’), suggesting that the CXCL12 chemokine might attract the angioblasts towards the future Pdx1-expressing pancreatic progenitor cells.

We then examined the expression of Cxcr4, the receptor for CXCL12. As shown in Fig. 2E-H’, Cxcr4 was expressed in both the mesoderm and endoderm (Fig. 2E-H’). This suggests that mesodermal Cxcr4-positive cells might be attracted by Cxcl12 expressed in the endoderm. We then examined the expression of Cxcr4 and Lmo2 from 4 ss to 10 ss, in the stomach and pancreatic endoderm region (Fig. S2 in the supplementary material). At 4 ss to 8 ss in the pancreatic region, Lmo2- and Cxcr4-double positive angioblasts were observed in the lateral plate mesoderm and at the endoderm border (Fig. S2B’, C’, F’, I’ in the supplementary material; yellow arrows). They were also detected in the stomach mesoderm at 6 ss and close to the future stomach endoderm at 4 to 8 ss (Fig. S2E’, H’ in the supplementary material). At 10 ss, almost all Cxcr4/Lmo2-positive cells had migrated to the endoderm border and Lmo2 expression decreased (Fig. S2K’, L’ in the supplementary material). Taken together, these results strongly suggest that
angioblasts in the mesoderm are recruited through CXCR4-CXCL12 signaling to the Cxcl12-expressing pre-pancreatic endoderm, which coordinates temporally with the pancreatic inducing activity in the mesoderm (Katsumoto et al., 2009).

Ectopic Cxcl12 expression in the endoderm attracts angioblasts and induces Pdx1 expression at an early somite stage.

To test whether Cxcl12 from endodermal cells attracts angioblasts, we ectopically expressed Cxcl12 by electroporating pCIG-Cxcl12 (a construct encoding CXCL12-IRES-GFP protein) into the pre-stomach or anterior lateral endoderm region at stage 6 (Fig. 3D-F). Electroporation of pCIG (GFP) was done as a control experiment (Fig. 3A-C). Embryos were analyzed at 11-12 ss. Angioblasts (Lmo2-positive) accumulated next to the cells that ectopically expressed Cxcl12 (Fig. 3D–F, n = 5/5, yellow arrow heads). This was not observed in control embryos (Fig. 3A–C, n = 5/6, blue arrow heads). To investigate whether the accumulated angioblasts can induce pancreatic progenitors, we performed electroporation into the pre-intestinal endoderm. When pCIG-Cxcl12 was electroporated at 4 ss (intestinal fate not yet being specified), but not at 8 ss (the intestine fate being specified), ectopic induction of Pdx1 expression was observed (Fig. 3 I, J, M, N). These results showed that ectopic Cxcl12 attracted angioblasts, which in turn induced ectopic Pdx1 expression in the pre-intestinal endoderm before intestinal fate decision occurred. In the above experiment, control ectopic pCIG expression was confirmed not to induce Pdx1 expression at 4 ss or 8 ss (Fig. 3G, H, K, L).

Double in situ hybridization for Pdx1 and Cxcl12 revealed that Cxcl12 is expressed in some of the endodermal cells (Fig. S3A-C" in the supplementary material). The Cxcl12 expression overlaps with that of Pdx1 in the pancreatic endoderm (Fig. S3B-B" in the supplementary material; yellow arrows). However, Cxcl12 expression is also observed in some intestinal endoderm, in which Pdx1 was not expressed (Fig. S3C-C" in the supplementary material). This result strongly suggests that Cxcl12 does not directly induce Pdx1 expression.

Blockade of the CXCR4 inhibits blood vessel formation and pancreatic fate specification

Next, we performed loss-of-function studies. Embryos treated at stage 6 with beads pre-adsorbed with a CXCR4 inhibitor, AMD3100, exhibited disturbed migration of the angioblasts and a reduction of Pdx1 expression (Fig. 4B, D, n = 5/6, Fig. S4C-D’ in the supplementary material). A defect in dorsal aorta formation was observed (Fig. 4G, H, n = 6/8, Fig. S4G-H’, in the supplementary material). These changes were not observed in control embryos treated with PBS (Fig. 4A, C, n = 6/6, Fig. 4E, F, n = 6/6, Fig. S4A-B’, E-F’, in the supplementary material). These results suggest that the CXCL12-CXCR4 chemokine signalling pathway regulates the migration and maturation of angioblasts, which in turn induced pancreatic differentiation.

To analyze the effects of AMD3100 on the angioblasts, we examined the expression of Lmo2 and Tal1 by in situ hybridization (Fig. S4 in the supplementary material). In embryos treated with PBS beads at stage 6 to 2 ss, Lmo2-positive angioblasts migrated to the endoderm border at 8 ss (Fig. S4B, B" in the supplementary material), and Tal1-, Lmo2- negative dorsal aorta were formed at 12 ss (Fig. S4F, F’ in the supplementary material). However, in embryos treated with AMD3100 beads at stage 6 to 2 ss, few Lmo2-positive angioblasts migrated and reached the endoderm border at 8 ss (Fig. S4D, D’, in the supplementary material). At 12 ss, dorsal aorta was formed and Lmo2 and Tal1 expression in the angioblasts disappeared in control embryos (Fig. S4F, F’ in the supplementary material). On the contrary, in AMD3100 treated embryos, migration and maturation of Lmo2-positive angioblasts was delayed and Lmo2 and Tal1 expression remained at 12 ss (Fig. S4H, H’ in the supplementary material). Later on, the angioblasts migration recovered and dorsal aortae were formed in AMD3100 treated embryos observed at 17 ss (Fig. S6E, E’, in the supplementary material, see below). The gross appearance of the vasculature seemed unaffected (KK unpublished). Therefore, the AMD3100 treatment delayed the migration and maturation of Lmo2-positive angioblasts, which resulted in an inhibition of Pdx1 expression.

CXCL12 potentiates pancreatic endocrine differentiation and AMD3100 inhibits it.

We next tested the effects of CXCL12
administration or CXCR4 inhibition at a later stage. First, embryos were treated with beads pre-adsorbed with in CXCL12 or control PBS in the pre-intestine region between stage 6 and 2 ss and cultured until stage 26 (Fig. 5A). Embryos treated with CXCL12 developed a bigger pancreatic bud and showed an increase in the area of Insulin-expressing cells (Fig. 5B-G). In embryos treated with CXCL12, the area of the Pdx1-expressing cells in the dorsal pancreas region revealed a 1.31-fold increase. Additionally, the Insulin-expressing cells showed a 1.24-fold increase compared to those of the PBS treated control embryos (Fig. 5B-G, Fig. 7).

In a mirror experiment, embryos were treated with AMD3100 or control PBS bead in the pre-pancreatic region between stage 6 and 2 ss and cultured until stage 26 (Fig. 6A). Embryos treated with AMD3100 beads exhibited a 0.79-fold decrease in the size of the dorsal pancreatic bud, quantified by measuring the areas expressing Pdx1. A 0.81-fold decrease in the area of Insulin-expressing endocrine cells was also observed as compared to that of the control embryos (Fig 6B-G, Fig. 7). This was not observed with embryos at a stage after the pancreatic fate was specified (8 ss) (data not shown). Representative images of the dorsal pancreatic region (Pdx1-positive region) and β cell region (Insulin-positive region) in control PBS, AMD3100 or CXCL12 treated embryo are shown in Figure 7A-C.

We also confirmed that the AMD3100 treatment resulted in an early down regulation of the pancreatic progenitor markers Pdx1, Sox9, and Nkx6-2 (Figs. S5, S6 in the supplementary material), whereas CXCL12 treatment resulted in the enlargement of the expression domain of Pdx1, Sox9 and Nkx6-2 (Figs. S5, S6 in the supplementary material). These results demonstrated that the differentiation of pancreas and endocrine precursor cells was inhibited by CXCR4 blockade, and potentiated by CXCL12 treatment (Figs. S5, S6 in the supplementary material). The dorsal aortae were observed in the CXCR4 inhibited embryos (Figs. S6E, E', in the supplementary material), demonstrating that the angioblast migration inhibition was a temporary event. Taken together, we conclude that CXCL12-CXCR4 signaling attracts the angioblasts to the vicinity of the pre-pancreatic endoderm, which potentiates the induction of Pdx1-expressing pancreatic progenitor cells and the differentiation into Insulin-expressing cells.

**DISCUSSION**

In this paper, we show that the CXCL12-CXCR4 chemokine signaling pathway plays an important role in establishing the fate of the pancreatic progenitors. This occurs prior to the formation of blood vessels, at a stage when the angioblasts are located in the lateral plate mesoderm. The chemokine ligand, Cxcl12, is expressed in the endoderm, before it starts to express Pdx1, the earliest pancreatic marker. CXCL12 then signals to the mesoderm and attracts the Cxcr4-, Lmo2-double positive angioblasts. The angioblasts then migrate to the endoderm border and signal back to induce Pdx1 expression in the gut endoderm. Over expression of Cxcl12 attracted the Lmo2-expressing cells (Fig. 3A-F), which then induced Pdx1-expressing pancreatic progenitors (Fig. 3G-N) and enhanced the differentiation into Insulin-expressing cells (Figs. 5, 7). Conversely, the blockade of CXCR4 resulted in the inhibition of the migration of angioblasts to the proximity of the endoderm (Fig. 4E-H, Fig. S4 in the supplementary material). This then resulted in a reduction of Pdx1-expressing pancreatic progenitors (Fig. 4A-D, Figs. S4A-D', S5A, B in the supplementary material) and a partial inhibition of their differentiation into Insulin-expressing endocrine cells (Figs. 6,7).

*Early angioblasts are capable of inducing Pdx1 expression in the early endoderm*

Endothelial cells were previously shown to play an inductive role in organ formation (Lammert et al., 2001; Matsumoto et al., 2001; Yoshitomi and Zaret, 2004; Zaret, 2008; Zaret and Grompe, 2008). At E8.5-9.5, the notochord is displaced from the endoderm by the fusing of the dorsal aorta, and the dorsal aorta contacts the pancreatic endoderm (Katsumoto et al.; Kume, 2005). The endothelial cells signal to the pancreatic endoderm and instruct them to adopt an endocrine fate. The endothelial cells signal to the pancreatic endoderm and instruct them to adopt an endocrine fate. The endothelial cells are also implicated in hepatic development (Matsumoto et al., 2001; Zaret, 2008; Zaret and Grompe, 2008).

However, Yoshitomi et al. showed that Flk1 (Kdr) mutant mice, which lack endothelial cells,
exhibit normal initial Pdx1 induction (Yoshitomi and Zaret, 2004). Flk1 is required for angioblasts to become mature endothelial cells (Shalaby et al., 1997; Shalaby et al., 1995). Flk1 might not be required for the formation of angioblasts and angioblasts may exist in the Flk1 mutants, which were capable of inducing the normal initial Pdx1 expression.

Similarly, Field et al. showed that in cloche zebrafish mutant embryos that lack endothelial cells, Pdx1, Insulin and trypsin expression were normal compared to the wild type embryo (Field et al., 2003). Angioblasts might also exist in the cloche mutant thereby accounting for the induction of the pancreas primordium markers. Although it remains controversial, a small number of primitive angioblasts were reported to exist in the cloche mutants (Thompson et al., 1998).

Here, we showed that Cxcl12 overexpression in early stage attracted angioblasts, which led to ectopic pancreatic development. However, there is possibility that Cxcl12 acts in an autocrine manner. To prove that the inducing signals do exert from the angioblasts but not due to direct effects of CXCL12 in the endoderm, future experiment using angioblast inhibitors might be useful.

Chemokine signals participate in the early pancreatic differentiation

The CXCL12/CXCR4 pathway was shown to participate in vascular formation. Mice bearing Cxcl12 or Cxcr4 gene inactivation die before birth and show defects in the vasculature of the gastrointestinal and nervous systems (Nagasawa et al., 1996; Nagasawa et al., 1998; Tachibana et al., 1998; Zou et al., 1998). In the mouse pancreas, CXCL12 and CXCR4 are expressed in the islets. CXCR4 is also expressed in and around the proliferating duct epithelium in the regenerating pancreas of non-obese diabetic (NOD) mice engineered to express IFN-γ driven by the Insulin promoter (Kayali et al., 2003). Inhibition of CXCR4 function in the IFN-γ NOD mouse resulted in diminished proliferation and increased apoptosis in pancreas ductal cells. This suggests that CXCR4-CXCL12 signaling participates in endocrine cell renewal in pancreas regeneration (Kayali et al., 2003), since CXCL12 is the only ligand for CXCR4 (Burns et al., 2006). Recently it was shown that CXCL12 facilitates β cell survival via the activation of Akt (Yano et al., 2007). CXCL12 (SDF1) was also shown to control morphological branching in the developing pancreas in vitro and in vivo (Hick et al., 2009). In the Cxcl12 mutant mice, a modest and transient branching inhibition was observed in the ventral but not dorsal pancreas (Hick et al., 2009).

In zebrafish, the reduction in cxcl12b (sdf1) and its receptor cxc4a by antisense morpholino oligonucleotide injection resulted in a duplication of the pancreas (Nair and Schilling, 2008). These data are compatible with a scenario in which CXCL12/CXCR4 signaling would affect the migration of angioblasts, so that these cells first associate with nearby dorsal endoderm cells before migrating to the correct region. The discrepancy between the phenotypes caused by CXCR4 inhibition could be interpreted by the differences between the size of the chick and zebrafish embryos. We hypothesize that the chick embryo is bigger and therefore the Cxcr4-positive mesodermal cells (angioblasts) have to cover a longer distance to reach the specific site at the endoderm border, whereas in the zebrafish they may cover the shorter distance. Our hypothesis is that their migration to the appropriate region of endoderm in the zebrafish may be disturbed, thereby yielding a duplicated pancreas. It was also shown in the zebrafish that endothelial cells are derived from cxc4a-positive anterior mesoderm, and that cxcl12 is expressed in the endoderm underlying the lateral aorta. Both cxc4a and cxcl12 are required specifically for the formation of the lateral aorta (Siekmann et al., 2009), in agreement with our observations in chick.

Retinoic acid is one of the candidate molecules secreted by the angioblasts

A defect in endocrine and exocrine pancreas formation was previously described in the zebrafish aldehyde dehydrogenase family 1 sub family A2 (Aldh1a2, also known as Raldh2) mutant neckless (nls) as well as in embryos treated with BMS493, a retinoic acid receptor (RAR) antagonist (Stafford and Prince, 2002). Aldh1a2 maternal activity participates in early pancreatic development (Alexa et al., 2009). In chick, retinoic acid (RA) also induces Pdx1 expression in endoderm in explants and in vivo
This role of RA is conserved in the mouse, quail and Xenopus (Chen et al., 2004; Martin et al., 2005; Molotkov et al., 2005; Stafford et al., 2004; Stafford and Prince, 2002). In the Aldh1a2 deficient mouse embryo, the expressions of Pdx1 and Prox1 were inhibited and dorsal but not ventral pancreas agenesis was observed (Molotkov et al., 2005).

Despite the multiple reports on the role of RA in early pancreatic development, no report definitively shows that RA acts as an instructive but not a maintenance signal for the expression of Pdx1. We observed Aldh1a2 expression in the angioblasts at an early somite stage, although the expression level was moderate compared to its high expression in the mesoderm (Fig. S7 in the supplementary material). Angioblasts might induce Pdx1 expression by adjusting the concentration of RA to an optimal one.

Timing of pancreas primordium induction by angioblasts

In the current literature, there is not much information on how the pre-pancreatic region is initiated. In this study we present evidence indicating that Pdx1 expression is initiated by the contact of endoderm cells with angioblasts. We previously showed that region-specific endodermal fates are determined sequentially in the order of the stomach (Sox2, 2 ss), intestine (CdxA, 5 ss), and then the pancreas (Pdx1, 8 ss) (Katsumoto et al., 2009). The angioblasts begin to appear around the pre-stomach region at 4 ss and contact the endoderm. However, the angioblasts cannot induce the expression of Pdx1 in the pre-stomach region because the pre-stomach region is already committed to expressing CdxA at 5 ss, and loses the responsiveness to the Pdx1 inducing signals from the angioblasts. Taken together, the timing of angioblast arrival, together with distinct responsiveness of different endoderm areas, restrict Pdx1 expression to the future pancreatic progenitor region.

In sum, in this study, the main discoveries are: (1) Pancreas induction is initiated by angioblasts by contact with the endoderm (Fig. 8). (2) The chemokine signals (CXCL12/CXCR4) spatiotemporally regulate angioblast migration to the correct position in contact with the endoderm, and induce Pdx1 expression (Fig. 8).

ACKNOWLEDGMENT

We thank Drs. Anne Grapin-Botton, Johan Ericson, Yoshio Wakamatsu and Ken Matsumoto for the kind gifts of the plasmids. We particularly thank Dr. Anne Grapin-Botton for critical reading of the manuscript, Drs. Sadao Yasugi, Kimiko Fukuda, Kenji Shimamura, Hideaki Tanaka, Kazuhiko Kume, Nobuaki Shiraki, Jun Tomita, Rika Miki, Norifumi Tatsumi, Daisuke Sakano, Yuichiro Higuchi, Kenjiro Katsu, Takashi Seki, Ms Kumi Matsuura, Yuki Kawamuro, Kahoko Umeda, Mr Akira Matsuo and Soichiro Ogaki for technical advice and critical comments. We also thank Ms Minako Kawabata and Mr Shingo Usuki for technical assistance. We thank members of the Gene Technology Center for their technical assistances. This work is supported by a Grant-in-Aid (no. 20790652 to K.K., no.21390280 to S.K.) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) Japan, by a grant (to S.K.) from the Project for Realization of Regenerative Medicine from MEXT and, in part, by the Global COE Program (Cell Fate Regulation Research and Education Unit), MEXT, Japan. K.K. is a research associate of the Global COE. S.K. is a Global COE member.
Figure 1. Angioblasts exist at the pre-stomach and pancreatic region but not at the intestine region at an early somite stage

To examine the tissues, which lie adjacent to the pre-pancreatic region at an early somite stage, paraffin sections were made at stages before (2 ss, 4 ss) or after (8 ss) pancreatic differentiation had occurred. We found that angioblasts located adjacent to the pre-pancreatic region during pancreatic differentiation.

(A) Whole mount in situ hybridization showed that \( Pdx1 \) (blue) is expressed in pre-pancreas endoderm at 8 ss. Yellow arrowheads indicate representative \( Pdx1 \) expression regions. (B) A transversal section at the pre-pancreatic region at 8 ss at the level of B (white broken line) in A is shown. Counter stain is done by eosin (red staining). (C, D) Para-sagittal sections at 2 ss, 4 ss or 8 ss, respectively, are shown. (B’, C’, D’, E’) Enlarged images of boxed areas in B, C, D, E, respectively, are shown. (C–E’) Sections are counter stained with hematoxylin and eosin. (A, C–E’) 1 to 8 indicate the somite level: 1-, 2-somite level (in blue), pre-stomach region; 3-7 somite level (in pink), pre-pancreatic region; 8-somite level (in green), pre-intestine region; ang, angioblast; en, endoderm; lpm, lateral plate mesoderm; ss, somite stage. Scale bar: 500 \( \mu \)m in A, 100 \( \mu \)m in B, 50 \( \mu \)m in B’, 250 \( \mu \)m in C–E’.

Figure 2. \( Pdx1 \)-expressing endoderm cells are in the proximity to \( Lmo2 \)-expressing angioblasts

Whole mount in situ hybridization analysis showed that the angioblasts that lie in the proximity of \( Pdx1 \)-expressing endoderm cells are \( Lmo2 \)-positive cells, thus indicating that they are angioblasts. Chemokine ligand Cxcl12 and chemokine receptor Cxcr4 are expressed in both the angioblasts and endoderm cells in the pre-pancreatic region at an early somite stage.

(A, C, E, G) Whole mount in situ hybridization for \( Pdx1/Lmo2, Cxcl12/Lmo2, Cxcr4 \) or \( Cxcl12/Cxcr4 \) are shown in A, C, E and G, respectively. \( Pdx1, Cxcl12, \) blue (A, C); \( Lmo2 \) red (A, C); \( Cxcr4, \) blue (E); \( Cxcr4, \) red fluorescence (H, H’). (B, D, F, H) Cross sections at the level of B, D, F, H (broken lines) in A, C, E, G, respectively, are shown. (B’, D’, F’, H’) Enlarged images indicated by boxed areas in B, D, F, H were shown in B’, D’, F’, H’, respectively. (A, C, E, G) Numbers indicate the somite level. ang, angioblast; en, endoderm; lpm, lateral plate mesoderm; ss, somite stage. Scale bar: 500 \( \mu \)m in A, C, E, G, 100 \( \mu \)m in B, D, F, H, 50 \( \mu \)m in B’, F’, H’, 20 \( \mu \)m in D’.
Figure 3. Cxcl12 expressed endoderm attracts the Lmo2-expressing angioblasts
In embryos electroporated with pCIG-Cxcl12 (a CXCL12-IRES-GFP construct) in the anterior endoderm region at an early somite stage, ectopic Lmo2-positive angioblasts migrated towards the region overexpressing pCIG-Cxcl12 (D-F, yellow arrow heads). No ectopic angioblasts were observed in control embryos electroporated with pCIG (GFP) (A-C, blue arrow heads). (A, D) Fluorescence images of GFP expression in embryos electroporated with pCIG (control, A) or pCIG-Cxcl12 (D) are shown. (B, C, E, F) Whole mount in situ hybridization with Cxcl12 (blue) and Lmo2 (red) probes are shown. Bright field images are shown in B and E. Fluorescence images are shown in C and F. (G-N) In embryos electroporated at 4 ss with pCIG-Cxcl12 in the intestinal endoderm region, the expansion of Pdx1 (blue) expression in the intestine (demarcated by a yellow bracket) was observed (I, J). However, the expansion of Pdx1 expression was not observed when embryos were electroporated at 8 ss (M, N). Embryos electroporated with control pCIG at 4 ss (G, H) or 8 ss (K, L) also did not showed an expansion of Pdx1 expression. (G, I, K, M) Fluorescence images of GFP expression in embryos electroporated with pCIG (control, G, K) or pCIG-Cxcl12 (I, M) are shown. Numbers in the images are embryos observed with phenotype versus total embryos examined. Solid black or white lines in G-N show the level of 10 somite. ss: somite stage. Scale bar: 500 μm.
Figure 4. The angioblasts participate in early pancreatic regionalization via chemokine signals
To examine whether CXCR4 participates in early pancreatic regionalization or not, we treated the embryos with beads adsorbed with a specific CXCR4 inhibitor, AMD3100, (B), or control PBS (A) at stage 6 (st 6). The embryos were cultured until they reached 16 ss. Whole mount in situ hybridization revealed that the Pdx1 (blue) expressing region was reduced in AMD3100 treated embryos (D), but not in control PBS treated embryos (C). The dorsal aorta formation was inhibited in AMD3100 treated embryos (G, H), but not in control PBS treated embryos (E, F).
(A-D, E, G) Views from the endoderm layer are shown. (F, H) Cross sections at levels of F or H (black broken line) in E or G, respectively, are shown. Sections are counter stained with hematoxylin and eosin. Control PBS (A, C, E) or AMD3100 (B, D, G) embryos at st 6 (A, B), 16 ss (C, D) or 12 ss (E-H) are shown. Numbers in the images are embryos observed with phenotype versus total embryos examined. 6 s and 11 s indicate the somite level. st: stage, ss: somite stage, da: dorsal aorta, *: a bead. Scale bar: 1 mm in A, B, E, G, 500 μm in C, D, 100 μm in F, H.

Figure 5. CXCL12 treatment results in an expansion of the pancreatic bud
Insulin and Pdx1 expression areas are expanded in CXCL12 treated embryos compared to those of the control PBS treated embryos. (A) A schematic drawing of the procedure of CXCL12 adsorbed beads treatment in ovo is shown. (B-G) Beads adsorbed with PBS (B-D) or CXCL12 (E-G) were applied to embryos at the posterior region at between st 6 and 2 ss. After 4 days culture, embryos were dissected at st 26. The dissected guts were analyzed by whole mount in situ hybridization for Insulin (red) (B, C, E, F), or by double whole mount in situ hybridization for Insulin (red) and Pdx1 (blue) (D, G). Insulin expression is indicated by red color fluorescence image (C, F). sto: stomach, dp: dorsal pancreas, int: intestine, vp: ventral pancreas, st: stage, ss: somite stage, *: nonspecific signal, Scale bar: 500 μm.

Figure 6. CXCR4 inhibition at an early stage results in a reduction of the pancreatic bud
Insulin and Pdx1 expression regions are reduced in AMD3100 treated embryos, compared to those of the control embryos. (A) A schematic drawing of the procedure of treatment with AMD3100 adsorbed beads in ovo is shown. (B-G) Beads adsorbed with PBS (B-D) or AMD3100 (E-G) were applied to the anterior part of the embryos at between st 6 and 2 ss. After 4 days culture, embryos were dissected at st 26. The dissected guts were analyzed by whole mount in situ hybridization for Insulin (red) (B, C, E, F), or by double whole mount in situ hybridization for Insulin (red) and Pdx1 (blue) (D, G). Insulin expression is indicated by red color fluorescence image (C, F). sto: stomach, dp: dorsal pancreas, int: intestine, vp: ventral pancreas, st: stage, ss: somite stage, *: nonspecific signal, Scale bar: 500 μm.
Figure 7. CXCL12/CXCR4 chemokine signaling participates in pancreatic differentiation
A reduction in pancreatic bud by AMD3100 treatment (CXCR4 function inhibition), and an expansion in pancreatic bud by CXCL12 treatment are observed, thus indicating that the CXCR4-CXCL12 chemokine signaling pathway plays a pivotal role in pancreatic regionalization.

(A-C) Pancreas buds treated with control PBS (A), AMD3100 (B) or CXCL12 (C) adsorbed beads were analyzed by sectioning through the buds. Representative images of double in situ hybridization for Pdx1 (blue) and Insulin (red) of the pancreas buds are shown. Broken white lines and pink lines demarcate the areas positive for Pdx1 or Insulin, which represent pancreas or β cell size, respectively. Insulin-positive areas are within Pdx1-positive areas. Total areas of Pdx1 or Insulin, encircled by broken lines of all sections, were measured and shown in (D).

(D) AMD3100 adsorbed beads applied embryos (AMD) resulted in a 0.79-fold decrease in the Pdx1-expressing area (average 29475.3 μm²; white bar), and a 0.81-fold decrease in Insulin-expressing area (average 23762.5 μm²; black bar), compared to those of the control PBS treated embryos (Pdx1-expressing area average 373786.8 μm², Insulin-expressing area average 29475.3 μm²). In contrast, embryos treated with CXCL12 developed a bigger pancreatic bud, with a 1.31-fold increase in the Pdx1-expression area (average 487971.6 μm²; white bar) and a 1.24-fold increase in the Insulin-expressing area (average 36672.0 μm²; black bar), compared to those of the control embryos. Numbers of embryos examined are showed in parentheses. Data are shown as means ± SD (standard deviation), the values show fold changes compared to the average of control PBS treated embryos. The differences were significant (*P<0.05; **P<0.01) for AMD3100 or CXCL12 treated embryos as shown in the graph, analyzed by two-tailed paired Student’s t test. Scale bar: 100 μm.

Figure 8. A working hypothesis for the early pancreatic regionalization
The angioblasts migrate to the endoderm border at an appropriate time and location via the chemokine signals. The angioblasts induce the Pdx1 expression in the endoderm. At an early somite stage, angioblasts are present in the pre-stomach and pancreatic region, but angioblasts cannot induce the Pdx1 expression in the pre-stomach region because the pre-stomach region has already committed and is not competent to the Pdx1-inducing signals. The pancreatic differentiation is triggered by the angioblasts, which lie in proximity to the endoderm cells. Pink: the pre-pancreatic region in the endoderm, Blue: the pre-pancreatic region in the mesoderm.
Supplementary Figure S1 Characterization of the Lmo2-positive angioblasts.
Whole mount in situ hybridization analyses of transversal frozen sections in the pancreatic region at the level of B, D, F are shown. Tal1, Cd34, Kdr, blue (A, C, E); Lmo2 red (A, C, E). The Lmo2-positive angioblasts also expressed Tal1 (A-B'), Cd34 (C-D') and Kdr (E-F') (yellow arrows show double positive cells). B', D', F' are enlarged images in boxed areas shown in B, D, F, respectively. (A, C and E) 3 to 6 indicate the somite level, ss: somite stage, Scale bars: 500 μm in A, C, E, 100 μm in B, D, F, 50 μm in B’, D’, F’.

Supplementary Figure S2 Lmo2-, Cxcr4- double positive cells reside in the lateral mesoderm and close to endoderm at 4 ss-10 ss.
Whole mount in situ hybridization analyses for Cxcr4 (blue) and Lmo2 (red) are shown in transversal frozen sections in the stomach (at the level of E, H, K) or pancreatic (B, C, F, I, L) region at 4 ss (A-C’), 6 ss (D-F’), 8 ss (G-I’) and 10 ss (J-L’), respectively. (B-C’, F, F’, I, I’) At 4 ss, 6 ss and 8 ss in the pancreatic region, the angioblasts (Lmo2-positive) expressing Cxcr4 (B’, C’, F’, I’; yellow arrows) were observed in the lateral mesoderm and abutting endoderm. A similar observation was made in the stomach region (E, E’, H, H’). (I) At 10 ss in the stomach and pancreatic regions, Lmo2 expression decreased (K, K’ L, L’). B’, C’, E’, F’, H’, I’, K’ L’ are enlarged images of the boxed areas in B, C, E, F, H, I, K, L, respectively. Scale bars: 500 μm in A, D, G, J, 100 μm in B, C, E, F, H, I, K, L, 50 μm in B’, C’, E’, F’, H’, I’, K’, L’.
Supplementary Figure S3 Cxcl12 is expressed in some endodermal cells.
Whole mount in situ hybridization analyses for Pdx1 (blue) and Cxcl12 (red fluorescence) are shown in transversal frozen sections. (A) Cxcl12 expression is observed in some of the endodermal cells at the level of B and C, revealed by in situ hybridization analyses. The Cxcl12 expression in pancreatic region (B), but not in the intestinal region (C) overlapped with that of the Pdx1 expression (yellow arrows). B’, B”, C’, C” are enlarged images in boxed area shown in B, C, respectively. (A) 3 to 8 indicate the somite level, ss: somite stage. Scale bars: 500 µm in A, 100 µm in B, C, 20 µm in B’-C”.

Supplementary Figure S4 Treatment of embryos with beads adsorbed with AMD3100, a CXCR4 inhibitor, showed a delay in the maturation and migration of the Lmo2-expressing angioblasts.
Whole mount in situ hybridization analyses for Pdx1 and Lmo2 or Tal1 are shown in transversal frozen sections at the level of B, F in control PBS (A-B’, E-F’) or at the level of D, H in AMD3100 (C-D’, G-H’) treated embryos at st 6 to 2 ss. Pdx1, Tal1, blue (A, C, E, G); Lmo2, red (A, C, E, G). Embryos at 8 ss (A-D’) or 12 ss (E-H’) are shown. In AMD3100 treated embryos, Lmo2-expressing embryos migration is delayed (D, D’), and fewer Pdx1-, Lmo2-expressing cells in the endoderm border were observed (D’, yellow arrows) compared to control PBS treated embryo (A-B’). Maturation is also delayed, and Tal1-, Lmo2-expressing angioblasts remain in the endoderm border were observed (H’, yellow arrow) compared to control PBS treated embryo (E-F’). Note that dorsal aortae (da) were observed in control PBS treated embryo (F, F’), but not in the AMD3100 treated embryo (H, H’). Numbers in the images are embryos observed with phenotype versus total embryos examined. B’, D’, F’, H’ are enlarged images in boxed areas shown in B, D, F, H, respectively. (A, C, E, G) 3 to 8 indicate the somite level, ss: somite stage. Scale bars: 500 µm in A, C, E, G, 100 µm in B, D, F, H, 50 µm in B’, D’, F’, H’. 
Supplementary Figure S5  AMD3100 treatment inhibited Pdx1 and Nkx6.2 expression, whereas CXCL12 treatment resulted in an expansion of Pdx1 and Nkx6.2 expression.

Whole mount in situ hybridization analyses for Pdx1 (blue) or Nkx6.2 (blue) in AMD3100 or CXCL12 treated embryos are shown. Embryos were treated with PBS (A–C'), AMD3100 (D–F') or CXCL12 (G–I') at st 6 to 2 ss. (B, C, E, F, H, I) Transversal frozen sections at the level of B, E, H show the pancreatic region, and those at the level of C, F, I show the intestinal region. Numbers in the images are embryos observed with phenotype versus total embryos examined. Black lines in A, D, G show the 10 somite level. (E, E') Expression of Sox9 in the pancreatic endoderm is inhibited in AMD3100 treated embryos. Expression of Sox9 in the intestinal endoderm is induced in CXCL12 treated embryos (I, I'). Note that the dorsal aortae (da) were observed in the control PBS, AMD3100 or CXCL12 treated embryos. This result indicates that the migration of the angioblasts recovered by this stage. B’, C’, E’, F’, H’, I’ are enlarged images in boxed areas shown in B, C, E, F, H, I, respectively. Scale bars: 500 μm in A, D, G, 100 μm in B, C, E, F, H, I, 50 μm in B’, C’, E’, F’, H’, I’.

Supplementary Figure S6  AMD3100 treatment inhibited Sox9 expression in the pancreatic endoderm, whereas CXCL12 treatment induced Sox9 expression in the intestinal endoderm.
Supplementary Figure S7  Expression of Aldh1a2 is observed in the Lmo2-expressing angioblasts at 6 ss. (A-C') In situ hybridization analyses revealed that Aldh1a2 (blue) is expressed in the angioblasts at 6 ss (yellow arrows). (D-E') Double in situ hybridization analyses revealed that these Aldh1a2 (blue)–expressing cells are also positive for Lmo2 (red) (yellow arrows). (B-C') Transversal paraffin sections at the level of B, C in A are shown. (E) Transversal frozen sections at the level of E in D are shown. (A, D) 3 to 6 indicate the somite level. B', C', E' are enlarged images in boxed areas shown in B, C, E, respectively. Scale bars: 500 μm in A, D, 100 μm in B, C, E, 20 μm in B', C', E'.

References


