Development 138, 1947-1955 (2011) doi:10.1242/dev.058719

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

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SUMMARY

We have discovered that angioblasts trigger an early inductive event in pancreatic 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 in blood vessel formation and pancreatic differentiation. The ectopic expression of *Cxcl12* in the endoderm attracted the angioblasts and induced ectopic *Pdx1* expression, resulting in an expanded pancreatic bud and an increased area of insulin-expressing cells. By contrast, in chick 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 in the potentiation of further differentiation into endocrine β-cells.

KEY WORDS: *Pdx1*, Angioblast, Pancreas, Chick

INTRODUCTION

The earliest pancreatic marker gene, pancreatic and duodenal homeobox 1 (*Pdx1*) (Jonsson et al., 1994; Offield et al., 1996), is expressed in the dorsal and ventral pancreatic bud and in a portion of the stomach and duodenal endoderm. PDX1-expressing precursor cells give rise to all three pancreatic lineages: the endocrine, acinar and duct cells (Gu et al., 2003; Gu et al., 2002). Analysis of mice with homozygous null mutations in *Pdx1* demonstrates that PDX1 plays a key role in pancreatic differentiation: 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 the 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 (E) 8.5] both in mouse and in the chick (Katsumoto et al., 2009; Kume, 2005; Matsuura et al., 2009). Fate mapping 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 DiI (1,10-dioctadecyl-3,3,30,30-tetramethyl indocarbocyanine perchlorate), we performed fate mapping of the endodermal, 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 the 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 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 mesoderm cells have been shown to play an important role in the development of endoderm regionalization (Wells and Melton, 2000). We previously showed by transplantation experiments that pancreatic fate specification occurs between 6 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 into 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 because they cannot induce the posterior non-pancreatic endoderm to express pancreatic marker genes (Kim et al., 1997a). Activin and bFGF (FGF2) are candidate molecules for the notochord signal that can direct dorsal pancreatic morphogenesis and

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Accepted 21 February 2011

maintain the expression of early pancreatic genes through the repression of sonic hedgehog (*Shh*) (Hebrok et al., 1998). The notochord subsequently separates 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 VEGFA, can signal back to attract blood vessels (Brissova et al., 2006; Lammert et al., 2001; Vasir et al., 1998).

Here, we aimed to determine the nature of the earliest pancreas-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 *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 arise 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, 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 soaked in 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 ventral side up in a 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 (350-NS-010, R&D Systems) were deposited in ovo above or underneath the embryo. At stage 6 to 2 ss, a window was opened in 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 (ectoderm) side of the embryo. 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 underneath 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 that was 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.

In situ hybridization

Embryos were fixed overnight with 4% paraformaldehyde at 4°C, washed with PBS three times and stored in cold methanol. Whole-mount in situ hybridization was performed 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*) (EST clone ChEST326d6), platelet/endothelial cell adhesion molecule 1 (*Pecam1*, also known as *Cd31*) 860 bp (a gift from Dr Ken Matsumoto, Kumamoto University), NK6 homeobox 2 (*Nkx6-2*) (Vallstedt et al., 2001), SRY-box containing gene 9 (*Sox9*) (Sakai et al., 2006) and aldehyde dehydrogenase family 1 subfamily 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 *Scl*) and kinase insert domain protein receptor (*Kdr*, also known as *Flk1*) were cloned by RT-PCR. The primers used were as follows (5′ to 3′): *Cxcl12* 558 bp, Fwd GTCGCCAGAATGGACCTC and Rev CTTGGGAGAACGGCTCTTTA; *Cxcr4* 1180 bp, Fwd ACTCGGTGCTCGGAGTATG and Rev TGGTCAGTCTTGTTTTTCTGAGAT; *Lmo2* 876 bp, Fwd ACAGGCACGCTACAAAGGAG and Rev GCACAATCCTAGTCTGCAGGT; *Tal1* 1035 bp, Fwd GGGCTCCTTGGACATAAAAA and Rev GTGCTCCACAGCCCTATGTT; *Kdr* 1000 bp, Fwd GGACCTGGCTGCTCGTAATA and Rev ACTGGTTTCGGCCTAGAGTG. For double whole-mount in situ hybridization, digoxigenin-labeled probe and fluorescently 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 with PBS and treated at 70°C for 1 hour to inactivate the first antibody, followed by blocking and second color development.

Measurement of the insulin-expressing area and the size of the pancreas

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

Electroporation

For *Cxcl12* overexpression experiments, we constructed the pCIG-*Cxcl12* plasmid (Megason and McMahon, 2002). First, we cloned chick *Cxcl12* by RT-PCR using primers *Cxcl12* Fwd 5′-GTCGCCAGAATGGACCTC-3′ and Rev 5′-CTTGGGAGAACGGCTCTTTA-3′. We then inserted the *Cxcl12* cDNA under the control of the chicken β-actin promoter. The plasmid was electroporated in ovo into the chick embryos at stage 6 or 4 ss. A window was opened in the side of the air chamber in the eggs using forceps. A solution of 5-10 μg/μl DNA with 0.01% Fast Green was injected into 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 mseconds were applied 999 mseconds 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). Transverse sections revealed that, although the 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 reveal that the angioblasts are situated adjacent to the stomach and pancreatic endoderm (1-7 somite level), but not adjacent to the intestinal endoderm (8 somite level) (Fig. 1E,E′).

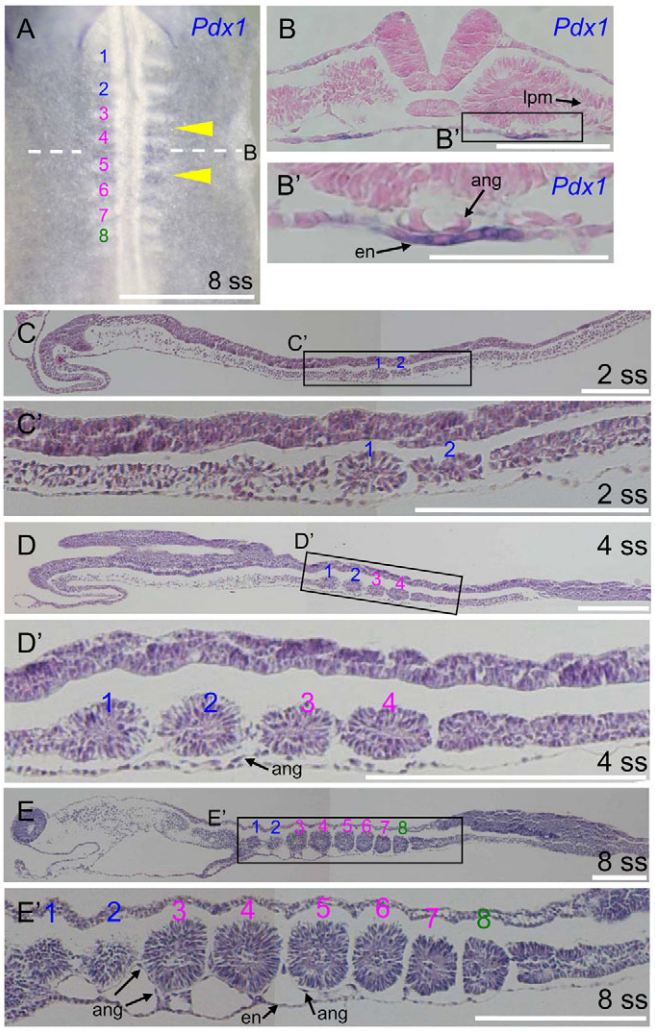

Fig. 1. Angioblasts exist at the chick pre-stomach and pancreatic region but not at the intestine region at an early somite stage. To examine the tissues that lie adjacent to the pre-pancreatic region at an early somite stage, paraffin sections were prepared at stages before (2 ss, 4 ss) or after (8 ss) pancreatic differentiation had occurred. We found that angioblasts were located adjacent to the pre-pancreatic region during pancreatic differentiation. (**A**) Whole-mount in situ hybridization showed that *Pdx1* (blue) is expressed in pre-pancreatic endoderm at 8 ss. Yellow arrowheads indicate representative *Pdx1* expression regions. (**B**,**B′**) Transverse section of the pre-pancreatic region at 8 ss (at the level of the white dashed line in A), counterstained with Eosin (red). The boxed region is enlarged in B′. (**C-E′**) Parasagittal sections at 2 (C), 4 (D) or 8 (E) ss, and enlargements of the boxed regions (C′,D′,E′). Sections are counterstained with Hematoxylin and Eosin. 1 to 8 indicate the somite level: 1-2 somite level (blue), pre-stomach region; 3-7 (pink), pre-pancreatic region; 8 (green), pre-intestine region. ang, angioblast; en, endoderm; lpm, lateral plate mesoderm; ss, somite stage. Scale bars: 500 μm in A; 100 μm in B; 50 μm in B′; 250 μm in C-E′.

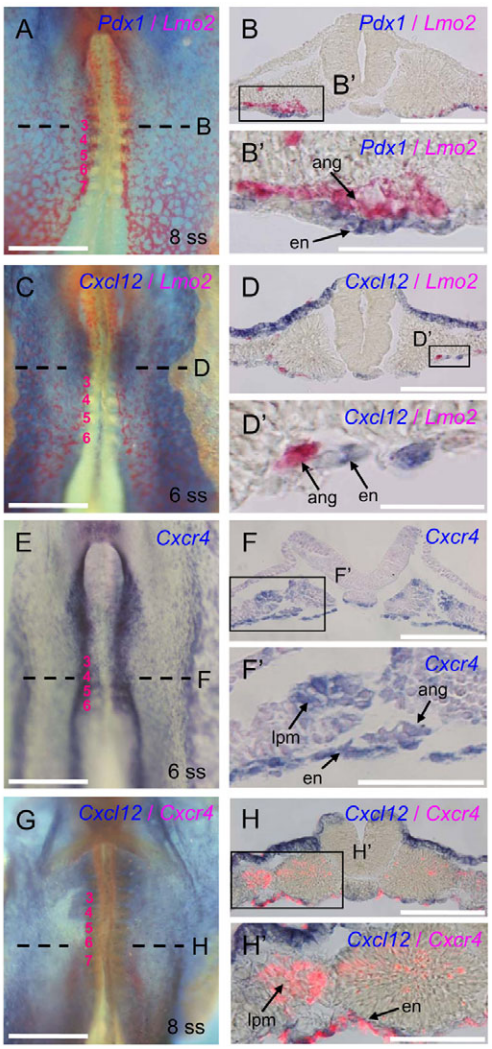

Fig. 2. *Pdx1*-expressing endoderm cells are in the proximity of *Lmo2*-expressing angioblasts. Whole-mount in situ hybridization analysis showed that the angioblasts that lie in the proximity of *Pdx1*-expressing endoderm cells are *Lmo2* positive, indicating that they are angioblasts. The chemokine ligand *Cxcl12* and 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* (A), *Cxcl12/Lmo2* (C), *Cxcr4* (E) or *Cxcl12/Cxcr4* (G). *Pdx1* (blue), *Lmo2* (red), *Cxcl12* (blue); *Cxcr4* (blue in E, red in G,H,H′). (**B**,**D**,**F**,**H**) Transverse sections (at the level of the dashed lines). (**B′**,**D′**,**F′**,**H′**) Magnifications of the boxed regions. Numbers indicate the somite level. ang, angioblast; en, endoderm; lpm, lateral plate mesoderm; ss, somite stage. Scale bars: 500 μm in A,C,E,G; 100 μm in B,D,F,H; 50 μm in B′,F′,H′; 20 μm in D′.

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 (K.K., unpublished) (Katsumoto et al., 2009), the emergence of angioblasts corresponds temporally and spatially with *Pdx1* expression.

The effect of 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 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 hemangioblasts (see

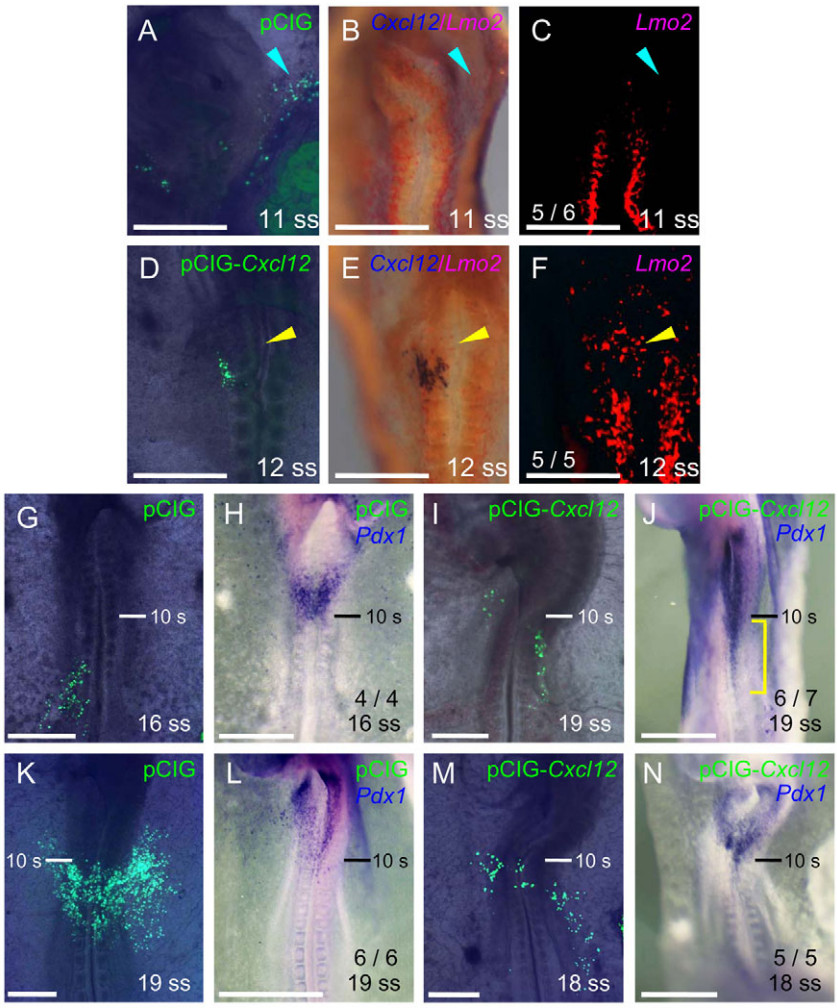

Fig. 3. *Cxcl12*-expressing endoderm attracts *Lmo2*-expressing angioblasts. (**A-F**) In chick embryos electroporated with pCIG-*Cxcl12* (a CXCL12-IRES-GFP construct) in the anterior endoderm region at an early somite stage, ectopic *Lmo2*-positive angioblasts migrated towards the region overexpressing pCIG-*Cxcl12* (D-F, yellow arrowheads). No ectopic angioblasts were observed in control embryos electroporated with pCIG (GFP) (A-C, blue arrowheads). (A,D) Fluorescence images of GFP expression in embryos electroporated with pCIG (control, A) or pCIG-*Cxcl12* (D). (B,C,E,F) Whole-mount in situ hybridization for *Cxcl12* (blue) and *Lmo2* (red). 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 show 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). The number of embryos showing the phenotype versus the total number examined is shown. Solid black or white lines in G-N show the level of somite 10. ss, somite stage. Scale bars: 500 μm.

Fig. S1A-B′ in the supplementary material). These cells were also positive for *Cd34* and *Kdr* (see Fig. S1C-F′ in the supplementary material) and negative for *Pecam1* (data not shown), 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) (see Fig. S1 in the supplementary material). Interestingly, the *Pdx1*-expressing cells resided 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 for 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 to 10 ss, in the stomach and pancreatic endoderm region (see Fig. S2 in the supplementary material). At 4 to 8 ss in the pancreatic region, angioblasts expressing both *Lmo2* and *Cxcr4* were observed in the lateral plate mesoderm and at the endoderm border (see 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 (see Fig. S2E′,H′ in the supplementary material). At 10 ss, almost all *Cxcr4* and *Lmo2* double-positive cells had migrated to the endoderm border and *Lmo2* expression had decreased (see Fig. S2K′,L′ in the supplementary material). Taken together, these results strongly suggest that angioblasts in the mesoderm are recruited through CXCL12-CXCR4 signaling to the *Cxcl12*-expressing pre-pancreatic endoderm, which coordinates temporally with the pancreas-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 performed as a

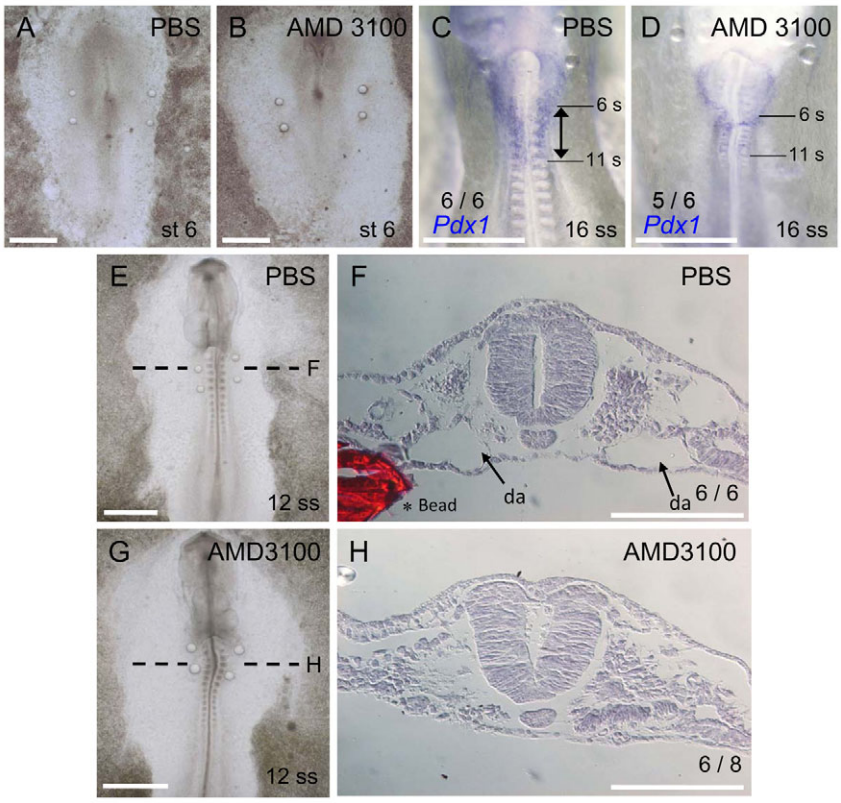

Fig. 4. The angioblasts participate in early pancreatic regionalization via chemokine signals. (**A**,**B**) To examine whether CXCR4 participates in early pancreatic regionalization, we treated chick embryos with beads soaked with the CXCR4 inhibitor AMD3100 (B) or with PBS as control (A) at stage 6. The embryos were cultured until they reached 16 ss. (**C**,**D**) Whole-mount in situ hybridization revealed that the *Pdx1*-expressing region (blue) was reduced in AMD3100-treated embryos (D), but not in PBS-treated embryos (C). (**E-H**) Dorsal aorta formation was inhibited in AMD3100-treated embryos (G,H) but not in PBS-treated embryos (E,F). (A-E,G) Views from the endoderm layer. (F,H) Transverse sections (at levels indicated by the dashed lines). Sections are counterstained with Hematoxylin and Eosin. The number of embryos showing the phenotype versus the total number examined is shown. 6 s and 11 s indicate the somite level. st, Hamburger and Hamilton stage; ss, somite stage; da, dorsal aorta. Asterisk indicates bead. Scale bars: 1 mm in A,B,E,G; 500 μm in C,D; 100 μm in F,H.

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, yellow arrowheads; *n*=5/5). This was not observed in control embryos (Fig. 3A-C, blue arrowheads; *n*=5/6). 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 specified), but not at 8 ss (intestinal fate being specified), ectopic induction of *Pdx1* expression was observed (Fig. 3I,J,M,N). These results showed that ectopic *Cxcl12* expression attracted angioblasts, which in turn induced ectopic *Pdx1* expression in the pre-intestinal endoderm before the intestinal fate decision occurred. Control ectopic pCIG expression was confirmed not to induce *Pdx1* expression at 4 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 (see Fig. S3A-C″ in the supplementary material). The *Cxcl12* expression overlapped with that of *Pdx1* in the pancreatic endoderm (see Fig. S3B-B″ in the supplementary material, yellow arrows). However, *Cxcl12* expression was also observed in some intestinal endoderm, in which *Pdx1* was not expressed (see Fig. S3C-C″ in the supplementary material). This result strongly suggests that *Cxcl12* does not directly induce *Pdx1* expression.

Blockade of 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; see Fig. S4C-D′ in the supplementary material). A defect in dorsal aorta formation was observed (Fig. 4G,H, *n*=6/8; see 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; see Fig. S4A-B′,E-F′ in the supplementary material). These results suggest that the CXCL12-CXCR4 chemokine signaling pathway regulates the migration and maturation of angioblasts, which in turn induces pancreatic differentiation.

To analyze the effects of AMD3100 on the angioblasts, we examined the expression of *Lmo2* and *Tal1* by in situ hybridization (see 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 (see Fig. S4B,B′ in the supplementary material), and at 12 ss dorsal aorta was formed and *Lmo2* and *Tal1* expression in the angioblasts disappeared (see 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 (see Fig. S4D,D′, in the supplementary material) and migration and maturation of *Lmo2*-positive angioblasts was delayed and *Lmo2* and *Tal1* expression remained at 12 ss (see Fig. S4H,H′ in the supplementary material). Later on, angioblast migration recovered and dorsal aortae were formed in AMD3100-treated embryos at 17 ss (see Fig. S6E,E′, in the supplementary material; see below). The gross appearance of the vasculature seemed unaffected (K.K., unpublished). Therefore, the AMD3100 treatment delayed the migration and maturation of *Lmo2*-positive angioblasts, which resulted in an inhibition of *Pdx1* expression.

CXCL12 potentiates whereas AMD3100 inhibits pancreatic endocrine differentiation

We next tested the effects of CXCL12 administration or CXCR4 inhibition at a later stage. First, embryos were treated with beads pre-adsorbed with CXCL12 or PBS in the pre-intestine region between stage 6 and 2 ss and cultured until stage 26 (Fig. 5A).

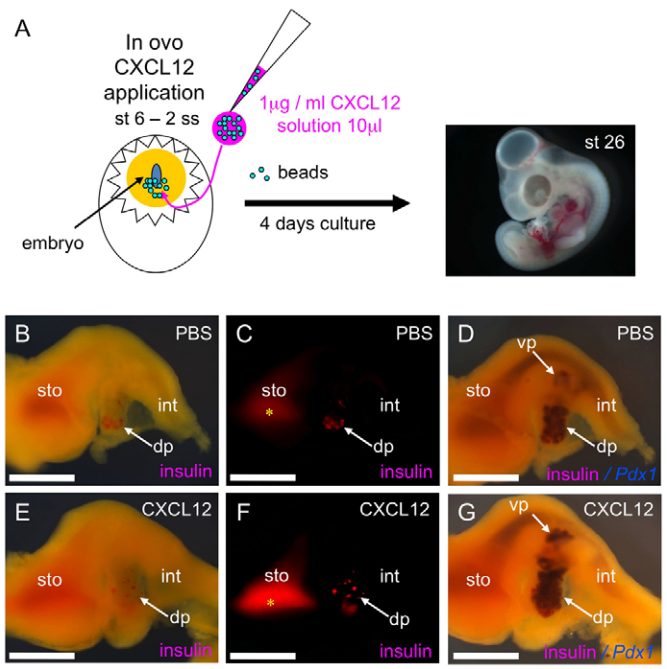

Fig. 5. CXCL12 treatment results in an expansion of the pancreatic bud. Insulin and *Pdx1* expression areas are expanded in CXCL12-treated chick embryos compared with those of PBS-treated control embryos. (**A**) Procedure of CXCL12-adsorbed bead treatment in ovo. (**B-G**) Beads adsorbed with PBS (B-D) or CXCL12 (E-G) were applied to embryos at the posterior region between stage 6 and 2 ss. After 4 days of culture, embryos were dissected at stage 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 shown alone in C,F (red). sto, stomach; dp, dorsal pancreas; int, intestine; vp, ventral pancreas; st, Hamburger and Hamilton stage; ss, somite stage. Asterisk indicates a nonspecific signal. Scale bars: 500 μm.

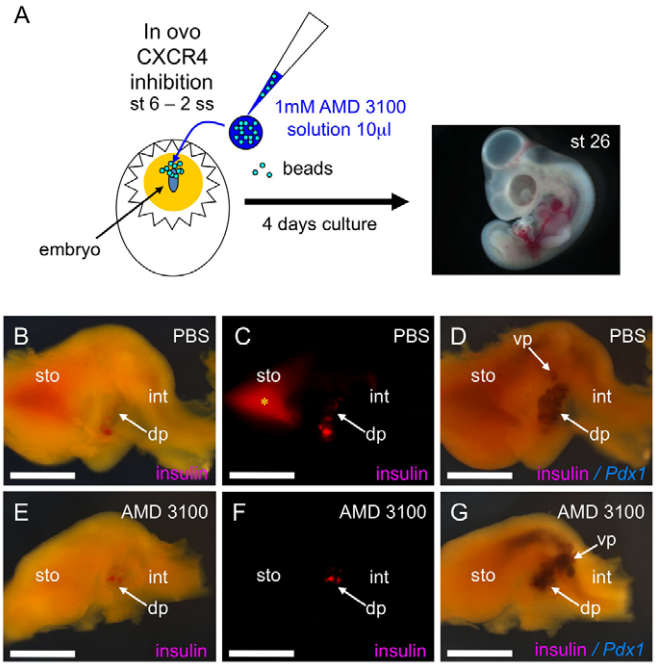

Fig. 6. CXCR4 inhibition at an early stage results in a reduction of the pancreatic bud. Insulin and *Pdx1* expression regions are reduced in AMD3100-treated chick embryos as compared with those of control embryos. (**A**) Procedure of treatment with AMD3100-adsorbed beads in ovo. (**B-G**). Beads adsorbed with PBS (B-D) or AMD3100 (E-G) were applied to the anterior part of the embryos between stage 6 and 2 ss. After 4 days of culture, embryos were dissected at stage 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 shown alone in C,F (red). sto, stomach; dp, dorsal pancreas; int, intestine; vp, ventral pancreas; st, Hamburger and Hamilton stage; ss, somite stage. Asterisk indicates a nonspecific signal, Scale bars: 500 μm.

Embryos treated with CXCL12 developed a larger pancreatic bud, showed a 1.24-fold increase in the area of insulin-expressing cells (Fig. 5B-G) and a 1.31-fold increase in the area of *Pdx1*-expressing cells in the dorsal pancreas as compared with PBS-treated control embryos (Fig. 5B-G, Fig. 7).

In a mirror experiment, embryos were treated with AMD3100 or PBS beads 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 21% decrease in the size of the dorsal pancreatic bud, as quantified by measuring the areas expressing *Pdx1*. A 19% decrease in the area of insulin-expressing endocrine cells was also observed, as compared with control embryos (Fig. 6B-G, Fig. 7). These decreases were not observed with embryos treated with AMD3100 at a stage after the pancreatic fate was specified (8 ss) (data not shown). Representative images of the dorsal pancreatic (*Pdx1*-positive) region and β-cell (insulin-positive) region in embryos treated with PBS, AMD3100 or CXCL12 are shown in Fig. 7A-C.

We also confirmed that AMD3100 treatment resulted in an early downregulation of the pancreatic progenitor markers *Pdx1*, *Sox9* and *Nkx6-2* (see Figs S5 and S6 in the supplementary material), whereas CXCL12 treatment resulted in an enlargement of all three expression domains (see Figs S5 and 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 (see Figs S5 and S6 in the supplementary material). Dorsal aortae were observed in the CXCR4-inhibited embryos (see Fig. S6E,E′ in the supplementary material), demonstrating that the inhibition of angioblast migration was a temporary event.

Taken together, we conclude that CXCL12-CXCR4 signaling attracts angioblasts to the vicinity of the pre-pancreatic endoderm, which potentiates the induction of *Pdx1*-expressing pancreatic progenitor cells and 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 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* and *Lmo2* double-positive angioblasts. The angioblasts then migrate to the endoderm border and signal back to induce *Pdx1* expression in the gut endoderm. Overexpression of *Cxcl12* attracted the *Lmo2*-expressing cells (Fig. 3A-F), which then induced *Pdx1*-expressing pancreatic progenitors (Fig. 3G-N) and

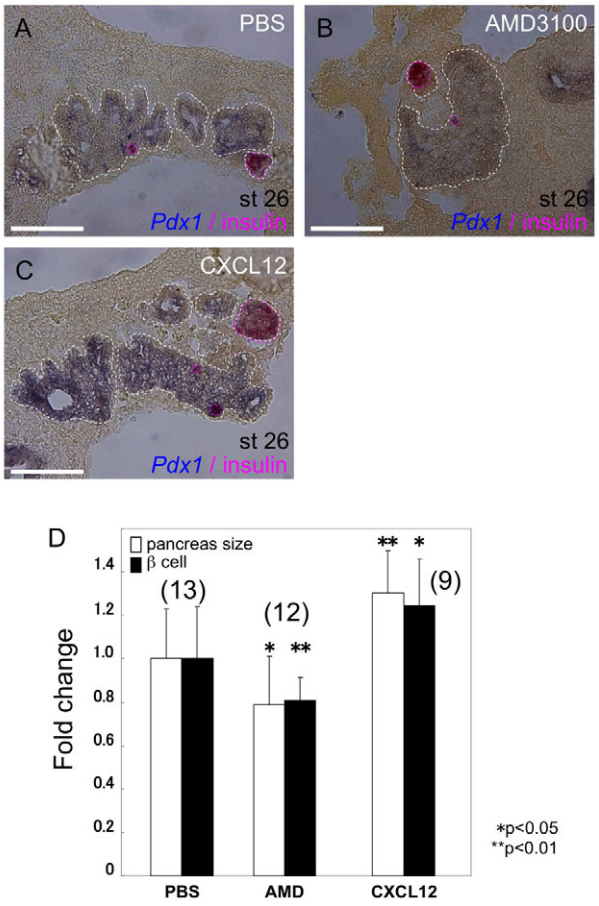

Fig. 7. CXCL12-CXCR4 chemokine signaling participates in pancreatic differentiation. A reduction in the pancreatic bud by AMD3100 treatment (inhibition of CXCR4 function) and an expansion in the pancreatic bud by CXCL12 treatment are observed, indicating that the CXCL12-CXCR4 chemokine signaling pathway plays a pivotal role in pancreatic regionalization. (**A-C**) Pancreas buds treated with 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. Dashed white 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. (**D**) Total areas positive for *Pdx1* or insulin. AMD3100-adsorbed beads applied to chick embryos (AMD) resulted in a 21% decrease in the *Pdx1*-expressing area (average 294,974.9 μm^2; white bar) and a 19% decrease in the insulin-expressing area (average 23,762.5 μm^2; black bar), as compared with those of the PBS-treated control embryos (*Pdx1*-expressing average 373,786.8 μm^2; insulin-expressing average 29,475.3 μm^2). By contrast, embryos treated with CXCL12 developed a larger pancreatic bud, with a 1.31-fold increase in the *Pdx1*-expressing area (average 487,971.6 μm^2; white bar) and a 1.24-fold increase in the insulin-expressing area (average 36,672.0 μm^2; black bar) as compared with those of the control embryos. The numbers of embryos examined are showed in parentheses. Data are shown as mean + s.d. Fold change is relative to the average of PBS-treated control embryos. The differences were significant for AMD3100-treated and CXCL12-treated embryos; *P<0.05, **P<0.01, two-tailed paired Student's t-test. Scale bars: 100 μm.

enhanced their differentiation into insulin-expressing cells (Figs 5 and 7). Conversely, blockade of CXCR4 inhibited the migration of angioblasts to the proximity of the endoderm (Fig. 4E-H; see Fig. S4 in the supplementary material). This resulted in a reduction in *Pdx1*-expressing pancreatic progenitors (Fig. 4A-D; see Fig. S4A-D′ and Fig. S5A,B in the supplementary material) and in a partial inhibition of their differentiation into insulin-expressing endocrine cells (Figs 6 and 7).

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

Endothelial cells 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., 2010; Kim et al., 1997b; Kume, 2005). 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 and Zaret 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 might exist in the *Flk1* mutants, which were capable of inducing normal, initial *Pdx1* expression.

Similarly, Field et al. showed that in *cloche* zebrafish mutant embryos, which lack endothelial cells, *pdx1*, *insulin* and *trypsin* expression were normal compared with the wild-type embryo (Field et al., 2003). Angioblasts might also exist in the *cloche* mutant, thereby accounting for the induction of the pancreatic primordium markers. Although it remains controversial, a small number of primitive angioblasts have been reported to exist in the *cloche* mutants (Thompson et al., 1998).

Here, we showed that *Cxcl12* overexpression in early stages attracts angioblasts, leading to ectopic pancreatic development. However, it is possible that *Cxcl12* acts in an autocrine manner. To prove that the inducing signals derive from the angioblasts and are not due to direct effects of CXCL12 in the endoderm, future experiments using angioblast inhibitors might be useful.

Chemokine signals participate in early pancreatic differentiation

The CXCL12-CXCR4 pathway participates 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 pancreatic ductal cells. This suggests that CXCL12-CXCR4 signaling participates in endocrine cell renewal in pancreas regeneration (Kayali et al., 2003) as 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 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* and its receptor *cxcr4a* 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 affects 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 explained by the difference in the size of chick and zebrafish embryos. We hypothesize that because the chick embryo is bigger, the *Cxcr4*-positive mesodermal cells (angioblasts) have to cover a greater distance to reach the correct site at the endoderm border, whereas in the zebrafish they only need cover a shorter distance. Our hypothesis is that angioblast migration to the appropriate region of endoderm in the zebrafish might be disturbed, thereby yielding a duplicated pancreas. It was also shown in the zebrafish that endothelial cells are derived from *cxcr4a*-positive anterior mesoderm, and that *cxcl12* is expressed in the endoderm underlying the lateral aorta. Both *cxcr4a* 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 as a candidate signal secreted by the angioblasts

A defect in endocrine and exocrine pancreas formation was previously described in the zebrafish *aldh1a2* mutant *neckless* (*nls*), as well as in embryos treated with BMS493, a retinoic acid receptor (RAR) antagonist (Stafford and Prince, 2002). Maternal *Aldh1a2* 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 (Bayha et al., 2009; Kumar et al., 2003). 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 expression of *Pdx1* and *Prox1* was inhibited and dorsal, but not ventral, pancreatic agenesis was observed (Molotkov et al., 2005).

Despite multiple reports on the role of RA in early pancreatic development, no report has definitively shown that RA acts as an instructive rather than 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 with its high expression in the mesoderm (see Fig. S7 in the supplementary material). Angioblasts might induce *Pdx1* expression by adjusting the concentration of RA to an optimal level.

Timing of pancreatic 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 this region is already committed to expressing *Sox2* at 2 ss, and loses the responsiveness to express *Pdx1*. Similarly, in the pre-intestine region, the angioblasts begin to appear at 10 ss but cannot induce the expression of *Pdx1* in this region because it 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 the distinct responsiveness of the different endoderm areas, restrict *Pdx1* expression to the future pancreatic progenitor region.

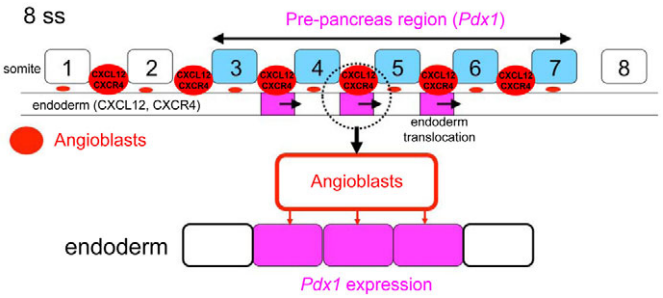

Fig. 8. A working hypothesis for early pancreatic regionalization. The angioblasts migrate to the endoderm border at an appropriate time and location in response to chemokine signals and induce *Pdx1* expression in the endoderm. At an early somite stage, angioblasts are present in the pre-stomach and pancreatic region, but angioblasts cannot induce *Pdx1* expression in the pre-stomach region because it has already committed and is not competent to respond to the *Pdx1*-inducing signals. 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.

In summary, in this study we have shown that pancreas induction is initiated by angioblasts by contact with the endoderm and that the chemokine signals (CXCL12-CXCR4) spatiotemporally regulate angioblast migration to the correct position in contact with the endoderm, and induce *Pdx1* expression (Fig. 8).

Acknowledgements

We thank Drs Anne Grapin-Botton, Johan Ericson, Yoshio Wakamatsu and Ken Matsumoto for the kind gifts of plasmids; 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; Ms Minako Kawabata and Mr Shingo Usuki for technical assistance; and members of the Gene Technology Center for their technical assistance. This work is supported by a Grant-in-Aid (20790652 to K.K., 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.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.058719/-/DC1

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