Critical role of connexin43 in zebrafish late primitive and definitive hematopoiesis

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Abstract In vitro studies have suggested that connexin43 (cx43) expression is of particular importance during establishment and regeneration of the mammalian hematopoietic system. However, little is known about its in vivo functions during hematopoiesis due to the embryonic lethality of mammalian knockout models. In this study, we observed that zebrafish cx43 is not only expressed in the eyes, cerebellum, heart, and vasculature, but also expressed, albeit at low levels, in intermediate cell mass (ICM, the primitive hematopoietic site). Knockdown of cx43 leads to vacuolization in the wedge of the ICM and an apparent reduction in the number of circulating blood cells, but does not affect their cellular morphology. Whole-mount in situ hybridization analysis revealed that the hemangioblastic marker flk-1 and the primitive hematopoietic markers lmo2 and scl are basically maintained at normal levels in cx43 morphant embryos at 12–13 h postfertilization (hpf) compared with the con-MO injected embryos. However, subsequent expression of the definitive hematopoietic stem cell (HSC) marker c-myb was severely downregulated in the ventral wall of the dorsal aorta of cx43-depleted embryos at 36 hpf. Furthermore, we confirmed this phenotype by injection of cx43-MO into Tg(gata1:EGFP) embryos. Together, our results show that cx43 contributes to late primitive and definitive hematopoiesis in zebrafish embryos.

Keywords Zebrafish • Gap junction • Connexin43 • Hematopoiesis • flk-1 • c-myb • gata1:EGFP

Introduction

Gap junctions are membrane channels that function as major conduits for cell–cell communication by mediating the intercellular passage of ions and small molecules (Bruzzone et al. 1996). Gap junctions play a critical role in the regulation of cell growth and development. Six connexins, as the subunits of gap junctions, assemble into a connexon, two of which from adjacent cells dock at the plasma membrane to form a gap junction channel. In human, dozens of connexin genes have been identified, with subsets of which expressed in different tissues (Sohl and Willecke 2004), and different connexin mutant genes caused distinct developmental defects, such as...
deafness, skin disorders, peripheral neuropathy, and cataracts (Bergoffen et al. 1993; Shiels et al. 1998; Kelsell et al. 1997). Although gap junctions are not usually present in circulating blood cells, connexin expression is known to occur in leukocytes during inflammatory reactions in blood vessels and gap junctions have been found in the hematopoietic bone marrow (Saez et al. 2000; Rosendaal 1995; Rosendaal et al. 1991). In vitro studies of cultured bone marrow or thymic stromal cells have shown that cx43 is the principal gap junction protein expressed by these primary cell types (Dorshkind et al. 1993; Rosendaal et al. 1994; Alves et al. 1995).

Owing to its optical clarity, genetics, and ease of manipulation, the zebrafish has been established as an ideal genetic system for analyzing hematopoietic development and disorders (Amatruda and Zon 1999; Dooley and Zon 2000; North and Zon 2003). Vertebrate hematopoiesis occurs in two developmental waves: a short primitive wave, predominantly producing erythrocytes and primitive myeloid cells, and a definitive wave, generating long-term hematopoietic stem cells (Galloway and Zon 2003). Primitive hematopoiesis in zebrafish occurs intra-embryonically above the yolk tube between the notochord and endoderm of the trunk in a region known as the ICM, which is analogous to the extraembryonic blood islands on the yolk sac of higher vertebrates and formed in two paraxial stripes of mesoderm that arise during gastrulation (Bahary and Zon 1998; Detrich et al. 1995; Hsia and Zon 2005). Subsequently, definitive hematopoiesis originates from the ventral wall of the dorsal aorta. The transcriptional mechanisms of hematopoiesis are evolutionarily well-conserved between mammals and zebrafish, and several zebrafish orthologs of key mammalian transcription factors have been identified, such as flk-1, scl, lmo2, gata1, gata2, c-myb, and runx1 (Hsia and Zon 2005).

Alignment analysis of the amino acid sequence of the zebrafish Cx43 protein confirmed the similarity to the relevant Connexin proteins of higher vertebrates (Liu et al. 2008; Chatterjee et al. 2005). Moreover, Iovine et al. (2005) have reported that down-regulation of cx43 significantly reduced the number of red blood cells in zebrafish, without further explanation for this phenomenon. Therefore, in the present study, we used the properties of the wild-type and Tg(gata1:EGFP) zebrafish to further understand the in vivo role of cx43 during hematopoiesis.

### Materials and methods

#### Zebrafish

Wild-type (AB* strain) zebrafish stocks were obtained from International Zebrafish Resource Center. Embryos were obtained from natural spawning of wild-type adults. Zebrafish were raised, maintained, and staged as previously described (Westerfield 2000; Kimmel et al. 1995). The Tg(gata1:EGFP) zebrafish line used in this study was a generous gift of Prof. Tingxi Liu from Shanghai Jiaotong University.

Confirmation of cx43-MO specificity and microinjection

A pair of morpholino-modified antisense oligonucleotides (cx43-MOs, Gene Tools, LLC) were designed against the 5'-untranslated region (5'-UTR) and the translational start site (ATG) to block cx43 translation, respectively, as follows:

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"cx43(ATG)-MO: 5'-TCCAGTCACC
CATCTTG
AGGGAGTT-3';
"cx43(5'-UTR)-MO: 5'-AAAGAAGTAAAGAGTG
GGAGAGCCCT-3'.
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The third MO was designed to control the microinjection (con-MO, standard control MO). The sequence of con-MO was 5'-CCTCTTACCTCAGTTACAATTTATA-3'.

Construction of cx43 anti-sense RNA probe and whole-mount in situ hybridization

To eliminate the chances of cross-reactivity of ORF probes during in situ hybridization, we used a probe against the 3'-untranslated region (UTR) of cx43 as it has little sequence homology. Briefly, the cx43 probe was generated by amplification of the 3'-UTR (1,001 bp) of cx43 and used as template for riboprobe
synthesis (forward: 5'-ACAGAGACATAAAATTA CTCACACTGCT-3'; reverse:5'-CGCTGGGCATATA TCTCTTAATATAACTG-3').

Whole-mount in situ hybridization experiments using the combination of digoxigenin-labeled antisense RNA probe and α-digoxigenin alkaline phosphatase-conjugated antibody (Roche) were performed as previously described (Westerfield 2000). In brief, embryos were permeabilized with Proteinase K (10 g/ml, Sigma) and hybridized overnight at 65°C with the DIG-labeled anti-sense riboprobes. After several washes at high stringent temperature, NBT/BCIP (Roche) staining was performed according to the manufacturer’s instructions. For antisense probes cx43, flk-1, scl, lmo2, and c-myb, the NBT/BCIP coloring reactions proceeded at about 20°C for 2–5 h.

O-dianisidine staining, isolation, and histological staining of embryonic blood cells

Staining for globin was completed as described (Detrich et al. 1995). Briefly, the dechorionated embryos were stained for 15 min with a solution consisting of o-dianisidine (0.6 mg/ml), sodium acetate (0.01 mol/l), 0.65% hydrogen peroxide, and 40% (v/v) ethanol in the dark.

Blood cells were collected by cardiac puncture on anesthetized embryos in a solution of 0.02% tricaine (Sigma), 1% bovine serum albumin in calcium- and magnesium-free phosphate-buffered saline, pH 7.4. Collected cells were putted onto slides, immediately fixed in methanol for 30 s, and stained by the Wright-Giemsa method.

Photography

Stained embryos were examined with Olympus BX61/SZX12 microscopes and photographed with a DP70 digital camera. Images were processed using Adobe Photoshop software.

Results

Spatio-temporal expression of cx43 during embryogenesis

To explore the role of cx43 in hematopoiesis, we analyzed the expression pattern of cx43 during embryogenesis. The first zygotic cx43 expression was detected in the notochord during early somitogenesis (data not shown). By 24 hpf, zebrafish cx43 is not only expressed in the eyes and vasculature, but also expressed, albeit at low levels, in the ICM (Fig. 1a, b, c). At 36 hpf, cx43 expression was observed in the cerebellum, heart, and pronephric ducts (Fig. 1d, e). By 72 hpf, in situ hybridization signals with the cx43 anti-sense probe can be detected in the lens epithelium, midbrain-hindbrain boundary, rhombomere and, hindbrain (Fig. 1f, g).

Characterization of the hematopoietic phenotype in cx43 morphants

Injection of morpholino oligonucleotides into zebrafish embryos is a well-established method to block translation of a targeted mRNA (Nasevicius and Ekker 2000). To analyze the loss-of-function

**Fig. 1** Expression pattern of cx43 in zebrafish embryos. **a, b, c** Expression of cx43 in wild-type embryos at 24 hpf. e eyes, v vasculature, icm intermediate cell mass. **d, e** Expression of cx43 in wild-type embryos at 36 hpf. c cerebellum, h heart, pd pronephric ducts. **f, g** Expression of cx43 in wild-type larvae at 72 hpf. l lens epithelium, mhb midbrain-hindbrain boundary. r rhombomere, hb hindbrain
phenotype of cx43, we knocked down cx43 using a pair of cx43-specific morpholino oligonucleotides (cx43(ATG)-MO and cx43(5' -UTR)-MO). Both of them caused very similar phenotypes. For later using, the cx43(5' -UTR)-MO was chosen. To confirm the efficacy of the morpholino approach, cx43-MOs were co-injected with a green fluorescent protein (GFP) reporter containing the partial 5' -UTR and the start site sequence of cx43. The cx43-MO specifically knocked down the expression of GFP from this RNA transcript, as revealed at 6.5 hpf in 100% of embryos (n = 44) (Fig. 2b). In comparison, there was no detectable knockdown of GFP when co-injected with a random control morpholino (n = 41) (Fig. 2a). This revealed the ability of the cx43-MO to inhibit the production of protein from its target sequence.

Although differences appeared to be undetectable before 15 hpf, the injection of cx43-MO (1.5–2.5 ng/embryo) causes vacuolization in the wedge of the ICM at 26 hpf, and is associated with the curved body phenotype (84%, n = 162) (Fig. 2c, d).

Fig. 2 Morpholino knockdown of zebrafish cx43. a, b Fluorescence microscopy images in 6.5 hpf zebrafish embryos. a Fluorescence microscopy image of zebrafish embryos co-injected with cx43-GFP plasmid and con-MO. GFP expression (green) is detectable in a mosaic pattern throughout injected embryos. b Fluorescence microscopy image of zebrafish embryos co-injected with cx43-GFP plasmid and cx43-MO. Note the absence of detectable GFP expression in all embryos. c, d Live embryos at 26 hpf. Arrows indicate that injection of cx43-MO (1.5–2.5 ng/embryo) causes vacuolization in the wedge of the ICM at 26 hpf and is associated with the curved body phenotype (84%, n = 162).

Fig. 3 Knockdown of cx43 causes an apparent reduction in the number of red blood cells, without changing their cellular morphology. a, b 36-hpf zebrafish embryos stained for hemoglobin with 0-dianisidine. Arrows indicate hemoglobinized blood cells (traversing cardinal veins of yolk sac). a con-MO-injected embryos; b cx43-MO-injected embryos. Note no or less hemoglobin staining in cx43 morphant embryos. Also, note the similar morphology of red blood cells in the con-MO and cx43-MO-injected embryos
Knockdown of cx43 affects hematopoietic transcription factor expression patterns

The isolation of zebrafish homologs of key hematopoietic transcription factor genes has allowed in situ hybridization studies to define the anatomy of hematopoiesis and vasculogenesis (Thompson et al. 1998). During early somitogenesis, hematopoietic stem cells and endothelial cells have similar gene and surface marker expression profiles, including CD34, SCL, and, Flk-1. To assess the effects of cx43 on a common progenitor of blood and endothelial cells, we showed that expression of the blood and vascular co-marker flk-1 is maintained at normal levels at the 6-somite stage (approximately 12 hpf) in cx43 morphants (Fig. 4a, b). In the zebrafish, c-myb and runx1 are expressed in the ventral wall of dorsal aorta after 31 hpf, indicating the presence of definitive HSCs (Thompson et al. 1998; Burns et al. 2002). To further investigate the role of cx43 in primitive and definitive hematopoiesis, we showed that at 13 hpf the primitive hematopoietic markers lmo2 and scl expression are detected at normal levels in cx43 morphants, similar to the con-MO injected or wild-type embryos (Fig. 4c, d, e, f), and the subsequent expression of definitive HSC marker c-myb was severely down-regulated in the ventral wall of dorsal aorta in cx43-depleted embryos at 36 hpf (70%, n = 30) (Fig. 4g, h).

To further confirm the result that knockdown of cx43 compromises hematopoiesis, we performed knockdown of cx43 expression in Tg(gata1:EGFP) zebrafish embryos (Fig. 5). This transgenic zebrafish line exhibits ectopic green fluorescence expression in the somites, but this does not disrupt assessment of circulating RBCs. We detected no gross defects to the early primitive hematopoiesis in cx43 morphant embryos at 14 hpf (Fig. 5a, b), but examination of the 26-hpf cx43 morphants revealed that the gata1+ population in the trunk was nearly undetectable except in the anterior part of the wedge of ICM (71%, n = 120) (Fig. 5c, d). At 48 hpf, the GFP+ cells were

![Fig. 4](image-url) In situ hybridization analysis using markers for hematopoiesis. a, c, e, g con-MO injected embryos at 12, 13, 13, and 36 hpf, respectively. (b, d, f, h) cx43 morphant embryos at 12, 13, 13, and 36 hpf, respectively. Arrows indicate expression of c-myb in the ventral wall of the dorsal aorta.
almost completely abolished in the circulation of cx43-depleted embryos (73%, $n = 124$) (Fig. 5e, f).

**Discussion**

Vertebrate hematopoiesis is a complicated process that proceeds in distinct phases and at various anatomic sites during embryogenesis. Although in vitro model system studies of cx43 have provided some insight into hematopoiesis, the in vivo function remains unclear. Major findings of this study are that knockdown of cx43 causes visual vacuolization in the primitive hematopoietic region and that cx43 is essential for blood formation in zebrafish embryos. Cx43 depletion in zebrafish does not disrupt expression of the hemangioblastic gene flk-1 and the hematopoietic genes scl and lmo2 at the early stages of primitive hematopoiesis, but does impair the late primitive and definitive hematopoiesis.

In previous studies, the expression of cx43 was detected using antibodies in both bone marrow and fetal liver cell lines (Cancelas et al. 2000). Several other studies have confirmed that cx43 gap junctions were expressed in hematopoietic tissues and that functional gap junction-mediated communication occurs between stromal cells (Dorshkind et al. 1993; Alves et al. 1995; Krenacs and Rosendaal 1998). Moreover, Rosendaal and his colleagues demonstrated that the number of gap junctions was increased at the sites of regeneration following cytoablative treatment and in the epiphyseal marrow of growing animals (Rosendaal et al. 1994; Krenacs and Rosendaal 1998). Together, these in vitro data support the hypothesis that cx43 is involved in hematopoiesis in mammals, including humans. In zebrafish, we have shown that cx43 is involved in the process of late primitive and definitive hematopoiesis, based on several pieces of evidence. First, cx43 expression is found in the vasculature and ICM; second, knock down of cx43 causes local vacuolization of the wedge of ICM; and third, down-regulation of cx43 results in the change of hematopoietic markers after the onset of vasculature. Taken together with the results of previous in vitro studies, these in vivo data indicate that the primary effects of cx43 on hematopoiesis may be mediated through local expression in the hematopoietic niche.

In summary, as hemangioblast originates from the ventral mesoderm and has two fates, namely the
HSCs and angioblasts, our present data, together with the previous finding of normal expression of the vascular marker flk1 in cx43 morphants, show that cx43 expression affects late primitive and definitive hematopoiesis but has no or little effect on proliferation of the hemangioblast (Liu et al. 2008). However, to further understand the mechanism(s) by which cx43 gap junctions regulate hematopoiesis after the onset of vasculature, some issues remain to be addressed.

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