Fgf21 is essential for haematopoiesis in zebrafish

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Fibroblast growth factors (Fgfs) function as key secreted signalling molecules in many developmental events. The zebrafish is a powerful model system for the investigation of embryonic vertebrate haematopoiesis. Although the effects of Fgf signalling on haematopoiesis *in vitro* have been reported, the functions of Fgf signalling in haematopoiesis *in vivo* remain to be explained. We identified *Fgf21* in zebrafish embryos. *Fgf21*-knockdown zebrafish embryos lacked erythroid and myeloid cells but not blood vessels and lymphoid cells. The knockdown embryos had haemangioblasts and haematopoietic stem cells. However, the knockdown embryos had significantly fewer myeloid and erythroid progenitor cells. In contrast, *Fgf21* had no significant effect on cell proliferation and apoptosis in the intermediate cell mass. These results indicate that *Fgf21* is a newly identified factor essential for the determination of myelo-erythroid progenitor cell fate *in vivo*.

Keywords: Fgf; haematopoiesis; erythroid cells; haematopoietic stem cells; zebrafish

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INTRODUCTION

Vertebrate haematopoietic cells are derived from self-renewing multipotential stem cells (Thisse & Zon, 2002). Haematopoiesis in vertebrates, from zebrafish to humans, is an evolutionarily conserved programme. The zebrafish is a powerful model system for the investigation of embryonic vertebrate haematopoiesis. In zebrafish embryos, primitive haematopoiesis occurs in the intermediate cell mass (ICM) and the anterior lateral mesoderm (ALM) and gives rise to progenitors that differentiate into embryonic blood cells. Definitive haematopoiesis occurs in the kidney marrow, which remains the principal site of this process throughout adulthood. Vascular cells and haematopoietic stem cells are derived from haemangioblasts that are derived from ventral mesoderm. Erythroid progenitor and lymphoid progenitor

cells are derived from haematopoietic stem cells. Myeloid progenitors are assumed to be derived from haematopoietic stem cells by analogy with amniote definitive haematopoiesis (Hsu *et al*, 2001; Lieschke *et al*, 2002; Davidson & Zon, 2004; Ransom *et al*, 2004).

Many key haematopoietic transcription factor genes have been identified (Davidson & Zon, 2004). Some key secreted signalling molecules in haematopoiesis have also been identified. Disruption of bone morphogenetic protein (Bmp) signalling leads to a reduction or a lack of ventral mesoderm, resulting in an absence of blood cells (Thisse & Zon, 2002). Hedgehog signalling is one of the mechanisms in the regulation of both vasculogenesis and haematopoiesis (Vokes *et al*, 2004; Detmer *et al*, 2005). Vascular endothelial growth factor (Vegf) is a crucial factor for vascular development. Vegf can stimulate endothelial cell differentiation and haematopoiesis (Liang *et al*, 2001). Fibroblast growth factors (Fgfs) also function as key secreted signalling molecules in many developmental events. The vertebrate Fgf family is large, comprising 22 members (Itoh & Ornitz, 2004). However, although the effects of Fgf signalling on haematopoiesis *in vitro* have been reported (Moroni *et al*, 2002), the functions of Fgf signalling in haematopoiesis *in vivo* remain to be explained.

The zebrafish animal model offers a unique opportunity to discover and study novel genes required for the control of normal vertebrate developmental events. Mouse *Fgf21* was expressed in the liver (Nishimura *et al*, 2000). However, roles of *Fgf21* in developmental events remain to be explained. We identified zebrafish *Fgf21* and inhibited *Fgf21* signalling in zebrafish embryos. *Fgf21*-knockdown zebrafish embryos lacked erythroid and myeloid cells but not blood vessels and lymphoid cells, indicating that *Fgf21* is essential for the determination of myelo-erythroid progenitor cell fate.

RESULTS AND DISCUSSION

We identified zebrafish *Fgf* genes by using the basic local alignment search tool (BLAST) to search for the zebrafish genomic sequences and expressed sequenced tag sequences with the amino-acid sequences of human FGFs. Then, we isolated their full-length complementary DNAs from zebrafish embryo cDNA. One of the cDNAs encodes a protein of 194 amino acids that is most homologous to human FGF21 among human FGFs. A sequence-based comparison of this protein with human FGFs by phylogenetic analysis also indicates that the protein is most

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Fig 1 | Molecular analysis of zebrafish *Fgf21*. (A) Phylogenetic tree comparing zebrafish *Fgf21* (zFgf21) with human FGFs (hFGFs). The apparent evolutionary relationships of zFgf21 and members of the hFGF family were examined using Clustal W. (B) Syntenic relationship between zebrafish chromosome 3 and human chromosome 19. Both zFgf21 and hFGF21 genes are closely linked to the zebrafish *dhfrs10* and *ca11* and human *DHRS10* and *CA11* genes, respectively. Mb, megabase.

closely related to human FGF21 (Fig 1A). However, although human FGF21 and mouse *Fgf21* are highly similar (~75% amino-acid identity; Nishimura *et al*, 2000), this zebrafish protein is less similar (~34% amino-acid identity) to human FGF21 (data not shown). Human *FGF21* is closely associated with carbonic anhydrase XI (*CA11*) and dehydrogenase/reductase member 10 (*DHRS10*) on chromosome 19 at q13.33 (Fig 1B). Therefore, we examined the location of this gene in the zebrafish genome. This gene was also closely associated with *ca11* and *dhfrs10* on chromosome 3 (Fig 1B). These results indicate that this gene is zebrafish *Fgf21* (accession number AB209932).

Injections of antisense morpholino oligonucleotides (MOs) have been demonstrated to inhibit the corresponding gene functions in zebrafish embryos efficiently and specifically (Nasevicius & Ekker, 2000). We injected *Fgf21* antisense and five-base-mismatched *Fgf21* antisense (control) MOs (10 ng per embryo) into zebrafish two-cell embryos. Control MO-injected embryos at 36 h post-fertilization (hpf) developed as well as did wild-type embryos (Fig 2Aa,b). In contrast, *Fgf21* MO-injected embryos developed abnormally, showing bent trunks (Fig 2Ac). Control MO embryos as well as wild-type embryos at 30 hpf had erythroid cells detected by α -dianisidine staining ($n=39/40$, 98%; Fig 2Ad,e).

However, *Fgf21* MO embryos had essentially no erythroid cells ($n=45/68$, 66%; Fig 2Af). We prepared capped zebrafish *Fgf21* RNA including the entire coding region but not the 5' non-coding region. As the *Fgf21* MO sequence (25 bases) includes 13 bases of the 5' non-coding region, the translation of the *Fgf21* RNA should be unaffected by the *Fgf21* MO. We examined whether the *Fgf21* RNA could rescue the phenotype of *Fgf21* MO embryos. The loss of erythroid cells in *Fgf21* MO embryos was definitely rescued by injection of the *Fgf21* RNA (10 pg per embryo; $n=27/32$, 84%; Fig 2Ag). In addition, we injected splice-site-targeted *Fgf21* MO (E12), the sequence (25 bases) of which corresponds to that between the second exon and intron of the coding region, into two-cell embryos (Fig 2B). *Fgf21* MO (E12) embryos also had essentially no erythroid cells ($n=19/30$, 63%; Fig 2Ah). To determine the expression of *Fgf21* in embryos, RNA was isolated from wild-type and *Fgf21* MO (E12) embryos at 24 hpf. *Fgf21* cDNA was amplified from the RNA by reverse transcription-PCR (RT-PCR; Fig 2B,C). The expression of *Fgf21* in *Fgf21* MO (E12) embryos was significantly decreased in comparison with that in wild-type embryos (Fig 2C). Furthermore, we also examined the effect of the Fgf receptor inhibitor SU5402 (Mohammadi *et al*, 1997) on erythroid cells. Embryos were treated with SU5402 during 11–13 hpf. The SU5402-treated embryos lacked erythroid cells, in a dose-dependent manner ($n=6/14$, 43% at 10 μ M; $n=66/70$, 94% at 100 μ M; Fig 2Ai). These results indicate that *Fgf21* is required for erythropoiesis.

Angiogenesis in the embryonic tail and trunk produces functional intersegmental vessels. With microangiography using fluorescein isothiocyanate-dextran (Weinstein *et al*, 1995), we visualized the functional vascular system in zebrafish embryos at 36 hpf. The blood vessels in the trunk and tail were generated in *Fgf21* MO embryos (Fig 2Aj,k). *flk1* is a marker gene for endothelial cells of the blood vessels (Thisse & Zon, 2002). The expression of *flk1* was observed in the blood vessels of wild-type embryos at 24 hpf. The expression was also observed in the blood vessels of *Fgf21* MO embryos (Fig 2Al,m). These results indicate that *Fgf21* is not essential for vasculogenesis.

The temporal expression of *Fgf21* during zebrafish embryonic development was first examined by RT-PCR (Fig 3A). The expression of *Fgf21* was weakly detected at 14 hpf and subsequently increased at least until 48 hpf. We examined the expression of *Fgf21* in zebrafish embryos during 12–48 hpf by whole-mount *in situ* hybridization using a digoxigenin-labelled antisense *Fgf21* probe. The definite expression of *Fgf21* was not detected in embryos at 12 and 14 hpf (data not shown). The expression of *Fgf21* was detected in the trunk adjacent to the ICM at 24, 36 and 48 hpf (Fig 3Ba–c). To determine the *Fgf21* expression region, the transverse sections of the trunk at 24 hpf were examined. *Fgf21* was expressed in the notochord adjacent to the ICM, where *gata1* was abundantly expressed (Fig 3Bd,e). However, some notochordless mutants (*ntl*, *flh* and *boz*) do not seem to have an obvious erythroid defect (Chin *et al*, 2000); therefore, the roles of *Fgf21* expressed in the notochord remain to be explained.

mpo and *I-plastin* are marker genes for heterophil granulocytes and macrophages, respectively (Fig 4A; Crowhurst *et al*, 2002). The expression of *mpo* and *I-plastin* was greatly decreased in the ICM of *Fgf21* MO embryos (Fig 4Ba–d). *rag1* is a marker gene for lymphoid cells (Davidson & Zon, 2004). The expression was essentially unchanged in the thymus of *Fgf21* MO embryos at

Fig 2 | Morphology and gene expression in *Fgf21* morpholino oligonucleotide embryos. (A) Two-cell embryos were injected with five-base-mismatched *Fgf21* morpholino oligonucleotide (MO; control) (b) and *Fgf21* MO (c) (~10 ng). The morphology of the embryos was observed at 36 hpf. Control MO embryos (b) as well as the wild-type (WT) embryos (a) developed well. In contrast, *Fgf21* MO embryos showed bent trunks (c). To visualize erythroid cells, the embryos were processed for *o*-dianisidine staining. Erythroid cells were detected in wild-type and control MO embryos (d,e). However, *Fgf21* MO embryos lacked erythroid cells (f). The loss of erythroid cells in *Fgf21* MO embryos was definitely rescued by the injection of *Fgf21* RNA (10 pg per embryo) (g). Splice-site-targeted *Fgf21* MO (E212) and SU5402-treated embryos also had essentially no erythroid cells (h,i). With microangiography using fluorescein isothiocyanate-dextran, the functional vascular system in wild-type embryos at 36 hpf was visualized (j). Blood vessels were also observed in the *Fgf21* MO embryos (k). The expression of *flk1*, a marker gene for endothelial cells, was observed in the blood vessels of wild-type embryos at 24 hpf (l). The expression was also observed in the blood vessels of *Fgf21* MO embryos (m). (B) The coding region of *Fgf21* was divided by three introns. Four blank boxes and three solid lines indicate four exons and three introns, respectively. The splice-site target is shown as E212. P1 and P4 indicate sites of P1 and P4 reverse transcription-PCR (RT-PCR) primers. (C) Zebrafish *Fgf21* complementary DNAs from wild-type and *Fgf21* MO (E212) embryos were amplified by RT-PCR using P1 and P4 primers. The cDNAs were analysed by 1.5% agarose gel electrophoresis. After electrophoresis, the gel was stained with ethidium bromide. The lower panel shows the results for zebrafish *elongation factor 1-α* (*ef1α*) gene expression as a control.

4 days post-fertilization (dpf; Fig 4Be,f). These results indicate that *Fgf21* is crucial for myelopoiesis but not lymphopoiesis.

Distinct types of blood cells are derived from haematopoietic stem cells as a result of a highly conserved gene programme (Thisse & Zon, 2002). Haematopoietic stem cells are derived from haemangioblasts. *scl* and *gata2* are marker genes for haemangioblasts and haematopoietic stem cells, respectively (Fig 4A). The expression of *scl* and *gata2* was observed in the ICM of wild-type embryos at 12 and 18 hpf, respectively (Fig 4Bg,i). Their expression was also observed in *Fgf21* MO embryos, indicating that *Fgf21* is not required for haemangioblasts and haematopoietic stem cells (Fig 4Bh,j). Erythroid progenitors are derived from haematopoietic stem cells. Myeloid progenitors are assumed to be derived from haematopoietic stem cells by analogy with amniote definitive haematopoiesis. Erythroid and myeloid cells are derived from myeloid and erythroid progenitors (Hsu et al, 2001; Lieschke et al, 2002; Davidson & Zon, 2004; Ransom et al, 2004). The interplay of *pu.1* and *gata1* determines the myelo-erythroid progenitor cell fate in zebrafish. *gata1* and *pu.1* determine whether myelo-erythroid progenitors become erythroid and

myeloid cells, respectively (Fig 4A; Galloway et al, 2005; Rhodes et al, 2005). The expression of *gata1* was greatly reduced in the ICM of *Fgf21* MO embryos at 13 and 24 hpf (Fig 4Bk-n). The expression of *pu.1* was also greatly reduced in the ICM of *Fgf21* MO embryos at 24 hpf (Fig 4Bo,p). These results indicate that *Fgf21* is required for the induction of myelo-erythroid progenitor cells. However, as we did not examine the expression of *pu.1* in the anterior ventral mesoderm at 13–14 hpf, where most of the *pu.1*-positive cells were born (Lieschke et al, 2002), we could not eliminate the possibility that the migration of *pu.1*-positive cells around the embryo was inhibited. We also examined the expressions of *runx1* and *c-myb*, marker genes for putative definitive progenitors, which were expressed in the dorsal aorta at 48 hpf (Davidson & Zon, 2004). Their levels were essentially unchanged in the dorsal aorta of *Fgf21* MO embryos (data not shown).

Proliferating cells, phosphohistone H3 (H3P)-positive cells, were examined in the ICM of zebrafish embryos by immunohistochemistry using an anti-H3P antibody. H3P-positive cells were detectable in the ICM of wild-type embryos at 13 and 24 hpf (Fig 5A,C). The rates of H3P-positive cells in the ICM were not significantly affected by the injection of *Fgf21* MO (Fig 5B,D–F). We also examined apoptosis in the ICM of wild-type and *Fgf21* MO embryos at 13 and 24 hpf by TdT-mediated dUTP nick end labelling (TUNEL) assay. A few and essentially no apoptotic cells were detectable in the ICM of embryo at 13 and 24 hpf, respectively (Fig 5G,I). The rates of apoptotic cells in the ICM were not significantly affected by injection of *Fgf21* MO (Fig 5H,J). In contrast, apoptotic cells were abundantly detectable in the

Fig 3 | The expression of *Fgf21* during zebrafish embryonic development. (A) Amplification of zebrafish *Fgf21* complementary DNA by reverse transcription-PCR from zebrafish embryonic cDNA at the indicated stages. The lower panel shows results for zebrafish *ef1α* gene expression as a control. (B) The expression of *Fgf21* in the trunk at 24, 36 and 48 hpf was examined by whole-mount *in situ* hybridization using a digoxigenin-labelled antisense *Fgf21* probe (a–c). The expression of *Fgf21* and *gata1* was examined using transverse sections of the trunk at 24 hpf (d,e). A bar indicates the position of the transverse section (a). An arrowhead indicates the notochord where *Fgf21* was expressed (d). *gata1* was expressed in the intermediate cell mass (d).

head region of *Fgf19* MO embryos at 16 hpf as a positive control (data not shown; Miyake *et al*, 2005). These findings indicate that *Fgf21* is not essential for cell proliferation and apoptosis in the ICM.

Four Fgf receptor genes (*Fgfr1–Fgfr4*) have been identified in zebrafish, and their expression profiles were compared in embryos (Tonou-Fujimori *et al*, 2002). No obvious erythroid defect was reported in *Fgfr1*-knockdown embryos (Scholpp *et al*, 2004). However, as the receptor specificity of *Fgf21* is not known, it remains to be explained which Fgf receptor mediates the activity of *Fgf21* in haematopoiesis.

Some key secreted signalling molecules have crucial roles in haematopoiesis. Bmp is required for ventral mesoderm, a lack of which results in an absence of blood cells (Thisse & Zon, 2002). Hedgehog signalling is crucial for both vasculogenesis and haematopoiesis (Vokes *et al*, 2004; Detmer *et al*, 2005). Vegf can stimulate endothelial cell differentiation and haematopoiesis (Liang *et al*, 2001). In contrast, *Fgf21* is essential for myelo-erythroid progenitor cell fates. *Fgf21* is a secreted signalling molecule that has a unique role in haematopoiesis. These findings indicate for the first time that Fgf signalling functions in developmental haematopoietic events *in vivo*.

Fig 4 | The expression of marker genes for blood cells in wild-type and *Fgf21* morpholino oligonucleotide embryos. (A) Schematic representation of haematopoietic differentiation in zebrafish embryos. Genes involved in the haematopoietic differentiation are indicated. (B) The expression of marker genes for blood cells was examined by whole-mount *in situ* hybridization using digoxigenin-labelled antisense complementary RNA probes: *mpo* for heterophil granulocytes; *l-plastin* for macrophages; *rag1* for lymphoid cells; *scl* for haemangioblasts; *gata2* for haematopoietic stem cells; *gata1* for erythroid progenitors; *pu.1* for myeloid progenitors.

METHODS

Fish maintenance. Zebrafish (*Danio rerio*) were maintained as described (Westerfield, 2000). Embryos were obtained by natural spawning and cultured at 28.5 °C in zebrafish Ringer's solution (Kimmel *et al*, 1995).

Isolation and characterization of zebrafish *Fgf21* cDNA. Using BLAST to search for zebrafish genomic DNA sequences with the amino-acid sequence of human *FGF21*, zebrafish *Fgf21* genomic DNA was identified. To determine the 5' non-coding region, zebrafish embryonic cDNA was analysed by the rapid amplification of cDNA ends method (Frohman *et al*, 1988). The full-length

Fig 5 | Cell proliferation and apoptosis in the intermediate cell mass. Proliferating cells, phosphohistone H3 (H3P)-positive cells, were examined in the intermediate cell mass (ICM) of wild-type and *Fgf21* morpholino oligonucleotide (MO) embryos at 13 and 24 hpf by immunohistochemistry using an anti-H3P antibody (A–D). The rates of H3P-positive cells in the ICM were quantitatively examined by counting H3P-positive and H3P-negative cells. The results are the means $\pm$ s.d. of three independent sections from three embryos (E,F). Apoptotic cells were examined in the ICM of wild-type and *Fgf21* MO embryos at 13 and 24 hpf by TdT-mediated dUTP nick end labelling assay (G–J).

cDNA was isolated by PCR with primers (5'-CTTAGTCAGTC ATGCTTTTGC-3', 5'-TGCTAATGTAAAGCATGTCTT-3') and the cDNA as a template. The apparent evolutionary relationships

of zebrafish *Fgf21* and members of the human FGF family were examined using Clustal W. The positions of zebrafish *Fgf21*, *dhrs10* and *ca11* on chromosome 3 were obtained from Ensembl Zebrafish Genome Browser. The positions of human *FGF21*, *DHRS10* and *CA11* on chromosome 19 were obtained from LocusLink.

Whole-mount *in situ* hybridization. Digoxigenin-labelled RNA probes were synthesized by *in vitro* transcription using T7 or SP6 RNA polymerase. A 0.59-kb *Fgf21* probe was synthesized using the full-length cDNA-containing plasmid. Other probes used were zebrafish *flk1* (AF487829), *scl* (AF045432), *gata2* (BC053131), *gata1* (U18311), *pu.1* (AF321099), *mpo* (AF34904), *l-plastin* (BC062381) and *rag1* (U71093). Whole-mount *in situ* hybridization was performed as described (Koshida *et al*, 1998). To detect *Fgf21* expression, whole-mount *in situ* hybridization was performed using the Tyramide Signal Amplification (TSA) system (Perkin Elmer).

***Fgf21* knockdown by *Fgf21* morpholino oligonucleotide.** MOs were synthesized by Gene-Tools, LLC (Corvallis, OR, USA). The sequences of the MOs used are as follows: *Fgf21* MO, the sequence (25 bases) of which corresponds to the 5' non-coding region (13 bases) and the coding region (12 bases), 5'-GGCA AAAAGCATGACTGACTAAGCT-3'; five-base-mismatched *Fgf21* (control) MO, 5'-GGaAAtAAGCATcACTGAgTAaCT-3'; splice-site-targeted *Fgf21* MO (E212), the sequence (25 bases) of which corresponds to that between the second exon and intron of the coding region, 5'-GCAGTGGTTGCTTACAATTAGATT-3'. Lowercase letters in the control *Fgf21* MO indicate mismatched bases in *Fgf21* MO. MOs were diluted in Danieau buffer (Nasevicius & Ekker, 2000). *Fgf21* MO (14 μ g/ μ l) and control *Fgf21* MO (14 μ g/ μ l) were injected at a volume of 0.70–0.80 nl into two-cell embryos. To see erythroid cells, the embryos were processed for α -dianisidine staining (Paffett-Lugassy & Zon, 2005). For whole-mount *in situ* hybridization, the embryos were fixed in 4% paraformaldehyde.

***Fgf21* morpholino rescue experiments with *Fgf21* RNA.** Capped zebrafish *Fgf21* sense RNA was synthesized using the mMESSAGE mMACHINE kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol from linearized pCS2+ plasmids containing the entire coding region of zebrafish *Fgf21* cDNA. For *Fgf21* morpholino rescue experiments, 10 pg of *Fgf21* sense RNA was injected separately, immediately after the injection of *Fgf21* MO into two-cell embryos. The embryos were processed for α -dianisidine staining (Paffett-Lugassy & Zon, 2005).

SU5402 treatments. SU5402 (Calbiochem, San Diego, CA, USA) was dissolved in dimethyl sulphoxide at a concentration of 20 mM. Embryos were incubated during 11–13 hpf in SU5402 diluted to a concentration of 10 or 100 μ M in zebrafish Ringer's solution.

Detection of proliferating and apoptotic cells. Proliferating cells were detected by immunohistochemistry using a rabbit polyclonal anti-phosphorylated histone H3 (H3P) antibody (Upstate Biotechnology, Lake Placid, NY, USA) as described previously (Maroon *et al*, 2002). Apoptotic cells were examined by TUNEL assay using the DeadEnd colorimetric detection kit (Promega, Madison, WI, USA) as described previously (Maroon *et al*, 2002).

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