Critical role of SPARC-matricellular protein in mediating erythroid progenitor cell development in zebrafish.

Rosa M. Ceinos^{1#}, Eva Torres^{1#}, Ruben Chamorro², Beatriz Novoa², Antonio Figueras²,
Neil M. Ruane³ & Josep Rotllant^{1*}

¹ Aquatic Molecular Pathobiology Lab. Instituto Investigaciones Marinas, CSIC. Vigo, Spain

² Immunology Lab. Instituto Investigaciones Marinas, CSIC. Vigo, Spain

³ Marine Institute, Rinnville, Oranmore, Co. Galway, Ireland.

These authors contribute equally to this work

* Corresponding Author. Tel. +351 986231930. E-mail: rotllant@iim.csic.es

Running title: Sparc in zebrafish haematopoiesis

Abstract

Sparc (Osteonectin) is a multifunctional matricellular glycoprotein expressed by many differentiated cells. Members of this family mediate cell-matrix interactions rather than acting as structural components of the extracellular matrix and therefore can influence many remodelling events, including haematopoiesis. We have investigated the role of *sparc* in embryonic haematopoiesis, using a morpholino antisense oligonucleotide-based knockdown approach. Knockdown of *sparc* function resulted in specific erythroid progenitor cell differentiation defects that were highlighted by changes in gene expression and morphology, which could be rescued by injection of *sparc* mRNA. Furthermore, a comparison of blood phenotypes of *sparc* and *fgfs* knockdowns with similar defects and the *sparc* rescue of *fgf21* blood phenotype places *sparc* downstream of *fgf21* in the genetic network regulating haematopoiesis in zebrafish. These results establish a role for an extracellular matrix protein (Sparc) as an important regulator of embryonic haematopoiesis during early development in zebrafish.

Keywords: *sparc*, Osteonectin, ECM, haematopoiesis, *gata 1*, *fgf21*, Zebrafish,

INTRODUCTION

Haematopoiesis is the biological process describing the formation and development of blood cellular components. Evolutionary comparisons have revealed that haematopoiesis is conserved within vertebrates, among whom the zebrafish *Danio rerio*, has been shown to be a valuable model organism for the study of haematopoiesis [Albacker et al., 2009].

From zebrafish to mammals, haematopoiesis occurs in two principal successive waves: the first (or primitive) wave supports the developing embryo while the second (or definitive) wave provides the organism with long term haematopoietic stem cells (HSC) to last its entire lifetime. In zebrafish, the primitive wave takes place between 12 and 24 hours post-fertilization (hpf), producing erythrocytes and myeloid cells. Primitive myelopoiesis takes place in the anterior lateral mesoderm (ALM), whereas primitive erythropoiesis occurs in the posterior lateral mesoderm (PLM) which later forms the intermediate cell mass (ICM). The definitive wave produces long term HSCs which will support the generation of all blood lineages. These differentiated lineages include not only erythroid and myeloid cells like the primitive wave, but also lymphocytes, thrombocytes and a larger variety of myeloid cells. In addition, it has been shown that the definitive wave may first generate committed erythromyeloid progenitors (EMPs) in the posterior blood island (PBI) between 1 and 2 dpf before HSCs arise [Bertrand et al., 2007]. Therefore, these erythromyeloid progenitors will serve as transient progenitors to initiate definitive haematopoiesis independently of HSCs and they represent a transient wave between primitive and definitive haematopoiesis.

Haematopoiesis is a complex developmental process controlled by a large number of factors that regulate stem cell renewal, lineage commitment and

differentiation. These regulatory molecules include hematopoietic growth factors (HGFs), hedgehog signalling molecules [Dyer et al., 2001], vascular endothelial growth factors (Vegfs) [Liang *et al.*, 2001], fibroblast growth factors (Fgfs) [Songhet et al., 2007; Yamauchi *et al.*, 2006] and bone morphometric proteins (BMPs) [Thisse & Zon, 2002] amongst others.

Furthermore, it is now known that activities of Vegf-A [Nozaki et al., 2006], Fgfs [Taiple et al., 1997; Whitehead et al., 2005] and other regulatory molecules are influenced by the interaction of cells with the ECM (extra cellular matrix). Matricellular proteins regulate cell-ECM communication and therefore can influence many remodelling events, including haematopoiesis. A recent study on morpholino antisense oligonucleotide (MO)-based functional screen in zebrafish showed a potential hematopoietic function of 14 genes [Eckfeldt et al., 2005]. Sparc, an extracellular matrix protein, also termed osteonectin was among them. Sparc is a multifunctional protein that modulates cell-matrix interaction and cell function, but does not seem to have a direct structural role in the matrix [Brekken & Sage, 2001]. Sparc is an evolutionary conserved matricellular protein [Laize et al., 2005; Rollant et al., 2008]. Within all vertebrates, *sparc* is expressed in a temporally and spatially specific manner with strong expression during embryogenesis in developing tissue such as the notochord, somites and embryonic skeleton [Holland *et al.*, 1987; Renn et al., 2006; Rollant et al., 2008;] and a marked reduction in *sparc* expression occurs once adulthood is reached. However, it re-emerges in response to tissue injury, remodelling and inflammation [Bornstein & Sage, 2002]. Therefore, its dynamic expression patterns during embryogenesis and its sequence homology with other vertebrates, suggests a conserved function of *sparc* in vertebrates [Rollant et al., 2008]. However, the precise function of *sparc*, in particular during early embryogenesis is largely unknown. Additionally, the apparent absence of

other *sparc* functional homologs in teleost fish compared with mammals [Rollant et al., 2008] may result in a greater understanding of the role of *sparc*, applicable to all vertebrates.

In the present study, it was demonstrated that zebrafish *sparc* plays a critical role in mediating erythroid progenitor cell development and additionally that *sparc* interacts with genes, in known genetic networks, thus further unveiling its novel function in the regulation of haematopoiesis.

RESULTS

Analysis of haematopoietic markers suggests a specific role of *sparc* in erythroid progenitor cell development.

To examine the spatial expression of zebrafish *sparc*, Whole-mount mRNA in situ hybridization was performed on 22, 25 and 35 hpf zebrafish embryos (Fig. 1). By 22 hpf, *sparc* transcripts were strongly expressed in the caudal fin fold, notochord, floor plate, somites and the PLM/ICM region (Fig. 1A). At 25 hpf, *sparc* messenger RNA was still detectable in the PLM/ICM region where *gata1* is strongly expressed. At 35 hpf, *sparc* was also expressed above the yolk extension at the AGM region (Fig. 1C). Embryos treated with control sense probes did not show any signal (data not shown).

To investigate the role of *sparc* during embryonic haematopoiesis, we took a loss-of-function approach. Two independent MOs, a translation blocker (ATG-MO) and a splicing blocker (E3I3-MO) were used to target *sparc*. A scrambled MO with no known target in zebrafish, *cMO*, was used as control as described previously [Rotllant et al., 2008]. The specificity and efficacy of the morpholinos were previously analyzed either by their ability to inhibit protein translation in an *in vitro* transcription-translation

assay or their efficacy at inhibiting transcription processing *in vivo* in 24hpf embryos [Rotllant et al., 2008]. To assess the functional duration of *sparc* inhibition *in vivo*, the test for disruption of *sparc* gene splicing by RT-PCR was performed. The splice junction morpholino (E3I3-MO targets the third coding exon-intron boundary (Figure. 1D). When injected into zebrafish embryos, the splicing morpholino induced the formation of a new transcript (401bp) due to the retention of the first 95 bp of intron 3 sequence (Fig. 1D). This leads to premature termination, producing a peptide that lacks the highly conserved C-terminal collagen and calcium binding domains [Rotllant et al., 2008]. Quantitative analysis showed that almost 100% of the *sparc* transcripts were incorrectly spliced in 1 nl, 1 mM E3I3-MO injected embryos up to 3 dpf, while at 5dpf almost all of the *sparc* transcripts were correctly spliced, compared with cMO injected or non-injected embryos (Fig. 1D).

Therefore, in this study, we injected *sparc* and scrambled control morpholinos into 1- to 2-cell stage embryos, and examined development for 3 days. Most injected embryos developed normally until 30 hpf, however they appeared smaller than control MO-injected embryos as previously shown by Rotllant et al, 2008. Eckfeldt *et al* (2005) used a similar ATG-MO against *sparc* in a morpholino antisense oligonucleotide (MO)-based functional screen in zebrafish to determine the haematopoietic function of 61 genes and they reported a reduced blood cell production identified by *gata1:DsRed* transgenic fish in more than 70% of *sparc*-MO injected embryos. Taken together, these results suggest a potential role of *sparc* in zebrafish haematopoiesis. The first 12 bases of their *sparc*-MO (5'ATCTTGAGTTTCAGCCTTCTGTCCG3') were identical to the last 12 bases of our independently designed ATG-*sparc*-MO (5'GATCCAAACCCTCATCTTGAGTTTC3') [Rotllant et al., 2008].

To better describe the effects of loss of *sparc* on zebrafish hematopoiesis, molecular markers that specify distinct stages of haematopoietic differentiation were analyzed.

The initial embryonic wave of blood production, the primitive wave, takes place in two locations, namely the ALM and the ICM. Primitive erythropoiesis takes place in the ICM, and primitive myelopoiesis in the ALM. It has been shown that the zinc-finger transcription factor *gata1* is crucial for primitive erythropoiesis and the myeloid – specific transcription factor *pu.1* for primitive myelopoiesis. Furthermore, *gata1* is co-expressed with *pu.1* in the ICM from 16 hpf to 24 hpf and it has been shown that the interplay of *pu.1* and *gata1* regulates the production of primitive erythroid and myeloid cells respectively [Rhodes et al., 2008]. Moreover, it has recently been shown that an additional transient wave exists between primitive and definitive haematopoiesis (24-48hpf) [Bertrand et al., 2007; Zon & Chen, 2009]. This wave is also known as the first wave of the definitive haematopoiesis, however it produces erythromyeloid progenitors (EMPs) that arise independently of HSCs and they exhibit an immature, blastic morphology and express only erythroid and myeloid genes.

Therefore, *gata1* and *pu.1* were used as gene markers for primitive and transient wave characterization. Additionally, other specific lineage markers like *βe3globin (Hbbe3)* for erythroid cells, *l-plastin (Lcp1)* for all myeloid cells, and *rag1* for lymphoid cells were also used.

As shown in Figure 2(A,C), *gata1* mRNA is expressed in the ICM at 19 and 24 hpf embryos, a crucial signalling centre for zebrafish primitive haematopoiesis [Berman et al., 2005] and in the PBI (posterior blood island) at 30 h (E) where the first hematopoietic progenitor cells with multilineage potential (EMPs) are found. The *gata1* gene expression level in these regions is markedly reduced in *sparc* morphant embryos

at 19, 24 and 30 hpf (Figs. 2 B,D,F). Unlike *gata 1*, the expression of *pu.1* a transcription factor that is necessary for myeloid progenitor cell development [Odenthal et al., 1996], was not significantly reduced in *sparc*-MO embryos at 19 and 30 hpf (Figs. 2 G-J). These results indicate that *sparc* is essential for mediating primitive and transient erythroid progenitor cell development but not the myeloid progenitor cells.

Blood defects identified were confirmed by ISH of the erythroid-specific *β3globin* (*hbee3*), the leukocyte specific *l-plastin* (*lcp1*) and the lymphoid-specific *rag1* in *sparc*-MO injected embryos compared to injected scrambled control morpholino embryos (Figs. 2K-P). A significant reduction of *β3globin* was observed in *sparc*-MO injected embryos (Fig.2 K,L). No significant differences in *l-plastin* and *rag-1* expression in *sparc* morphants were observed (Fig. 2 M-P). Thus, suggesting a critical role of *sparc* in erythropoiesis but not in myelopoiesis and lymphopoiesis. These results were also established by standard reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR; Fig. 2Q)

Following the hematopoietic conserved gene program [Davidson & Zon, 2004], definitive wave specific markers such as *c-myb* and *runx1* were used for *in situ* hybridization (ISH). The expression patterns for specific HSC markers *c-myb* and *runx1* revealed non-significant changes at 48h and 72h indicating that loss of *sparc* does not affect the emergence of haematopoietic stem cell (Fig. 3C-D; Fig 4A-D) and the proliferation and differentiation of haematopoietic stem cell (Fig.3E-F; Fig.4E-F). The slightly difference found in *c-myb* expression at 24h (Fig. 3A-B) is likely due to residual *c-myb* expression in primitive erythrocytes.

***Sparc* knockdown has no effect on angiogenesis**

To further confirm the *sparc* knockdown effect on haematopoiesis, we examined its effect on angiogenesis as both processes arise from a common precursor, the haemangioblast. Furthermore, we also examined vasculogenesis to rule out the possibility that the haematopoietic defect observed in the *sparc*-MO was secondary to a vascular defect.

We injected *sparc*-MO into *fli-EGFP* transgenic (Tg) zebrafish. Allowing for the general dysmorphic appearance of the embryo at 48 h, neither vasculogenesis nor angiogenesis were affected as shown by intact axial and intersegmental vessels (Supplementary data : Fig. 1A,B,C)

***Sparc* morphant zebrafish embryos do not show altered cell death but do show altered cell proliferation in posterior haematopoietic tissues.**

To examine *sparc*-deficient ICM cell proliferation and apoptosis, wild-type and *sparc*-deficient embryos were analyzed. Fluorescent immunohistologic analysis of 22 hpf larvae for BrdU incorporation revealed an increase in the number of ICM region BrdU positive cells in morphant samples when compared with controls (Fig. 5A,B,E). These results demonstrate that *sparc* could regulate cell proliferation in zebrafish, supporting the well characterized role of *sparc* as a modulator of cell proliferation in other organism [Brekken & Sage, 2001].

The frequency of apoptotic cells in the ICM region as measured by TUNEL reaction was low and was unaltered between *sparc*-MO and scrambled control-MO injected embryos (Fig. 5 C, D and E). However, apoptotic cells were significantly increased in the trunk region of *sparc*-MO at 22 h (Fig. 5D).

***Sparc* mRNA rescues defects caused by *sparc* MOs**

To verify the specificity of the defects produced by *sparc* knockdown, we rescued ATG-*sparc*-MO-injected embryos by co-injecting synthetic *sparc* mRNA [Rotllant et al., 2008]. Nearly complete rescue was achieved. The severe reduction in *gata1* ICM expression after *sparc* knockdown (Fig. 6 A,B) was rescued by co-injection of *sparc* mRNA (Fig. 6 D, E) although the body shape was still somewhat abnormal. The rescue success was dose dependent with 27% (13 from 48 embryos) of the co-injected embryos (0.5 mM ATG-*sparc*MO plus 320 µg/ml *sparc* mRNA) showing some *gata1* expression (Fig. 6D) compared to 69% (33 from 48 embryos) of the co-injected embryos (0.5 mM ATG-*sparc*MO plus 750 µg/ml *sparc* mRNA) showing *gata1* expression (Fig. 6E). These results demonstrate that exogenous *sparc* is sufficient to correct the blood defects caused by *sparc*MO, consistent with the interpretation that these haematopoietic defects are due to reduced levels of *sparc* protein function.

To provide further evidence that *sparc* morphant phenotypes are *Sparc*-specific and not due to non-specific off-target effects (e.g. *p53* mediated apoptosis), we also analyzed *gata-1* expression in *sparc* morphants that were coinjected with a *p53* morpholino [Robu et al., 2007]. *Gata1* mRNA expression was dramatically reduced in embryos coinjected with *sparc* and *p53* morpholinos (Fig. 6C), with *p53* morpholino injected embryos exhibiting gene expression patterns similar to control embryos (data not shown), indicating that *sparc* protein is critical for *gata1* expression.

***Sparc* expression is dependent on Fgf signalling**

The regulation of *sparc* gene expression by members of the *Fgf* family of signalling molecules [Taiple & Keski-Oja, 1997; Whitehead et al., 2005] and the resemblance between *sparc* morphant blood phenotype and *fgf21* morphant blood phenotype (characterized by a severe disruption of erythroid progenitor cell development

[Yamauchi et al., 2006]) led us to investigate whether Fgf signalling may mediate the decrease of *sparc* activity. This in turn, acts by modulating the lineage-specific transcription factor *gata1* expression levels or activity. Injection of *fgf21* MO (10ng embryo) [Yamauchi et al., 2006] induced a significant reduction of *sparc* expression in nearly 80 % of the injected 24 h embryos (Fig. 7A,B). In addition, erythroid-specific *gata 1* and *β3globin* and the myeloid-specific *pu.1* and *l-plastin* were analyzed by Q-RT-PCR in *fgf21* morphant embryos. A significant reduction of *gata 1* and *β3globin* (*Hbbe3*) was seen on *fgf21*-MO injected embryos (Fig.7C). On the other hand, no significant differences in *pu.1* and *l-plastin* (*lcp1*) expression in *fgf21* morphants were observed (Fig.7C). These results demonstrate the similarity between *fgf21* morphant blood phenotype (Fig. 7C) and *sparc* morphant blood phenotype (Fig. 2Q), which are characterized by a severe disruption of erythroid specific cell makers. No differences in *sparc* gene expression were found when other fgf's family members (*fgf3* and *fgf8*) were knocked-down (data not shown).

***Sparc* rescues the haematopoietic defect induced by fgf21 knockdown**

The disruption of expression of *gata-1* and *β3globin* mRNA in *sparc* (Fig.2B,D,F,L,Q) and *fgf21* (Fig. 7C, 8B, 9B,) morphants together with the significant reduction of *sparc* in *fgf21* morphants (Fig.7B) raises the possibility that *sparc*'s effects on haematopoiesis may, in part, be due to perturbed *fgf21* signalling. Therefore, to test this possibility, we examined whether exogenous *sparc* can rescue *gata1* and *β3globin* deficiency in gene targeted *fgf21* zebrafish embryos. The severe reduction in *gata1* and *β3globin* ICM expression after *fgf21* knockdown (10ng per embryo) (Fig.8B and Fig. 9B) was partially rescued by co-injection of *sparc* mRNA (~0.75 ng) (Fig. 8C and 9C). The rescue success was 30.5 % (n=56) and 36.3 % (n=40) respectively, with an *fgf21*/MO efficiency

(the rate of embryos showing a decreased *gata1* or β 3globin levels in the injected embryos) of 78% and 67%, respectively. No rescue was achieved when a different mRNA were co-injected with *fgf21MO* (data not shown).

DISCUSSION

In this study, we examined the role of *sparc* during embryonic haematopoiesis in zebrafish. We have previously demonstrated that *sparc* is dynamically expressed in skeletal and non-skeletal tissues from early development to adulthood in zebrafish, suggesting a potentially wide range of action [Rotllant et al., 2008]. While its specific role remains elusive, the high degree of similarity of zebrafish Sparc protein to Sparc protein of other vertebrates [Laize et al., 2005] and analysis of conserved syntenies [Rotllant et al., 2008] suggests a strong evolutionary pressure to conserve this protein.

Injection of *sparc* morpholinos into one-cell embryos resulted in specific inner ear [Rotllant et al., 2008], cartilage [Rotllant et al., 2008] and blood defects [Eckfeldt et al., 2005], suggesting a role for *sparc* in zebrafish development and haematopoiesis. We extended these studies to investigate in more detail *sparc*'s function in zebrafish haematopoiesis.

Results demonstrated that *sparc* knockdown using antisense MOs significantly reduced embryonic haematopoiesis at the lineage-committed cellular level. In particular, genes associated with primitive and transient erythroid progenitor cell development (*gata 1* and *β 3globin*) were down-regulated in the *sparc* morphants. Conversely, genes associated with primitive and transient myeloid progenitor cell development and genes associated with definitive haematopoiesis were not deregulated. This suggests a critical role of *sparc* in mediating erythroid progenitor cell development probably modulating the lineage-specific transcription factor *gata 1* expression levels or activity. Specificity

of gene targeting was confirmed both *in vivo* by RT-PCR and *in vitro* by transcription-translation assay [Rotllant et al., 2008] as well as by successful *sparc* mRNA rescue (Fig. 6). We have also demonstrated that *sparc* knockdown had no effect on endothelial cell specification as shown by the intact vasculature in *fli-EGFP* transgenic (Tg) zebrafish embryos injected with *sparc*MO. Proliferating and apoptotic cells were also examined in the ICM of control and *sparc* morphant zebrafish embryos. The rates of apoptotic cells in the ICM were not affected by injection of *sparc*MO. In contrast, cell proliferation was increased in the ICM region of *sparc* morphant embryos. These results demonstrate that while *sparc* may not be essential for apoptosis, it could regulate cell proliferation, supporting the well characterized role of *sparc* as a modulator of cell proliferation [Brekken et al., 2001].

We have previously shown that temporal expression of *sparc* during zebrafish embryonic development is initially detected by 14 hpf and the expression subsequently increased and persisted [Rotllant et al., 2008]. Whole-mount mRNA *in situ* hybridization showed that by 22 and 25 hpf, *sparc* transcripts were strongly expressed in the notochord, fin fold and the PLM/ICM region where *gata1* is strongly expressed (Fig.1A,B). However, it has been shown that some notochordless mutants (*bozozok* (*boz*), *floating head* (*flh*) and *no tail* (*ntl*)) do not seem to have an apparent blood defect [Odenthal et al., 1996; Chin et al., 2000] consequently; the possible role of *sparc* in the notochord is unclear.

Our evidence suggests that *sparc* acts by modulating the lineage-specific transcription factor *gata 1* expression levels or activity. However, this assumption raises a puzzling question of how a matricellular protein can regulate expression of transcription factor genes. The role of *sparc* in cell-matrix interactions may hold the answer; *sparc* may mediate or trigger signal transduction pathways required for

activation or maintenance of target genes transcription. This concept could be explored by identifying extracellular signalling molecules that act upstream of these genes encoding for *gata1* and *sparc*.

It is known that members of the Fgf family of signalling molecules can regulate *sparc* gene expression [Whitehead et al., 2005; Brekken & Sage, 2001]. Furthermore, the expression of *gata1* transcription factor gene is regulated by Fgf signalling pathways [Songhet et al., 2007; Nakazawa et al., 2006], as altered *fgf* expression leads to perturbation of expression of this gene [Yamauchi et al., 2006]. The disruption of expression of *gata1* mRNA in *sparc* morphants raises the possibility that *sparc*'s effects on haematopoiesis may at least in part be due to perturbed *fgf* signalling. This hypothesis is supported by the fact that the *sparc* morphant blood phenotype is very similar to the *fgf21* morphant blood phenotype, which is characterized by a severe disruption of erythroid/myeloid progenitor cell development [Yamauchi et al., 2006]. Therefore, to test this hypothesis, we examined whether *sparc* gene expression was perturbed in *fgf21* morphants and if exogenous *sparc* could rescue *gata1* deficiency in gene targeted *fgf21* zebrafish embryos. We found that *sparc* expression was substantially reduced or missing in *fgf21* morphant embryos (Fig. 7B). Furthermore, injection of ~0.75 ng of synthetic *sparc* mRNA together with ~10ng *fgf21*-MO per embryo resulted in the partial rescue (30.5 %, n=56) of *gata1* expression in the ICM of *fgf21* morphant embryos (Fig. 8C). In addition, we also tested the *fgf21* gene expression in *sparc* morphants and found that *fgf21* mRNA expression was not altered (data not shown).

Our findings therefore suggest that *sparc*, at least in part, acting downstream of the *fgf21* signalling pathway, is critically required in mediating erythroid progenitor cell development in zebrafish.

In mammals, although it is well known that *Sparc* gene expression is regulated by members of the *Fgf* family and in turn the *fgf* pathway regulates primitive hematopoiesis by modulating *gata1* expression level and activity. Its function in hematopoiesis is not clear. Surprisingly, no mutations in *Sparc* have been identified in humans, although mouse deficient in *Sparc* have no severe developmental alterations including hematopoietic defects. It has been hypothesized that the presence of more *Sparc* functional homologs in mammals functionally compensates for the lack of *sparc* expression, possibly leading to mild defects in *sparc*-null mice. However, studies carried out in other organisms such as *C. elegans* and zebrafish, where there is less redundancy, reduction in *Sparc* produces much more significant defects. Consequently, our observations in zebrafish likely uncover the significant roles of *Sparc*.

In summary, our study shows that *Sparc* has a critical role in embryonic haematopoiesis during zebrafish early development. In this process, it functions as a modulator of lineage-specific transcription factors *gata1* expression levels or activity. Our results also suggest that *sparc*'s effects on erythroid progenitor cell development may at least, in part, be due to a perturbed *fgf* signalling. However, the detailed mechanism on how *sparc* affects the lineage-specific transcription factors *gata1* (potentially via *fgf* signalling) and its functional conservation in other vertebrates remains to be elucidated.

EXPERIMENTAL PROCEDURES

Animals

Zebrafish embryos were cultured as previously described [Westerfield, 2007] and staged according to [Kimmel et al., 1995]. Experiments were performed using standard wild type strain (AB, Zebrafish International Resource Center (ZIRC)). To inhibit embryo pigmentation, embryo medium was supplemented with 0.003% (w/v) 2-phenylthiourea [Westerfield, 2007]. Dechorionated embryos were collected for total RNA extraction and cell proliferation assays or fixed overnight at 4°C in 4% paraformaldehyde in 1XPBS, washed in PBS, and dehydrated through a methanol series and stored at -20°C in 100% methanol for *in situ* hybridization and TUNEL assay. Ethical approval (N011011) for all animal studies was obtained from the Institutional Animal Care and Use Committee of the IIM-CSIC Institute in accordance with the National Advisory Committee for Laboratory Animal Research Guidelines licensed by the Spanish Authority (1201/2005).

RNA isolation and Q-RT-PCR

Embryos at 19, 24 and 30 hpf were dechorionated and total RNA was extracted using Trizol reagent according to manufacturer's protocol (Invitrogen). cDNA was made from total RNA using superscript III (Invitrogen) according to manufacturer's recommendations. Primer sequences are given in Table 1. All expression levels were normalized to *actin* using the $2^{-\Delta\Delta T}$ method [Livak et al., 2001]. Quantitative real time PCR (qRT-PCR) reactions were performed using an AB 7300 Real-Time PCR System and SYBR green incorporation (Applied Biosystems). The PCR cycles for all primer sets were: denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15s and 60 °C for 1 min. All samples were done in triplicate and each condition was repeated 3 times.

Morpholino knockdown

Two independent MOs, a translation blocker (ATG-MO: 5'GATCCAAACCCTCATCTTGAGTTTC3') and a splicing blocker (E3I3-MO: 5'GAAAAATGAACTCACTCTCAGCAAT3'), were used to target *sparc* [Rotllant et al., 2008]. Additionally, *fgf21* (*fgf21*-MO) [Yamauchi et al., 2006] and/or *p53* (*p53*-MO) [Robu et al., 2007] specific antisense morpholino oligonucleotides were also used to target *fgf21* and *p53* genes respectively. A scrambled MO with no known target in zebrafish, *cMO*, 5'-CCTCTTACCTCAGTTACAATTTATA-3' was used as control. All antisense oligos were synthesized by GeneTools, LLC (Corvallis, OR). The MOs were resuspended in 1x Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂, 5 mM HEPES, pH 7.6) to a final concentration of 0.5 mM for (ATG *sparc*-MO) or 1mM for (splicing (E3I3-*sparc*-MO), 1 mM (*fgf21*-MO), 1mM (*p53*-MO) and 1mM (control-MO). Subsequently, ~1 nl was injected into one- or two-cell stage embryos. To determine morpholino functional duration, the splicing blocker (E3I3) morpholino was used. To test for disruption of splicing, RT-PCR was performed (primers: exon 1 forward, 5'GCTGAAACTCAAGATGAG-3'; exon 4 reverse, 5'-TCCAATCGGAGACTTCGAGCA-3'). Total RNA from 2 pools of 10 uninjected (WT) 1dpf embryos, 2 pools of 10 injected with 1nl of 1mM *cMO* 1dpf embryos and 2 pools of 10 1dpf, 2dpf, 3dpf and 5 dpf embryos injected with 1 nL of 1 mM E3I3-MO were collected, the cDNA transcribed following the above protocol.

***In situ* hybridization, mRNA synthesis and rescue**

Whole-mount in situ hybridization was performed by using digoxigenin-labeled antisense probes as previously described [Rotllant et al., 2008]. *Sparc* Antisense riboprobe was made from linearized partial length *Danio rerio sparac* cDNA containing a 3'UTR fragment (GenBank Accession number: BC071436) (primers: forward 5'

GATGAAGCCATTGAGGTCGT 3'; reverse 5' AATCCACCACCAAAGAGTGC 3')

Other antisense RNA probes used in this study were *gata1*, *cmyb* and *β3globin* [Gardiner et al., 2007]; *runx1* [Murayama et al., 2006]; pu.1 & *l-plastin* [Bennet et al., 2001]; *rag1* [Trede et al., 2008].

For *in vitro* mRNA synthesis, the pCS2+*-sparc* was linearized with EcoRI. Capped RNA was transcribed *in vitro* using the SP6 Message Machine Kit (Ambion). The PCS2⁺-*sparc* construct used in the rescue experiments includes a Kozak sequence upstream of the ATG, instead of the endogenous zebrafish sequence, resulting in five mismatches between the antisense sequences and the rescue mRNA. Thus, the capped mRNA rescue construct were not susceptible to the ATG-*sparc*-MO. Rescue mRNA was injected into one- or two-cell stage embryos either alone or in the presence of an antisense MO. For each rescue experiment, the amount of mRNA injected was titrated for the maximal dose which could be injected [Rotllant et al., 2008]. For *sparc* rescue experiments, approximately 1 nL of 0.5 mM (4μg/μL) ATG-*sparc*-MO or or 1 mM (8μg/μL) *E3l3*-MO was injected together with 1 nL of two different *sparc* RNA concentrations (325 μg/mL or 750 μg/mL) per embryo. Approximately 150 embryos were used. For the *fgf21* morphant phenotype rescue, approximately 1 nL of 1 mM (8μg/μL) of *fgf21*-MO was injected together with 1 nL of *sparc* RNA (750 μg/mL) per embryo (approximately 200 embryos were used).

Detection of apoptotic and proliferating cells

Cell proliferation was measured with the *in situ* Cell Proliferation Kit, FLUOS (Roche, Mannheim, Germany) as described previously [Flores et al., 2008]. Apoptotic cells were examined by TUNEL assay using the *in situ* cell death detection kit POD (Roche, Mannheim, Germany).

Statistics

Data are expressed as mean $\pm$ SEM (calculated by dividing the standard deviation by the square root of the number of replicate experiments). Comparisons between numerical data were evaluated by paired Student *t* tests. A *P* value less than 0.05 (*) was considered statistically significant.

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REFERENCES

Albacker, C.E., L. Zon (2009) Use of Zebrafish to Dissect Gene Programs Regulating Hematopoietic Stem Cells. VK Rajasekhar, MC Vemuri (eds.), *Regulatory Networks in Stem Cells, Stem Cell Biology and Regenerative Medicine*, 101 DOI 10.1007/978-1-60327-227-89.

Bennett C.M., J.P. Kanki, J. Rhodes , T.X. Liu, B.H. Paw M.W., Kieran, D.M. Langenau, A. Delahaye-Brown, L.I. Zon, M.D. Fleming, A.T. Look (2001) Myelopoiesis in the zebrafish, *Danio rerio*. *Blood* 98(3):643-51.

Berman JN, Kanki JP, Look AT. (2005) Zebrafish as a model for myelopoiesis during embryogenesis. *Experimental Hematology* 33:997–1006.

Bertrand JY, Kim AD, Violette EP, Stachura DL, Cisson JL, Traver D. (2007) Definitive hematopoiesis initiates through a committed erythromyeloid progenitor in the zebrafish embryo. *Development*. 134: 4147-4156.

Bornstein P, Sage EH. (2002) Matricellular proteins: extracellular modulators of cell function. *Curr. Opin. Cell Biol.* 14:608-616 .

Brekken RA, Sage EH. (2001) SPARC, a matricellular protein: at the crossroads of cell-matrix communication. *Matrix Biol.* 19(8):816-27.

Chin, AJ, Tsang M, Weinberg ES. (2000) Heart and gut chiralities are controlled independently from initial heart position in the developing zebrafish. *Dev Biol* 227: 403–421.

Davidson AJ, and Zon LI. (2004) The 'definitive' (and 'primitive') guide to zebrafish hematopoiesis. *Oncogene* 23: 7233–7246.

Dyer MA, Farrington SM, Mohn D, Munday JR and Baron MH. (2001) Indian hedgehog activates hematopoiesis and vasculogenesis and can respecify prospective neurectodermal cell fate in the mouse embryo. *Development* 128: 1717-1730.

Eckfeldt CE, Mendenhall EM, Flynn CM, Wang TF, Pickart MA, Grindle SM, Ekker SC, Verfaillie CM. (2005) Functional analysis of human hematopoietic stem cell gene expression using zebrafish. *PLoS Biol.* 3(8):e254.

Flores MV, Hall C, Davidson AJ, Singh PP, Mahagaonkar AA, Zon LI, Crosier K, Crosier PS. (2008) Intestinal Differentiation in Zebrafish Requires Cdx1b, a Functional Equivalent of Mammalian Cdx2. *Gastroenterology* 135:1665–1675.

Gardiner MR, Gongora MM, Grimmond SM, Perkins AC. (2007) A global role for zebrafish *klf4* in embryonic erythropoiesis. *Mech Dev.* 124(9-10):762-74.

Holland PW, Harper SJ, McVey JH, Hogan BL. (1987) In vivo expression of mRNA for the Ca⁺⁺-binding protein SPARC (osteonectin) revealed by in situ hybridization. *J. Cell Biol.* 105(1):473-82.

Kimmel CB, Ballard WW, Kimmel SR, Ullmann , Schilling, TF. (1995) Stages of embryonic development of the zebrafish. *Dev Dyn.* 203(3):253-310.

Laize V, Pombinho AR, Cancela ML. (2005) Characterization of *Sparus aurata* osteonectin cDNA and in silico analysis of protein conserved features: evidence for more than one osteonectin in Salmonidae. *Biochimie.* 87(5):411-20.

Liang D, Chang JR, Chin AJ, Smith A, Kelly C, Weinberg ES, Ge R. (2001) The role of vascular endothelial growth factor (VEGF) in vasculogenesis, angiogenesis, and hematopoiesis in zebrafish development. *Mech. Dev.* 108(1-2): 29-43.

Livak KJ, Schmittgen TD. (2001) Analysis of relative gene expression data using real-time quaitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408.

Murayama E, Kissa K, Zapata A, Mordelet E, Briolat V, Lin HF, Handin RI, Herbomel P. (2006) Tracing hematopoietic precursor migration to successive hematopoietic organs during zebrafish development. *Immunity* 25(6):963-975.

Nakazawa F, Nagai H, Shin M, Sheng G. (2006) Negative regulation of primitive hematopoiesis by the Fgf signaling pathway. *Blood* 108(10):3335-43.

Nozaki M, Sakurai E, Raisler BJ, Baffi JZ, Witta J, Ogura Y, Brekken RA, Sage EH, Ambati BK, Ambati J. (2006) Loss of SPARC-mediated VEGFR-1 suppression after

injury reveals a novel antiangiogenic activity of VEGF-A. *J. Clin. Invest.* 116(2): 422-429.

Odenthal J, Haffter P, Vogelsang E, Brand M, van Eeden FJ, Furutani-Seiki M, Granato M, Hammerschmidt M, Heisenberg CP, Jiang YJ, Kane DA, Kelsh RN, Mullins MC, Warga RM, Allende ML, Weinberg ES, Nüsslein-Volhard C. (1996) Mutations affecting the formation of the notochord in the zebrafish, *Danio rerio*. *Development* 123:103-15.

Renn J, Schaedel M, Volff JN, Goerlich R, Schartl M, Winkler C. (2006) Dynamic expression of *sparc* precedes formation of skeletal elements in the Medaka (*Oryzias latipes*). *Gene* 372:208-18.

Rhodes J, Hagen A, Hsu K, Deng M, Liu TX, Look AT, Kanki JP. (2005) Interplay of *pu.1* and *gata1* determines myelo-erythroid progenitor cell fate in zebrafish. *Dev Cell* 8: 97–108.

Robu ME, Larson JD, Nasevicius A, Beiraghi S, Brenner C, Farber SA, Ekker SC. (2007) p53 activation by knockdown technologies. *PLoS Genet* 3:e78.

Rotllant J, Liu D, Yan YL, Postlethwait JH, Westerfield M, Du SJ. (2008) *Sparc* functions in morphogenesis of pharyngeal skeleton and inner ear. *Matrix Biology* 27(6): 561-572.

Songhet P, Adzic D, Reibe S, Rohr B. (2007) Fgf1 is required for normal differentiation of erythrocytes in zebrafish primitive hematopoiesis. *Dev. Dyn.* 236(3): 633-643.

Taipale J, Keski-Oja J. (1997) Growth factors in the extracellular matrix. *FASEB J.* 11:51-59.

Thisse C, Zon LI. (2002) Organogenesis—heart and blood formation from the zebrafish point of view. *Science* 295: 457–462.

Trede NS, Ota T, Kawasaki H, Paw BH, Katz T, Demarest B, Hutchinson S, Zhou Y, Hersey C, Zapata A, Amemiya CT, Zon LI. (2008) Zebrafish mutants with disrupted early T-cell and thymus development identified in early pressure screen. *Dev Dyn.* 237(9):2575-84.

Westerfield, M. (2007) *The Zebrafish Book: A guide for the laboratory use of Zebrafish (Danio rerio). 5th Edition. Univ. of Oregon Press, Eugene.*

Whitehead GG, Makino S, Lien CL, Keating MT (2005) Fgf20 is essential for initiating zebrafish fin regeneration. *Science* 310(5756):1957-60.

Yamauchi, H., Hotta Y, Konishi M, Miyake A, Kawahara A, Itoh N (2006) Fgf21 is essential for haematopoiesis in zebrafish. *EMBO Rep.* 7(6): 649–654.

Zon L.I., T. Chen (2009) Zebrafish blood stem cells. *Journal of Cellular Biochemistry* 108:35-42.

LEGEND TO THE FIGURES

Figure 1. In situ hybridization showing the expression pattern of *sparc* in the trunk and tail region. (A) 22 hpf, (B) 25hpf, (C) 35hpf zebrafish embryos. (D) Efficacy and functional duration of *sparc* inhibition in vivo. Location of the splice blocker E3I3-MO at the exon 3/intron 3 junction. E3I3-MO blocks the splicing of *sparc* transcript. RT-PCR shows the defective splicing induced by the E3I3-MO. PCR results from non-injected and cMO injected embryos show a single band (306 bp) (lane 1 and 2). A single band (401bp) was also detected in 1dpf (lane 3), 2 dpf (lane 4) and 3 dpf (lane 5) 1mM E3I3-MO-injected embryos. At 5 dpf (lane 6) 1mM E3I3-MO-injected embryos two bands (306 bp and 401 bp) are detected. In lane 3, 4 and 5 the 401 bp band, which is the major PCR product, is a result of defective splicing from using a cryptic splice donor located 95 bp 3' of the normal exon3/intron 3 splice site in intron 3 as shown by DNA sequencing (data not shown). Abbreviations: PLM, posterior lateral mesoderm; ICM, intermediate cell mass; AGM, aorta-gonad-mesonephros; PBI, posterior blood island; n, notochord; ff, fin fold. (A,B,C) anterior to the left, dorsal to the top. Scale bars: 100 μ m.

Figure 2. *Sparc* is required for normal erythroid progenitor cell development in zebrafish embryos. Expression of haematopoietic markers in wild type (*Control-MO*) (A,C,E,G,I,K,M,O) and *sparc* morpholino (*Sparc-MO*) (B,D,F,H,J,L,N,P) injected embryos. Reduced embryonic *gata1* and β *E3globin* gene expression but not *pu.1*, *l-plastin* and *rag1* in *sparc* morphants. The expression was examined by whole-mount *in situ* hybridization (A-P), and confirmed by qRT-PCR of *gata1*, *pu.1*, β *E3 globin* (*hbbe3*) and *l-plastin* (*lcp1*) (Q). *Gata 1* for erythroid progenitors; *pu.1* for myeloid progenitors; *l-plastin* (*lcp1*) for late myelomonocytic lineages, β *e3globin* (*hbbe3*) for

erythrocytes and *rag1* for lymphoid cells. Abbreviations: ICM, Intermediate Cell Mass; PBI, posterior blood island; t, timus; e, eye. (A-P) lateral views, anterior to the left. Scale bars: 100 μ m (A-N), 150 μ m (O-P).

Figure 3. Knockdown of *sparc* does not affect genes associated with definitive haematopoiesis. Whole-mount mRNA *in situ* hybridization with *c-myb* antisense probe.. (A-F) lateral views, anterior to the left. (A-B) 22 hpf, (C-D) 48 hpf and (E,F) 72hpf. Abbreviations: PLM, posterior lateral mesoderm; ICM, intermediate cell mass; AGM, aorta-gonad-mesonephros; CHT, caudal haematopoietic tissue. Scale bars: 100 μ m.

Figure 4. Knockdown of *sparc* does not affect genes associated with definitive haematopoiesis. Whole-mount mRNA *in situ* hybridization with *runx1* antisense probe. (A-F) lateral views, anterior to the left. (A-B) 24 hpf, (C-D) 48 hpf and (E,F) 72hpf. Abbreviations: PLM, posterior lateral mesoderm; ICM, intermediate cell mass; AGM, aorta-gonad-mesonephros; CHT, caudal haematopoietic tissue. Scale bars: 100 μ m.

Figure 5. Proliferation and apoptosis in *sparc*-deficient 22 hpf embryos. (A–D) Lateral views (anterior to left) of trunk region of control-MO injected (A and C) and *Sparc*-MO injected embryos (B and D) processed for fluorescent labelling of BrdU incorporation (A and B) and TUNEL reaction (C and D). (E) Graph depicting a comparison of BrdU and TUNEL-positive cells in the Intermediate Cell Mass (ICM) of uninjected (n: 20) and morphant (n: 26) embryos. Numbers represent average counts of labelled ICM cells per embryo. Mean $\pm$ SEM, *P<0,05.

Figure 6. *Sparc* RNA can rescue the haematopoietic defect (*gata1* expression) in *sparc* morphants. Sense *sparc* mRNA 1 nl of 350 or 750 µg/ml was co-injected with 1-2 nl of 0.5 mM *Sparc*-MO and the embryos were fixed for *in situ* hybridization. (A) control-MO 24hpf injected embryos, (B) *sparc*-MO 24hpf injected embryos, (C) *sparc*-MO plus *p53*-MO (2 nl, 1mM) injected embryos, (D) *sparc*-MO plus *sparc* mRNA (0.325 ng) injected embryos and (E) *sparc*-MO plus *sparc* mRNA (0.750 ng) injected embryos. Whole-mount *in situ* hybridization analysis of *gata 1* expression (A-E). Knockdown of *sparc* causes haematopoietic developmental abnormalities independent of *p53* dependent apoptosis (C). Abbreviations: ICM, Intermediate Cell Mass. (A-E) lateral views, anterior to the left. Scale bars: 100 µm.

Figure 7. *Fgf21* morphants show altered *sparc*, *gata1* and β 3globin (*hbbe3*) expression. A-B: Early *Sparc* expression (19hpf) is decreased in *fgf21* morphants. *Sparc* expression was examined by whole-mount *in situ* hybridization. C: haematopoietic defects were quantified by qRT-PCR for the expression of *gata1*, *pu.1*, *β 3globin* (*hbbe3*) and *l-plastin* (*lcp1*) transcripts in *fgf21* MO-target embryos relative to un-injected 19hpf embryos. The figure shows the average fold change in expression calculated from 3 independent experiments, with samples (n: 10 pools of 5 embryos each) analyzed each time in triplicate. Samples were normalized to β -actin, and control set to 1. Data are expressed as mean $\pm$ SEM. Comparisons of numerical data were evaluated by paired Student t tests. *P<0.05. Abbreviations: ov, otic vesicle; n notochord; ff, fin fold. Scale bars: 100 µm.

Figure 8. *Sparc* RNA partially rescues *gata1* expression in *fgf21* morphant haematopoietic phenotypes. Sense *sparc* mRNA 1 nl 750 µg/ml was co-injected with 1

nl of 1mM *fgf21*-MO and the embryos were fixed for *in situ* hybridization. (A) control-MO 22hpf injected embryos, (B) *fgf21*-MO 22hpf injected embryos and (C) *fgf21*-MO plus *sparc* mRNA (0.750 ng). Whole-mount *in situ* hybridization analysis of *gatal* expression (A-C). Abbreviations: ICM, Intermediate Cell Mass. (A-E) lateral views, anterior to the left. Scale bars: 100 μ m.

Figure 9. *Sparc* RNA partially rescues β 3globin (*hbbe3*) expression in *fgf21* morphant haematopoietic phenotypes. Sense *sparc* mRNA 1 nl 750 μ g/ml was co-injected with 1 nl of 1mM *fgf21*-MO and the embryos were fixed for *in situ* hybridization. (A) control-MO 30hpf injected embryos, (B) *fgf21*-MO 30hpf injected embryos and (C) *fgf21*-MO plus *sparc* mRNA (0.750 ng). Whole-mount *in situ* hybridization analysis of *gatal* expression (A-C). Abbreviations: ICM, Intermediate Cell Mass. (A-E) lateral views, anterior to the left. Scale bars: 100 μ m.

Supplementary data

Figure 1. Phenotypes seen with wild type non-injected (control), injected control-morpholino (control-MO) and injected *sparc*-morpholino (*sparc*-MO) embryos at 48h using *flil*-EGFP transgenic fish (EGFP in vascular endothelial cells). The embryos do not display major vascular defects. Abbreviations: ISV, intersegmental vessels; AC, axial circulation. Scale bar: 500 μ m.

Control

sparc MO

Q

Fold increase (% control)

0 20 40 60 80 100 120 140

Gata 1

*

Pu.1

Hbbe3

*

Lop1

Control

sparc MO

Control

sparc MO

Control

sparc MO **E**

gata 1

Table 1. qRT-PCR primer sequences.

genes	forward primer sequence (5'-3')	reverse primer sequence (5'-3')
<i>Gata1</i>	TACTGCCACCCGTTGATG	ACTTGGCGAACTGGACTG
<i>Pu.1</i>	CAGAGCTACAAAGCGTGCAG	GCAGAAGGTCAAGCAGGAAC
<i>hbbe3</i> ($\beta e3$ globin)	TTTCCGGCTGTTAGCGGACT	TTGCCTTCTGAGGGCTGACA
<i>lcp1</i> (<i>l-plastin</i>)	CCTGACGGATGAAAAGAAGC	GTTTCAGGCGTATAATGGAG
<i>actin</i>	AGCACGGTATTGTGACTAACTG	TCGAACATGATCTGTGTCATC