Regulation of endoderm formation and left-right asymmetry by miR-92 during early zebrafish development

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SUMMARY
microRNAs (miRNAs) are a family of 21-23 nucleotide endogenous non-coding RNAs that post-transcriptionally regulate gene expression in a sequence-specific manner. Typically, miRNAs downregulate target genes by recognizing and recruiting protein complexes to 3’ UTRs, followed by translation repression or mRNA degradation. miR-92 is a well-studied oncogene in mammalian systems. Here, using zebrafish as a model system, we uncovered a novel tissue-inductive role for miR-92 during early vertebrate development. Overexpression resulted in reduced endoderm formation during gastrulation with consequent cardio and viscera bifida. By contrast, depletion of miR-92 increased endoderm formation, which led to abnormal Kupffer’s vesicle development and left-right patterning defects. Using target prediction algorithms and reporter constructs, we show that gata5 is a target of miR-92. Alteration of gata5 levels reciprocally mirrored the effects of gain and loss of function of miR-92. Moreover, genetic epistasis experiments showed that miR-92-mediated defects could be substantially suppressed by modulating gata5 levels. We propose that miR-92 is a critical regulator of endoderm formation and left-right asymmetry during early zebrafish development and provide the first evidence for a regulatory function for gata5 in the formation of Kupffer’s vesicle and left-right patterning.

KEY WORDS: Kupffer’s vesicle, gata5, Left-right asymmetry, miR-92 (miR92), Zebrafish

INTRODUCTION
microRNAs (miRNAs) are short non-coding RNAs that post-transcriptionally downregulate gene expression by pairing with complementary sequences in the 3’ UTR of target mRNAs (Bartel, 2004; He and Hannon, 2004; Kim et al., 2009). In animals, the pairings are usually imperfect and typically result in translation inhibition and/or mRNA destabilization (Chelulaeva and Filipowicz, 2009; Filipowicz et al., 2008). miRNAs play crucial roles in differentiation, cell specification, proliferation and the response to stress, but the exact function of many individual miRNAs remains to be elucidated (Ambros, 2004; Flynn et al., 2007; Flynn et al., 2009; Hatfield et al., 2005; Liu and Olson, 2010; Shkumatava et al., 2009; Takacs and Giraldez, 2010). In part, this is because of the imperfect pairing with target mRNAs, which complicates the identification of bona fide targets given the large number of possible pairing interactions. Nucleotides 2-7 at the 5’ end of miRNAs constitute the seed region, which plays a crucial role in target pairing (Lewis et al., 2003). However, the seed sequence is not the only determinant, as other nucleotides of the miRNA as well as the sequence context within the 3’ UTR also play important roles (Grimson et al., 2007; Li et al., 2008).

Previous work has shown that miR-92 functions as an oncogene (reviewed by Croce, 2009). In humans, two different miR-92 loci, MIR-92A and MIR-92B, are encoded in the polycistronic MIR-17-92 and MIR-106A-363 clusters, respectively (reviewed by Petrocca et al., 2008a). Amplification of the MIR-17-92 cluster is frequently observed in hematopoietic malignancies and other solid tumors (Ota et al., 2004; Petrocca et al., 2008b; Volinia et al., 2006). Also, both clusters are common insertion sites in multiple types of retroviral-induced murine leukemias (Cui et al., 2007; Joosten et al., 2002; Landais et al., 2007; Lund et al., 2002; Mikkers et al., 2002; Suzuki et al., 2002; Wang et al., 2006). In a mouse B-cell lymphoma model, enforced overexpression of the miR-17-92 cluster significantly accelerated disease onset and progression (He et al., 2005). Consistent with a role in cancer, transcriptional activation of the miR-17-92 cluster is directly regulated by c-Myc and E2F3 (O’Donnell et al., 2005; Sylvestre et al., 2007; Woods et al., 2007). Multiple downstream targets of the miR-17-92 cluster have been identified that contribute to its tumorigenic function, including E2F1 (Lazzerini Denchi and Helin, 2005; O’Donnell et al., 2005; Woods et al., 2007), p21/CDKN1A (Ivanovska et al., 2008; Petrocca et al., 2008b, Bim/BCL2LI1, Pten (Koralov et al., 2008; Petrocca et al., 2008b; Ventura et al., 2008; Xiao et al., 2008), TSPI (thrombospondin 1) and CTGF (connective tissue growth factor) (Dews et al., 2006). Control of these factors illustrates the important role that miRNAs can play in differentiated cells and during tumorigenesis. By contrast, the exact role of these and other miRNAs during development is less well understood (Fontana et al., 2007; Koralov et al., 2008; Takacs and Giraldez, 2010; Ventura et al., 2008; Xiao et al., 2008). Here, we focus on the significant issue of normal miRNA function in controlling differentiation and patterning during early vertebrate embryogenesis.

During early vertebrate development, the formation of embryonic germ layers and of body axes are the result of the complex integration and precise regulation of multiple signaling pathways (Hamada et al., 2002; Zorn and Wells, 2009). In zebrafish, Nodal signaling is both necessary and sufficient for mesendoderm specification (the common progenitors of both endoderm and mesoderm) (Rodaway et al., 1999; Schier et al., 1997). The transcription factor Gata5 (Evans et al., 1988) apparently acts directly downstream of Nodal signaling to specify endodermal fate via the activation of another transcription factor,
sox22 (casanova), which induces expression of the essential endodermal marker sox17 (Alexander et al., 1999; Warga and Nusslein-Volhard, 1999). Depletion of Gata5 in mutants and morphants impairs endoderm formation, whereas excess Gata5 causes an expansion of endoderm (Reiter et al., 1999; Reiter et al., 2001). Here, we reveal a central role for miR-92 as a regulator of endoderm specification via control of gata5. Further, we show that loss of miR-92 inhibits the formation and function of Kupffer’s vesicle, a transient organ equivalent to the embryonic node in annelids that plays a crucial role in establishing left-right asymmetry (Bakkers et al., 2009; Essner et al., 2002; Fliegauf et al., 2007; McGrath et al., 2003; Nonaka et al., 1998; Nonaka et al., 2005; Raya and Izpisua Belmonte, 2006; Tabin and Vogan, 2003; Tanaka et al., 2005). In contrast to the large number of studies that have examined a role for miR-92 in cancer, our results reveal a novel and essential role for miR-92 during early vertebrate development.

MATERIALS AND METHODS

Zebrafish lines and maintenance

Wild-type AB and sox17:gfp (Sakaguchi et al., 2006) lines of zebrafish (Danio rerio) were used. Embryos were grown at 28°C in egg water and staged by morphology (Kimmel et al., 1995) and age [hours post-fertilization (hpf)].

Microinjection

miRNAs were microinjected at the one-cell stage with miRNAs, morpholinos (MOs) or mRNA reporters. miR-92a, miR-92b and control (ctrl) miRNAs were prepared by annealing single-stranded RNAs [synthesized by Integrated DNA Technologies (IDT)]: miR-92a sense, 5'-UAUUGACACUUGCCCGCCUGUU-3'; miR-92a antisense, 5'-ACAGGGCGGAACAGGCGAAUUAUU-3'; miR-92b sense, 5'-UAUUGACACUUGCCCGCCUGCU-3'; miR-92b antisense, 5'-GGAGGGCGGAGGACAGGCGAAUUAUU-3'; ctrl mRNA sense, 5'-CUCUAGGUAACACUCCCGGUU-3'; and ctrl mRNA antisense, 5'-UUAGAGAUCAAUUGAGGACC-3'.

Annealing was performed by mixing equal amounts of sense and antisense strands, heating to 65°C for 5 minutes, and gradually cooling to room temperature. Annealed duplexes were aliquoted and stored at −80°C. Unless otherwise indicated, 0.5 ng of miR-92a and miR-92b were injected. Control miRNAs were injected at 1 ng per embryo. In Fig. 5G, 0.2 ng of miR-92a and miR-92b were used to avoid general toxicity caused by injection of large amounts of RNA. All MOs were obtained from Gene Tools: MO92a1, 5'-ACAGGGCGGAACAGGCGAAUUAUU-3'; MO92a2, 5'-CACAACACATGCATTCAATCCCAA-3'; MO92a2', 5'-CACAAGACATTGCGGCCGATCCCAA-3'; MO92b1, 5'-GGAGGGCGGAGGACAGGCGAAUUAU-3'; MO92b2, 5'-TGACAACACTGCAACACACTCCCAA-3'.

Unless otherwise indicated, 1 ng of MO92a1 and MO92b1 was injected per embryo and are jointly referred to as MO1; 3 ng of MO92a2, MO92a2' and MO92b2 were injected per embryo and are referred to jointly as MO2. Standard control MOs (GeneTools) were injected at 10 ng per embryo; 1 pmol of a gata5 translation blocker MO (5'-AAGATAAAGCCA-GGCTCGAATACAT-3') was injected per embryo (Holzinger and Evans, 2007). In vitro transcribed, capped GFP reporter mRNA and gata5 mRNAs were injected at 25 pg and 20 pg per embryo, respectively.

Dorsal forerunner cell (DFC) injections were performed as described (Amack and Yost, 2004). For delivery into DFCs, embryos were injected with 1 ng of a miR-92a and miR-92b mixture at 3 hpf.

Molecular cloning

The gata5 (NM_131235.2) 3’UTR was amplified by RT-PCR using forward 5’-CCACCGAATTCGATGCGAGCC-3’ and reverse 5’-GGAGGCTGAGGAAAAGCATATAATTCC-3’ primers. The resulting cDNA was cloned downstream of the GFP open reading frame in the pCS2+ vector (Rupp et al., 1994). Deletion of both MREs was created by reverse PCR (Coolidge and Patton, 1995) using the following primers: gata5 D1F, 5’-TCCACAAAATATGTTGGATG-3’; gata5 D1R, 5’-ACATCATAGATGCCCACAAATCAA-3’; gata5 D2F, 5’-GACC-CGCAGGCGTT-3’; and gata5 D2R, 5’-GGATACATACACACT-TGACAGATC-3’. All clones were verified by DNA sequencing.

In situ hybridization

Embryos were fixed in 4% paraformaldehyde (PFA) in 1× PBS. Digoxigenin-labeled RNA probes were synthesized using a Roche DIG RNA Labeling Kit. cDNA templates included foxa3 (Field et al., 2003), cmic2 (Yelon et al., 1999), sox20 (Amack and Yost, 2004), †nf (Schulte-Merker et al., 1994), †irx (Essner et al., 2005), fso1 (Neugebauer et al., 2009) and fga (Yamuchi et al., 2009). Whole-mount in situ hybridization was performed as described (Thiess and Thiess, 2008). Embryos were mounted in 100% glycerol and images were obtained using a Zeiss Axiohot camera. The number of sox17-expressing cells was manually counted over a large number of images, as indicated in the relevant figure legends.

Northern blotting

Total RNA from zebrafish embryos was separated on 12% polyacrylamide gels and electrophoretically transferred to positively charged nylon membranes. DNA oligonucleotides complementary to miR-92 were labeled with [γ-32P]ATP using StarFire Labeling Kits (IDT). Hybridizations were carried out in 7% SDS and 0.2 M NaPO4 (pH 7.2) for 16 hours followed by washes in 2× SSPE containing 0.1% SDS.

Quantitative PCR

Total RNA from embryos at 90% epiboly was isolated using TRI reagent (Molecular Research Center). cDNAs were prepared from 20 ng total RNA and quantitative PCR was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems) on an iCycler iQ Multicolor machine (Bio-Rad) using primers 5’-GGTTGTTGCGCAAGATGACG-3’ (forward) and 5’-CTCGTACAGTGGCCTGCCC-3’ (reverse), with annealing at 60°C.

Immunoblotting

Proteins were extracted from deyolked embryos at 1 day post-fertilization (dpf) in lysis buffer [25 mM HEPES (pH 7.5), 5 mM MgCl2, 300 mM NaCl, 1 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 10% glycerol, 1.0% Triton X-100, 1 mM PMSF]. Then, 20 μg total protein was separated on 10% SDS-PAGE gels and transferred to PVDF plus membranes (GE Osmonics). Rabbit polyclonal antibodies against GFP (Torrey Pines Biolabs) and α-tubulin (Abcam) were used at 1:1000 and 1:500 dilution, respectively. Anti-rabbit HRP-conjugated secondary antibodies (GE Healthcare) were then used for visualization with ECL reagents (Perkin Elmer). For quantification, GFP levels were normalized to α-tubulin control levels, after which the ratio of GFP in the presence versus absence of miR-92 was determined.

Immunohistochemistry

Embryos were fixed in 4% PFA in 1× PBS, permeabilized in 0.5% Triton X-100 in 1× PBS for 1 hour, then incubated in blocking buffer (5% donkey serum, 5 mg/ml BSA, 1% DMSO, 0.1% Tween 20 in 1× PBS) at room temperature for 2 hours. Mouse polyclonal primary antibodies against acetylated tubulin (Sigma) were diluted 1:800. Cy3-conjugated secondary antibodies against mouse IgG (Jackson Immunoresearch) were used at 1:100. Embryos were mounted in GVA mount (Invitrogen). Samples were imaged on a Zeiss LSM 510 META confocal microscope.

RESULTS

Overexpression of miR-92 results in partial visceral and cardia bifida

To characterize the function of miR-92 during early vertebrate development, we performed both gain- and loss-of-function experiments. miR-92 is among the earliest expressed miRNAs detected during zebrafish development following analysis by deep sequencing (C. Wei and J.G.P., unpublished). It localizes to the
developing gut, liver and heart by 2-3 dpf (Wienholds et al., 2005). Gain-of-function experiments were performed by injecting miR-92 into zebrafish embryos at the one-cell stage, followed by assessment of the effects on gut and liver tissues by in situ hybridization with the pan-endodermal marker forkhead box a3 (foxa3) (Reiter et al., 1999). In non-injected controls (NICs), foxa3 localized to the developing gut tube, liver and pancreas primordia at 50 hpf, with liver on the left and pancreas on the right-hand side of the midline (Fig. 1A,B). Strikingly, miR-92 injection caused over 70% of the embryos to display aberrant foxa3 localization, showing a bifurcated gut tube with duplication of liver primordia (Fig. 1A,B). A similar phenotype has been observed previously and is referred to as viscera bifida (Nair and Schilling, 2008). For the remaining embryos, 20% showed localization of foxa3 along the midline, indicating a lack of gut looping, and another 3% had undetectable levels of foxa3, suggesting possible defects in endodermal specification.

The developing heart is also enriched for miR-92 and its development depends indirectly on the proper establishment of endodermal fates (Wienholds et al., 2005; Reiter et al., 1999; Alexander et al., 1999; Kitkuchi et al., 2000; Schier et al., 1997). Thus, we also tested the effects of miR-92 overexpression on heart development by examining the localization of the cardiac marker cmcl2 (cardiac myosin light chain 2; myl7 – Zebrafish Information Network) (Reiter et al., 1999). In NICs, cmcl2 localized to cardiac primordia on the left side of the embryo at 30 hpf, as expected (Fig. 1C,D). By contrast, injection of miR-92 caused over 85% of the embryos to display bilateral cmcl2 expression, indicating a failure of heart fusion that is referred to as cardia bifida (Reiter et al., 1999; Reiter et al., 2001; Holtzinger and Evans, 2007; Stainier, 1996). The remaining 15% of embryos showed cmcl2 along the midline, indicating an inability of the heart tube to undergo normal looping. The specificity of these gain-of-function experiments was supported by an absence of changes in foxa3 or cmcl2 expression upon injection of unrelated and control miRNAs (Fig. 1B,D). Also, the effects of miR-92 were dose dependent (see Table S1 in the supplementary material).

**Overexpression of miR-92 results in a reduction of endoderm**

Viscera and cardia bifida result from the failure to coalesce the relevant mesendodermal organ progenitors at the midline during the segmentation stages and may be secondary to a variety of
earlier defects in endoderm or mesoderm formation (Alexander et al., 1999; Kikuchi et al., 2000; Nair and Schilling, 2008; Ober et al., 2004; Reiter et al., 1999; Reiter et al., 2001; Schier et al., 1997).

To address this issue, we examined the effects of miR-92 gain of function on the expression of genes that act early in the specification and determination of endoderm and mesoderm. No significant defects were detected in mesoderm formation with miR-92 injection, as indicated by the normal expression pattern of ntl (see Fig. S1I-P in the supplementary material). By contrast, miR-92 injection caused a dramatic decrease in the specification of early endoderm cells, as indicated by the decreased numbers of sox17- and sox32-expressing cells at the end of gastrulation (Fig. 1E-I and see Fig. S1A-H in the supplementary material). This suggests a selective impairment in the formation of endodermal cells with no effect on the formation of the mesodermal germ layer.

Depletion of miR-92 results in aberrant left-right patterning of internal organs

We next performed loss-of-function experiments using antisense morpholinos (MOs) to block miR-92 activity during early development (see Figs S2 and S3 in the supplementary material). In NICs, the localization of foxa3 to the developing liver was primarily on the left side at 50 hpf (Fig. 2A). However, injection of MOs against miR-92 resulted in 20-40% of the embryos displaying abnormal left-right localization of foxa3 in the developing liver. Similarly, 40% of the morphants showed localization of cmcl2 to either the right or the middle, as compared with normal left-sided heart patterning in NICs (Fig. 2B). Thus, loss of miR-92 resulted in a significant incidence of aberrant left-right patterning.

Several lines of evidence suggest that the observed left-right asymmetry defects are specific to depletion of miR-92. First, two independent MOs (MO1 and MO2) yielded identical results (Fig. 2A,B). Second, synergistic and similar effects were observed upon co-injection of both MOs at much lower doses than the individual injections. Third, the defects were dose dependent (see Table S2 in the supplementary material). Fourth, injection of control MO did not affect the left-right localization of either marker (Fig. 2A,B).

Depletion of miR-92 alters Kupffer’s vesicle function

Specification of left-right patterning is highly regulated and propagated through several stages of embryogenesis in a complex genetically controlled program (Bakkers et al., 2009; Hamada et
al., 2002; Raya and Izpisua Belmonte, 2006). In zebrafish, motile cilia in Kupffer’s vesicle (KV) appear to be crucial for left-right asymmetry, analogous to their counterparts in the node in amniotes (Amack et al., 2007; Amack and Yost, 2004). Evidence gathered from both zebrafish and mouse embryos suggests a strong connection between node/vesicle structures and proper left-right patterning, especially with regard to the flow-generating function of primary cilia (Amack et al., 2007; Amack and Yost, 2004; Kreiling et al., 2008; Marszalek et al., 1999; Murcia et al., 2000; Nonaka et al., 1998; Okada et al., 1999; Schneider et al., 2008; Supp et al., 1999; Takeda et al., 1999). We therefore tested for KV defects in the miR-92 morphants. To visualize KV, we used a sox17:gfp transgenic line to label KV cells with GFP. A substantial reduction in cell numbers within KV was observed in the miR-92 morphants (Fig. 2C-E,G). To evaluate further the structural deficits in KV function in the miR-92 morphants, we examined the number and length of monocilia within KV by immunohistochemistry with antibodies against acetylated tubulin (Essner et al., 2005). A significant decrease in cilia number and a modest, but significant, decrease in their length were observed in miR-92 morphants (Fig. 2G and see Fig. S4 in the supplementary material). These results suggest that miR-92 is required for the proper development of KV and cilia within KV.

To ensure that the KV defects were specific to the loss of miR-92, we performed rescue experiments in which exogenous miR-92 was expressed in dorsal forerunner cells (DFCs) (Amack and Yost, 2004). DFCs are the progenitor cells that form KV, and if loss of miR-92 from these cells is responsible for the observed defects, we should be able to rescue such defects by targeted miR-92 gain of function in the DFCs of morphant embryos. Indeed, specific restoration of miR-92 expression in DFCs was able to partially, but significantly, rescue KV size in morphant embryos (Fig. 2F,G). In addition, expression of miR-92 in DFCs also rescued left-right patterning defects when examined using the cmle2 marker (see Fig. S5 in the supplementary material). These results indicate that the observed KV defects are specific to miR-92 depletion in DFCs.

Depletion of miR-92 results in increased endoderm
Normal KV development from DFCs requires both endodermal and mesodermal signals, in addition to genes involved in general ciliogenesis (Alexander et al., 1999; Amack et al., 2007; Amack and Yost, 2004; Neugebauer et al., 2009; Oteiza et al., 2008). We therefore examined the expression and localization of markers involved in both general ciliogenesis and the formation of endoderm and mesoderm. No significant defects in general endoderm or mesoderm formation were detected, as indicated by markers such as ntl, dnah9, fgf8, and sox17, as well as the normal development of motile cilia in the pronephros and inner ear (see Figs S1 and S6 in the supplementary material). By contrast, loss of miR-92 caused a dramatic increase in the number of sox17-expressing endodermal cells and these cells were much more spread out at the end of gastrulation, indicating defects in endoderm formation (Fig. 2H-L). Consistent with increased cell number, there was an overall 1.6-fold increase in sox17 mRNAs in the miR-92 morphants, as detected by quantitative PCR. This increase was suppressed by co-injection of miR-92 (see Fig. S7 in the supplementary material). Taken together, these results suggest that miR-92 is required for proper endoderm specification and for the development of structures that are crucial for controlling fundamental aspects of organogenesis and overall body patterning.

gata5 is a target of miR-92
Online target prediction algorithms [TargetScan (http://www.targetscan.org/), MicroCosm Targets (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) and PicTar (http://www.pictar.org/j)] were used to search for potential targets of miR-92. The zinc-finger transcription factor gata-binding protein 5 (gata5) was identified as a potential target as it contains two potential miRNA recognition elements (MREs) in its 3’UTR (Fig. 3A). Gata5 is a critical regulator of vertebrate endoderm development, as overexpression of Gata5 increases endodermal cell numbers, whereas reduced Gata5 expression causes a reduction in endodermal cell numbers (Reiter et al., 1999; Reiter et al., 2001). Also, gata5 localizes to DFCs during early zebrafish development (data not shown). Hence, gata5 is a compelling miR-92 target during early zebrafish development.

To determine whether gata5 is a bona fide target of miR-92, we analyzed the interaction between the gata5 3’UTR and miR-92 using GFP reporter assays. The full-length gata5 3’UTR and a deletion construct lacking both MREs were cloned downstream of the GFP open reading frame. RNA transcripts from these constructs

3’UTR of gata5. Base pairing between miR-92 and two miRNA recognition elements (MREs) in its 3’UTR (Fig. 3A).
were then injected into single-cell zebrafish embryos in the presence or absence of miR-92. The following day, GFP expression levels were monitored by fluorescence microscopy and by western blotting with antibodies against GFP. In both assays, GFP levels were reduced by miR-92 co-injection, and this was dependent on the presence of intact miR-92 MREs (Fig. 3). Importantly, co-injection of miR-92 antisense MOs rescued expression of GFP in the presence of miR-92 (Fig. 3D,G-I). Injection of a control MO injection of miR-92 antisense MOs rescued expression of GFP in the presence of intact miR-92 MREs (Fig. 3). Importantly, co-blotting with antibodies against GFP. In both assays, GFP levels were monitored by fluorescence microscopy and by western blotting with antibodies against acetylated tubulin (red). Scale bars: 5 μm. (J) KV cell number was counted as above. Error bars represent s.e.m. *, P<0.01 between injected embryos and NIC; Student’s t-test. (H,I) Confocal z-stacks of KV in NIC and gata5-injected embryos at the 10 somite stage. KVs were labeled using a sox17:gfp (green) transgenic line as above. Motile cilia were labeled with antibodies against acetylated tubulin (red). Scale bars: 5 μm. (J) KV cell number was counted as above. Error bars represent s.e.m. *, P<0.01 between injected embryos and NIC; Student’s t-test.

Regulation of gata5 by miR-92 during early zebrafish development

The hypothesis that miR-92 targets gata5 is not only supported by the reporter assays but also by previous work that showed that altered levels of gata5 control endoderm formation, as monitored by changes in sox17-expressing cell numbers (Reiter et al., 1999; Reiter et al., 2001). Also, loss of gata5 has been shown to cause cardia and viscera bifida (Reiter et al., 1999; Reiter et al., 2001). However, no prior reports have implicated gata5 in KV defects or consequent left-right asymmetry defects. Thus, we sought to test directly whether miR-92 control of gata5 would induce KV defects and changes in body plan. If gata5 were regulated by miR-92 during early zebrafish development, then altered expression of Gata5 should reciprocally mirror the effects caused by gain and loss of miR-92, which would then enable powerful genetic epistasis experiments to verify the interaction between miR-92 and gata5.

First, we confirmed that knockdown of gata5 causes a reduction of sox17-expressing cells at the end of gastrulation and that injection of gata5 mRNA causes an increase in sox17-expressing cells (Fig. 4A-G) (Reiter et al., 1999; Reiter et al., 2001). Second, we confirmed that gata5 knockdown causes cardia and viscera bifida defects, as observed in gata5 mutants (faust) and gata5 morphants (Holtzinger and Evans, 2007; Reiter et al., 1999; Reiter et al., 2001) (Fig. 4K,L). After confirming that our experimental system recapitulated earlier work, we then tested the effects of excess Gata5 expression on organogenesis and left-right patterning. When we injected gata5 mRNA and determined the localization of foxa3 and cmc12, we found that 45% and 7% of embryos displayed altered left-right patterning of cardia and viscera primordia, respectively (Fig. 4K,L). These results show that the increase or reduction of endodermal cell numbers caused by raising or lowering Gata5 levels, together with the finding that increased and decreased levels of miR-92 have converse effects that can be offset by altered gata5 expression, support the idea that the miR-92-gata5 regulatory interaction is involved in allocating correct endodermal cell numbers and maintaining proper left-right patterning.

Because we discovered a link between miR-92 expression in DFCs and KV formation, it was important to determine whether the gata5-induced left-right asymmetry defects could be explained by defective KV formation. Using the sox17:gfp transgenic line that allows visualization of KV formation, we found a dramatic reduction in KV cell number after gata5 overexpression (Fig. 4H-J and see Fig. S4 in the supplementary material).
miR-92-mediated defects can be partially suppressed by modulation of Gata5

Genetic epistasis experiments were performed to test the hypothesis that miR-92 regulates gata5. If miR-92 acts as a negative regulator of gata5, co-injection of gata5 mRNA with miR-92 should suppress the miR-92 gain-of-function defects. Likewise, blocking gata5 function should suppress the miR-92 loss-of-function defects. As shown in Fig. 5, the reduction in the number of sox17-expressing cells caused by overexpression of miR-92 could be suppressed by co-injection of gata5 RNA. Similarly, co-injection of gata3 MOs with miR-92 MOs suppressed the increase in sox17-expressing cells (Fig. 5A-F).

For cardiac morphogenesis, we observed a partial, but significant, rescue of cmlc2 localization upon co-injection of miR-92 and gata5 RNAs that resulted in a reduction of cardia bifida from 40% to 6% (Fig. 5G). Similarly, blockage of gata5 function in miR-92 morphants resulted in a significant suppression (from 40% to 10%) of the left-right patterning defects (Fig. 5H). Thus, the effects of altered miR-92 levels can be partially suppressed by modulating gata5 levels. These results strongly support the hypothesis that miR-92 regulates endoderm formation and left-right asymmetry by controlling gata5 expression.

DISCUSSION

Our data suggest a model in which miR-92 acts as a critical regulator of early zebrafish development by precisely controlling gata5 expression. By repressing gata5 expression, excess miR-92 causes a reduction in endoderm during the blastula and gastrula stages and, at later stages, cardia/viscera bifida. By contrast, reduced levels of miR-92 cause increased endoderm formation, defects in KV development and, at later stages, abnormal left-right patterning of internal organs. We have uncovered a novel function for miR-92 in controlling endoderm formation and left-right asymmetry by modulating gata5.

Function of miR-92 during early development

The miR-92 family is among the earliest expressed miRNAs during zebrafish development, beginning at the mid-blastula stage. Spatially, miR-92 is expressed somewhat broadly during early development but with increased expression in DFCs and KV (data not shown). Only a limited number of miRNAs have been analyzed for their roles during these early stages when the germ layers develop and the body axes are patterned (Choi et al., 2007; Martello et al., 2007; Rosa et al., 2009). Here, we have shown a novel role for miR-92 in early zebrafish development in endoderm formation, KV function and left-right patterning via the control of gata5. To our knowledge, this is the first time that a miRNA has been identified as a regulator of endoderm formation, vertebrate left-right asymmetry and KV development.

Regulation of gata5 by miR-92

The precise regulation of Gata5 expression is not well understood. During zebrafish endoderm formation, gata5 is transcriptionally upregulated by phosphorylated Smad2 (Shivdasani, 2002). Hypermethylation of the GATA5 promoter region has been reported in various human cancer cell lines, concomitant with downregulation of GATA5 transcription (Akiyama et al., 2003; Guo et al., 2004; Guo et al., 2006; Hellebrekers et al., 2009). Our results suggest that gata5 is subject to post-transcriptional regulation by miR-92. Three lines of evidence support this hypothesis: (1) miR-92 silencing of GFP reporter expression is dependent on an intact gata5 3’UTR (Fig. 3); (2) the effects of gain and loss of function with gata5 are opposite to those observed with miR-92 (Fig. 4); and (3) miR-92-induced defects could be substantially suppressed by changing concentrations of gata5 in epistasis experiments (Fig. 5).

As a downstream effector of Nodal signaling and a regulator of endoderm specification, Gata5 needs to be precisely controlled, both temporally and spatially. In zebrafish, gata5 mRNA is found within five cell diameters of the blastoderm margin at the onset of gastrulation and overlaps with definitive endodermal cells during gastrulation (Reiter et al., 2001; Warga and Nusslein-Volhard, 1999). Excess expression or depletion of Gata5 alters endodermal cell numbers at the end of gastrulation (Reiter et al., 2001). We hypothesize that miR-92 contributes to the establishment of the proper gata5 expression patterns during the blastula and gastrula stages that guide the differentiation and allocation of proper endodermal cell numbers during gastrulation.

Interestingly, the MIR-17-92 cluster is upregulated in lung cancer cell lines, whereas GATA5 is downregulated (Guo et al., 2004; Volinia et al., 2006). It is tempting to hypothesize that both transcriptional regulation via DNA methylation and post-
transcriptional regulation via MIR-92 contribute to the silencing of GATA5 in lung cancer. It will be interesting to determine whether gata5 is a general target of miR-92 in other types of tumor.

**Misregulation of Gata5**

We observed a decrease in sox17-expressing cell numbers upon miR-92 overexpression or gata5 knockdown (Figs 1 and 4). We also observed both cardia and viscera bifida in these embryos. The mechanisms underlying cardia and viscera bifida are not fully understood. It will be interesting to elucidate how endoderm defects cause cardia/viscera bifida. An increase in endodermal formation was observed upon miR-92 knockdown or gata5 overexpression (Figs 2 and 4), but it remains unclear whether endodermal cell numbers increase at the expense of reduced mesoderm cell numbers. Endoderm and mesoderm share common progenitors; endodermal cells become specified from mesodermal cells as a result of stronger Nodal signaling (Schier et al., 1997; Warga and Nusslein-Volhard, 1999). Perhaps miR-92 regulation of gata5 affects endoderm versus mesoderm fate decisions or reflects control of overall cell numbers within the endodermal lineage.

Examining the localization of cmlc2 and foxa3 surprisingly revealed left-right patterning defects in internal organs upon loss of miR-92 or overexpression of gata5. This is the first evidence, to our knowledge, for a regulatory function for gata5 in left-right asymmetry and also the first data to demonstrate defects in viscera morphogenesis upon gata5 overexpression. Previously, overexpression of gata5 was found to cause the expansion and ectopic development of cardiac tissue (Holtzinger and Evans, 2007; Reiter et al., 1999). Here, we did not observe significant expansion of cmlc2 expression or ectopic localization, except for on the right-hand side or midline in both the gata5 gain-of-function and miR-92 loss-of-function experiments. A possible explanation for these differences could be in the use of different dosages for gata5 RNA injection in the two studies.

The exact causes of the observed KV defects upon altered miR-92 and gata5 levels remain to be determined. Development of KV from DFCs is a complex procedure that is not fully understood (Oteiza et al., 2008). Both cell-autonomous and non-autonomous signals contribute to the induction, migration, proliferation and polarization of DFCs (Amack and Yost, 2004; Choi et al., 2007; Oteiza et al., 2008). We observed impaired KV development upon miR-92 loss of function that could be suppressed by restoration of miR-92 expression specifically within DFCs of miR-92 morphants. This suggests that the requirement for miR-92 during KV development is cell-autonomous, although the involvement of other signaling pathways or mechanisms cannot be excluded. Previously, diminished KVs were found in embryos with decreased endodermal cell numbers (Alexander et al., 1999). Here, both the miR-92 loss-of-function and gata5 gain-of-function experiments resulted in an increase in endodermal cell numbers and smaller KVs, but it remains unclear at what stage of development the KV defects arise.

**miR-92 targets**

One explanation for the partial suppression of miR-92 defects in epistasis experiments with gata5 is that gata5 is not the only target of miR-92. Other genes important for endoderm formation and left-right asymmetry might also be subject to regulation by miR-92. Many genes involved in cell cycle control, proliferation or apoptosis are predicted to be targets of miR-92 by online target prediction algorithms. During normal gastrulation movements, endodermal cells migrate dorsally in a characteristic pattern. Besides an increase in endodermal cell number, depletion of miR-92 caused endodermal cells to spread out more during migration, which might indicate additional defects in such as cell adhesion. This phenotype was not observed in gata5-overexpressing embryos, suggesting that miR-92 might regulate genes involved in cell adhesion or migration. If true, this could be another way in which miR-92 contributes to cancer by controlling cell migration and metastasis.

**Acknowledgements**

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**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/doi/10.1242/dev.056697-D/DC1

**References**


Calcium fluxes in dorsal forerunner cells antagonize beta-catenin and alter left-right patterning. Development 135, 75-84.


**cas 90% epiboly**

A

NIC

miR-92

C

MO1

G

MO2

**ntl 70% epiboly**

I

NIC

J

MO1

K

MO2

**ntl tail bud**

M

NIC

N

MO1

O

MO2

P
pre-miR-92a-1

pre-miR-92a-2

pre-miR-92b

MO1 = MO92a1 + MO92b1
MO2 = MO92a2 + MO92a2' + MO92b2
NIC MO1 2ng

NIC MO2 9ng

U6

miR-92a
Localization of cmlc2

MO + miR-92 DFC
A

B

C

D

E

F

G

H

I

J

K

L

M

N

lrdr 90% epiboly

foxj1 100% epiboly

fgf8 6-somite

30hpf

Acetylated Tubulin(cilia)  SYTO59(nucleus)
**Relative sox17 expression**

NIC  MO1  MO2  Ctrl MO  MO+miR-92
Table S1. Dose-dependent effects of miR-92 gain of function

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>L</th>
<th>R</th>
<th>M</th>
<th>B</th>
<th>A</th>
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<tbody>
<tr>
<td>foxa3</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>NIC</td>
<td>188</td>
<td>98.4</td>
<td>1.6</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
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<tr>
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<td>72</td>
<td>23.6</td>
<td>22.2</td>
<td>22.2</td>
<td>26.4</td>
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<td>37</td>
<td>2.7</td>
<td>0.0</td>
<td>18.9</td>
<td>75.7</td>
<td>2.7</td>
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<tr>
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<td>0.0</td>
<td>14.9</td>
<td>70.3</td>
<td>14.9</td>
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<td>Ctrl miRNA</td>
<td>90</td>
<td>92.2</td>
<td>3.3</td>
<td>4.4</td>
<td>0.0</td>
<td>0.0</td>
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</tbody>
</table>

<p>| | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>NIC</td>
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<tr>
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<td>14.3</td>
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<td>98.1</td>
<td>1.9</td>
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</tr>
</tbody>
</table>

Percentages of left (L), right (R), midline (M) and bilateral (B) localization of foxa3 or cmlc2 in non-injected controls (NIC), miR-92-injected and control miRNA-injected embryos. Occasionally, no foxa3 was detected (A, absent).

n, the total number of embryos analyzed in each condition.
Table S2. Dose-dependent effects of miR-92 loss of function

<table>
<thead>
<tr>
<th>foxa3</th>
<th>n</th>
<th>L</th>
<th>R</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIC</td>
<td>188</td>
<td>98.4</td>
<td>1.6</td>
<td>0.0</td>
</tr>
<tr>
<td>MO1 400 pg</td>
<td>10</td>
<td>100.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>MO1 1 ng</td>
<td>59</td>
<td>94.9</td>
<td>3.4</td>
<td>1.7</td>
</tr>
<tr>
<td>MO1 2 ng</td>
<td>55</td>
<td>60.0</td>
<td>14.5</td>
<td>25.5</td>
</tr>
<tr>
<td>MO1 4 ng</td>
<td>35</td>
<td>57.1</td>
<td>22.9</td>
<td>20.0</td>
</tr>
<tr>
<td>MO2 1.5 ng</td>
<td>59</td>
<td>88.1</td>
<td>11.9</td>
<td>0.0</td>
</tr>
<tr>
<td>MO2 3 ng</td>
<td>49</td>
<td>98.0</td>
<td>2.0</td>
<td>0.0</td>
</tr>
<tr>
<td>MO2 6 ng</td>
<td>48</td>
<td>87.5</td>
<td>8.3</td>
<td>4.2</td>
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<tr>
<td>MO2 9 ng</td>
<td>15</td>
<td>80.0</td>
<td>6.7</td>
<td>13.3</td>
</tr>
<tr>
<td>MO1+2</td>
<td>126</td>
<td>59.5</td>
<td>17.5</td>
<td>23.0</td>
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<tr>
<td>Ctrl MO</td>
<td>56</td>
<td>94.6</td>
<td>5.4</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Percentages of left (L), right (R) and midline (M) localized foxa3 in non-injected controls (NIC), miR-92 MO-injected and control MO-injected embryos.

n, the total number of embryos analyzed in each condition.