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miR-142-3p Controls the Specification of Definitive Hemangioblasts during Ontogeny

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SUMMARY

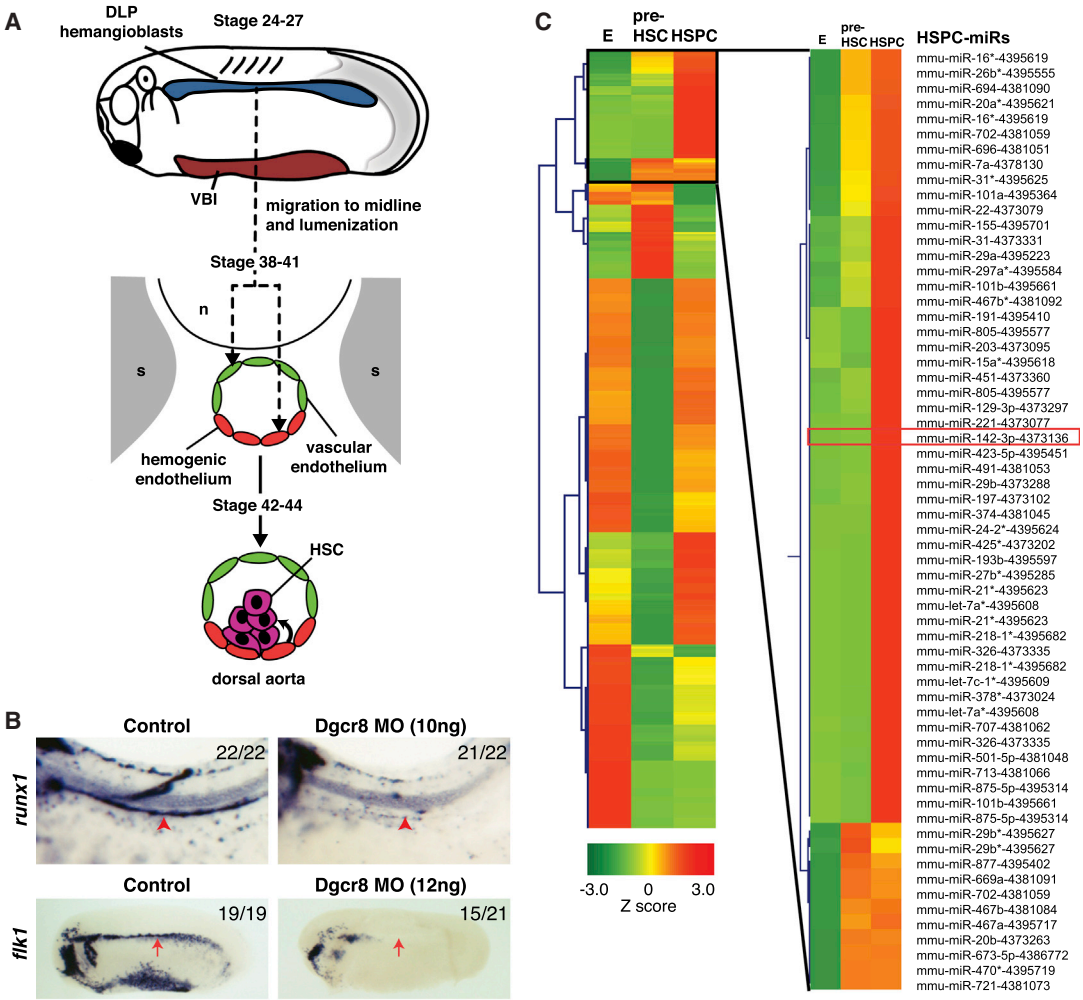
Hematopoietic stem cells (HSCs) emerge during embryogenesis from hemogenic endothelium, but it remains unclear how the HSC lineage is initially established from mesoderm during ontogeny. In *Xenopus*, the definitive hemangioblast precursors of the HSC lineage have been identified in dorsal lateral plate (DLP) mesoderm, and a transcriptional gene regulatory network (GRN) controlling hemangioblast programming has been elucidated. Herein, we identify an essential role for microRNAs (miRNAs) in establishing the mesodermal lineage leading to both HSC emergence and vasculogenesis and determine that a single miRNA, miR-142-3p, is primarily responsible for initiation of definitive hemangioblast specification. miR-142-3p forms a double-negative gate unlocking entry into the hemangioblast program, in part by inhibiting TGFβ signaling. Our results table miR-142-3p as a master regulator of HSC lineage specification, sitting at the apex of the hierarchy programming the adult hemangioblast, thus illustrating that miRNAs can act as instructive determinants of cell fate during development.

INTRODUCTION

Hematopoietic stem cells (HSCs) are formed during embryogenesis and are required to maintain the hematopoietic system throughout life (Medvinsky and Dzierzak, 1996). Understanding how HSCs are programmed will inform protocols for ex vivo production and expansion of HSCs for regenerative medicine. The emergence of HSCs occurs through an endothelial to hematopoietic transition (EHT) of hemogenic endothelial cells lining the dorsal aorta (DA) in the aorta-gonad-mesonephros (AGM) region (Bertrand et al., 2010; Boisset et al., 2010; Kissa and Herbomel, 2010). However, the origin of the hemogenic endothelium and the mechanism by which the HSC lineage is first established is less well understood (Medvinsky et al., 2011). Current protocols for ex vivo hematopoietic differentiation of mammalian pluripotent cells are able to mimic an EHT but are incapable of producing definitive, long-term reconstituting HSCs (McKinney-Freeman and Daley, 2007). It is therefore important to understand how the precursors of the HSC lineage, the definitive hemangioblasts, are programmed during embryonic development in order to achieve the production of functional HSCs in vitro.

Lineage tracing in the mouse has suggested that both hemogenic and vascular endothelial cells in the DA originate from the lateral plate mesoderm (Wasteson et al., 2008; Zovein et al., 2010), and in *Xenopus*, in marked contrast to the mouse, this definitive hemangioblast population has been prospectively identified (Ciau-Uitz et al., 2000; Walmsley et al., 2002). The dorsal lateral plate (DLP) mesoderm contains hemangioblasts that coexpress blood and endothelial genes and give rise to both endothelial cells of the major vessels as well as the hemogenic endothelium in the ventral wall of the DA, from which HSCs emerge (summarized in Figure 1A). The hemangioblasts in the DLP migrate to the midline in response to VEGF signaling, coalesce, and lumenise to form the DA, whereas some remain in the lateral plate and produce the posterior cardinal veins (PCV) (Ciau-Uitz et al., 2000; Cleaver and Krieg, 1998). Transplantation experiments showed that the DLP region, but not the ventral blood island (VBI), gives rise to adult blood, demonstrating that this tissue contains the precursors of the adult blood stem cell lineage (Maéno et al., 1985).

Genetic analysis in *Xenopus* has begun to define a transcriptional road map for programming the blood stem cell lineage from definitive hemangioblasts through to HSCs (Ciau-Uitz et al., 2010, 2013; Liu et al., 2008; Walmsley et al., 2002). The Ets transcription factor (TF) Fli1, sits at the top of this hierarchy and promotes expression of a cascade of TFs and signaling molecules, including Gata2, Flk1, and Etv2, that collaborate to activate the key hemangioblast determinant, Scl, which then activates a battery of downstream effector genes in order to produce both hemogenic and vascular endothelium (Ciau-Uitz et al., 2013). BMP and VEGF signaling have been found to promote the specification of this hemangioblast population during embryogenesis, but it is not known how this genetic cascade is initiated (Ciau-Uitz et al., 2010, 2013; Walmsley et al., 2002). Interestingly, many of the same TFs that regulate adult HSC function are required for HSC development in the embryo and recent studies



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in mouse hematopoietic cells have begun to explore the direct transcriptional connections between these TFs and their target genes using chromatin immunoprecipitation sequencing (ChIP-seq) analysis (Pimanda and Göttgens, 2010; Wilson et al., 2010; G. May, S.S., and T.E., unpublished data). Ultimately, these gene regulatory networks (GRNs) will provide important clues for recapitulating embryonic development in vitro in order to produce functional, long-term reconstituting HSCs from pluripotent stem cells for regenerative medicine.

We set out to investigate how microRNAs (miRNAs) contribute to the emergence of the blood stem cell lineage during embryonic development. miRNAs are a new class of gene regulatory molecules that act at the posttranscriptional level to repress multiple target genes via inhibition of translation and destabilization of mRNA (reviewed in Nimmo and Slack, 2009). miRNA function has been studied by globally ablating miRNA production by knocking out genes required for miRNA processing, such as Dicer, Drosha, and Dgcr8. These studies have shown that miRNAs are fundamentally required for embryogenesis in mice (Bernstein et al., 2003; Chong et al., 2010; Murchison et al., 2005; Wang et al., 2007), and evidence abounds for miRNA regulation of adult hematopoiesis (Alemdehy and Erkeland, 2012; Baltimore et al., 2008). Conditional Dicer knockout in adult bone marrow using the Mx-Cre system results in a catastrophic loss of HSCs, illustrating that miRNAs have a major role in adult HSC homeostasis (Guo et al., 2010). It is not yet known which miRNAs are responsible for this defect, and furthermore, the contribution that miRNAs make to the development of HSCs and their hematoendothelial precursors during embryogenesis has yet to be investigated.

By knocking down the miRNA-processing factor, Dgcr8, in *Xenopus* embryos, we have discovered a role for miRNAs in early specification of the HSC lineage in lateral plate mesoderm. The failure to correctly specify hemangioblasts in the DLP results in defects in vasculogenesis and disruption of DA formation, resulting in a complete ablation of HSC emergence. Using loss- and gain-of-function approaches, we identify a single hematopoietic miRNA, miR-142-3p, that is both necessary for specification of definitive hemangioblasts and sufficient to rescue hemangioblast programming in embryos globally depleted of miRNAs by Dgcr8 knockdown. Genetic analysis positions this miRNA at the top of the regulatory hierarchy controlling hematoendothelial programming in the DLP, acting in part via modulation of TGFβ signaling.

RESULTS

Identification of a miRNA Required for Definitive Hemangioblast Specification

We first asked whether miRNAs are required for HSC formation by inhibiting miRNA processing. miRNAs are produced by the action of two main processing complexes: the Microprocessor complex components Drosha and Dgcr8 excise the pre-miRNA hairpin from the primary transcripts, and the terminal loop is then removed by Dicer to release the mature miRNA duplex, one strand of which is loaded into the silencing complex RISC (Kim et al., 2009). We depleted *Xenopus* embryos of miRNAs using a *dgcr8* translation blocking morpholino (MO) (Agrawal et al., 2009) to assess on a global scale whether miRNAs are required for HSC formation. To minimize nonspecific effects and to optimize viability, the MO dosage was titrated to an optimal range of 10–15 ng per embryo. Using *runx1* as a marker, we observed a complete ablation of hemogenic endothelium in the *dgcr8* morphants even at the lowest (10 ng) dose, at which the overall morphology and survival of the embryos was good (Figure 1B). Interestingly, earlier in development at stages 22–26, the number of Flk1+ definitive hemangioblasts in the DLP was severely reduced in *dgcr8* morphants, suggesting that the failure to produce HSCs is caused by defective specification of hemangioblast precursors in lateral plate mesoderm (Figure 1B; Figure S1A available online).

Having established that miRNA production is crucial for the specification of the hemangioblast precursors of HSCs, we then asked which miRNA, or miRNAs, are responsible. Candidate miRNAs associated with the development of the definitive hematopoietic lineage were identified by miRNA expression profiling of embryonic HSC precursors. The mouse system affords the ability to prospectively isolate hematopoietic

Figure 1. Identification of a Role for miRNAs in Specification of the HSC Lineage

(A) Schematic diagram showing the stepwise differentiation of the blood stem cell lineage in *Xenopus*, from DLP hemangioblast precusors through to HSCs via hemogenic endothelium. The DLP hemangioblasts give rise to the DA, including the hemogenic endothelium in the ventral wall from which stem cells emerge as part of hematopoietic clusters. The hematopoietic clusters in the DA at stages 42–44 of the *Xenopus* embryo are analogous to the clusters found in the E11.5 AGM of the mouse.

(B) miRNAs are required for HSC lineage specification. *dgcr8* morphants do not produce hemogenic endothelium because of failure to correctly specify hemangioblasts in the DLP. Top: WISH showing *Runx1* expression in the DA (arrowheads) at stage 39 is dependent on Dgcr8. Bottom: WISH showing loss of *Flk1* expression in the DLP (arrows) of *dgcr8* MOs at stage 24. Numbers of embryos represented by each panel, out of the number analyzed, are indicated in the top right corner. The anterior of the embryo is to the left and dorsal to the top in all images.

(C) Heatmap showing hierarchical clustering of miRNA expression profile of sorted cell populations from E11.5 mouse AGM. E: Ter119$^-$ CD31$^+$ c-Kit$^-$ CD45$^-$ endothelial cells (including hemogenic endothelium), presumptive pre-HSCs: Ter119$^-$ CD31$^+$ c-Kit$^+$ CD45$^-$ HSC precursors, and HSPC: Ter119$^-$ CD31$^+$ c-Kit$^+$ CD45$^+$ hematopoietic stem and progenitors. Red indicates high expression and green indicates low expression as represented by the Z score. The "HSPC-miRs" cluster is expanded to show miRNA names. All miRNA qRT-PCR array data are available in Table S1. See Figure S1D for gating strategy of the sort and Figure S1E for schematic of AGM populations.

(D) Ranking of miRNAs by scoring peaks within 5 kb up- and downstream of the genomic location of the pre-miRNA in ChIP-seq data for ten hematopoietic TFs from hematopietic progenitor cells (Wilson et al., 2010). Graph showing rankings for the "HSPC-miRs" cluster; only miRNAs with a score greater than zero are shown.

(E and F) Relative expression of the 5′ and 3′ strands of mir-142, miR-142-5p, and miR-142-3p. (E) qRT-PCR expression analysis on sorted mouse BM adult HSPC (Flt3$^-$KLS) and mouse AGM E, pre-HSC, and HSPC populations. (F) qRT-PCR expression analysis on dissected stage 26 DLP and, for comparison, stage 14 whole embryos. ΔCt values relative to U6 are shown on a log scale. (Error bars represent mean ± SD.)

See also Figure S1 and Table S1.

populations from the AGM region by surface marker phenotype during the transit of HSC precursors from hemogenic endothelium to HSCs (Ruiz-Herguido et al., 2012; Rybtsov et al., 2011; Yokomizo and Dzierzak, 2010). We fluorescence-activated cell sorting (FACS) isolated E11.5 AGM-derived populations containing hematopoietic stem and progenitors (HSPCs: Ter119$^-$ CD31$^+$ c-Kit$^+$ CD45$^{+)}$, the immature pre-HSCs (pre-HSC: Ter119$^-$ CD31$^+$ c-Kit$^+$ CD45$^-$), and endothelial cells (including hemogenic endothelium) (E: Ter119$^-$ CD31$^+$ c-Kit$^-$ CD45$^-$) (Figures S1D and S1E). These AGM populations represent a later stage in HSC development than the hemangioblast and are instead equivalent to the cells found in the hematopoietic clusters in the dorsal aorta in stage 43 *Xenopus* embryos. A global comparison of miRNA expression was performed using TaqMan Rodent MicroRNA Arrays (from miRbase [v. 10.1]) (Table S1). We hypothesized that miRNAs involved in HSC lineage specification would be expressed at all stages of HSC development from hemogenic endothelium through to pre-HSC and HSPCs and would be upregulated as the cells progressed to the hematopoietic fate. Based on these criteria, we used hierarchical clustering to identify a group of HSPC-associated miRNAs, termed "HSPC-miRs" (Figure 1C; Table S1).

Recently, it was shown that combinatorial binding of key hematopoietic TFs is enriched at genes expressed in hematopoietic progenitor cells and is a useful tool to predict novel HSPC regulators (Hannah et al., 2011; Wilson et al., 2010). Therefore, we ranked this cluster of "HSPC-miRs" based on the number of binding peaks within 5 kb of the pre-miRNA locus for ten major hematopoietic TFs (Scl, Lyl1, Lmo2, Runx1, Gata2, Meis1, Erg, Fli1, Pu.1, and Gfi1b) using existing ChIP-seq data obtained in multipotential hematopoietic progenitor cells (Wilson et al., 2010) and identified miR-142-3p as the top-ranked hematopoietic miRNA in this analysis (Figure 1D). All three of the AGM populations analyzed expressed miR-142-3p, but it is strongly upregulated in the HSPC population (Figure 1E). High-level expression is maintained in adult bone-marrow-derived HSPCs (Flt3$^-$KLS) (Figure 1E), consistent with the prior observations that miR-142-3p is abundant specifically in hematopoietic cells (Bissels et al., 2011; Chen et al., 2004). miR-142-3p is absolutely conserved across vertebrates and has been previously reported to modulate the maturation of the myeloid, erythroid, and T cell lineages (Chen et al., 2004; Nishiyama et al., 2012; Wang et al., 2012b), but its role in the ontogeny of HSCs has not been investigated. miR-142-3p is likely to be the major functional form of this miRNA in HSC development as the opposite strand of miR-142, miR-142-5p, is virtually undetectable in endothelium and pre-HSCs and is expressed at a much lower level than miR-142-3p in embryonic HSPCs (almost 1,000-fold lower expression, Figure 1E).

We next asked whether this miRNA is also expressed in the precursors to the hemogenic endothelium in lateral plate mesoderm, because the HSC defect in our *dgcr8* morphants originated in an earlier failure to specify this hemangioblast population. The profiling of AGM hematopoietic and endothelial populations identified miRNAs expressed proximal to HSC emergence, but their hemangioblastic anlage could not be analyzed in murine embryos as this population has not yet been prospectively isolated. However, in *Xenopus*, definitive hemangioblasts have been shown to be located in the DLP of tailbud stage embryos (Ciau-Uitz et al., 2000; Walmsley et al., 2002) (shown schematically in Figure 1A). qRT-PCR analysis on DLP tissue dissected from stage 26 embryos revealed that both strands of miR-142 are expressed in the definitive hemangioblast population with miR-142-3p present at a much higher level than miR-142-5p, similar to our observations in the mouse AGM (Figure 1F). Therefore, miR-142-3p is expressed in all stages of the HSC lineage during development, including definitive hemangioblasts, and the miR-142 locus is subject to combinatorial binding by multiple major hematopoietic TFs, tabling this miRNA as a candidate effector of hematopoietic programming during development.

To directly test the hypothesis that this miRNA is responsible for specification of the precursors of the blood stem cell lineage, we carried out a combination of genetic approaches in *Xenopus* embryos (summarized in Figure 2). Initially, we performed a miR-142-3p loss-of-function (LOF) experiment using an antisense MO to inhibit miR-142-3p activity (Figure 2). miR-142-3p morphants failed to form hemogenic endothelium as judged by a complete loss of *runx1* expression in the midline of stage 39 embryos (Figure 3A), thus phenocopying the hematopoietic defect seen in *dgcr8* morphants. In contrast, a negative control MO, with six mismatches compared to the miR-142-3p MO, had no effect on the expression of *runx1* (Figure 3B). Similar to *dgcr8* morphants, the HSC defect associated with miR-142-3p MO could be traced back to the failure to correctly specify hemangioblasts as the morphants had highly reduced *flk1* expression in the DLP at stage 26, with a complete ablation of *flk1* staining in the posterior region of the DLP (Figure 3A). In contrast, neither the control mismatch MO nor a MO targeting the less abundant strand, miR-142-5p, had any effect on *flk1* expression in the DLP (Figures 3B and 3C). The miR-142-3p MO therefore phenocopies the effect of the *dgcr8* MO on definitive hemangioblast specification, strongly suggesting that depletion of miR-142-3p is responsible for the failure to activate the hematoendothelial program in the DLP of *dgcr8* morphants.

miR-142-3p Is Sufficient to Rescue Definitive Hemangioblast Specification in Dgcr8 Morphants

To test the hypothesis that depletion of miR-142-3p is responsible for the definitive hemangioblast specification defect in *dgcr8* morphants, we performed a rescue experiment with a synthetic miR-142-3p mimic that does not require prior processing by the Drosha/Dgcr8 complex and is instead optimized for direct loading into RISC (summarized in Figure 2). Coinjection of 10 or 12.5 fmol mimic with *dgcr8* MO was sufficient to restore hemangioblast specification in *dgcr8* morphants as assessed by expression of *flk1* and *scl* in the DLP at stage 26 (Figure 3D). We also tested the other top candidates from our miRNA screening, miR-26, miR-24, and miR-191, as well as the minor strand of miR-142, miR-142-5p, for their ability to rescue *dgcr8* morphants, but none of these were able to restore *flk1* expression in the DLP (Figure S2A). Interestingly, the miR-142-3p mimic was unable to restore formation of hemogenic endothelium from these hemangioblasts (Figures S2B and S2C), suggesting that other miRNAs are required for later stages of hemangioblast differentiation. Taken together, these data strongly support the hypothesis that miR-142-3p is the primary miRNA governing definitive hemangioblast specification in lateral plate mesoderm.

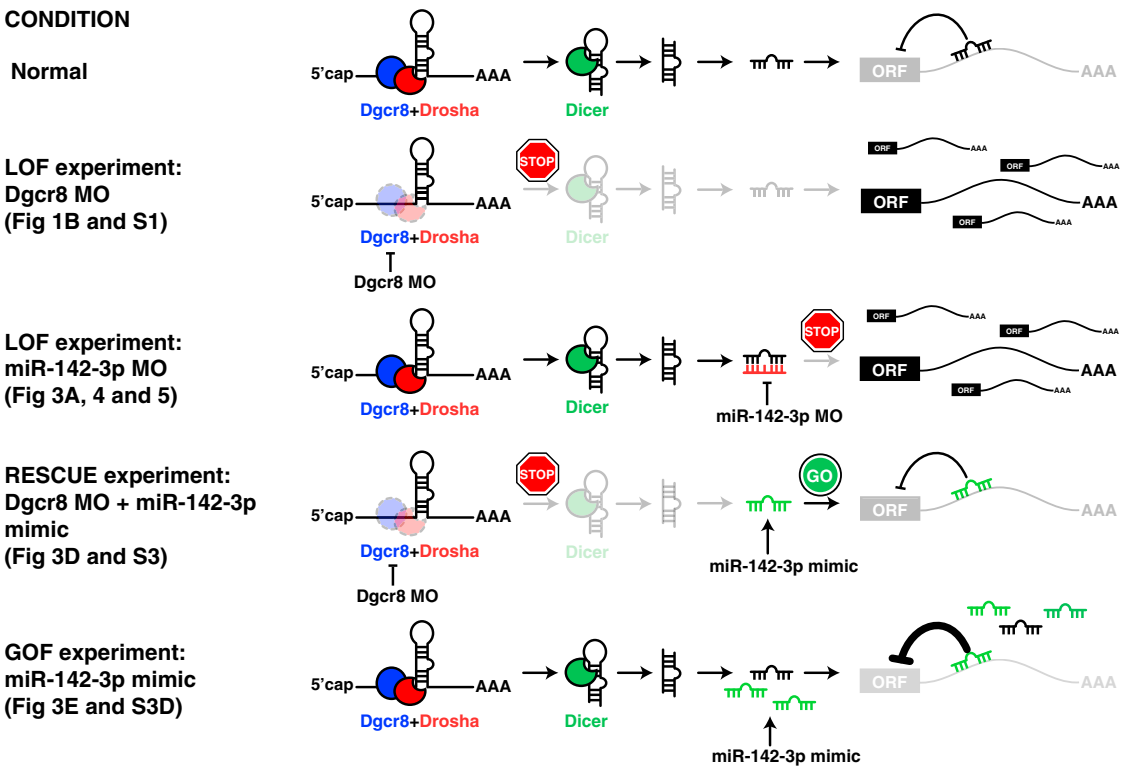

Figure 2. A Combination of Genetic Approaches to Investigate miRNA Function in *Xenopus* Embryos

Schematic diagram summarizing the different approaches used to show that miR-142-3p is necessary and sufficient for hemangioblast specification during establishment of the HSC lineage. A combination of loss-of-function (LOF), gain-of-function (GOF), and rescue experiments were used to investigate whether miR-142-3p controls definitive hemangioblast specification.

We next performed a gain-of-function (GOF) experiment to investigate whether overexpression of miR-142-3p is sufficient to enhance hemangioblast and HSC specification (summarized in Figure 2). Hemogenic endothelium is normally restricted to the trunk region of the DA, but in the embryos injected with miR-142-3p mimic, expression of *runx1* is also found ectopically in the tail region at stage 39 (indicated with red dashed line in Figure 3E), suggesting that miR-142-3p overexpression enhances HSC programming in the DA. In addition, there is a mild posterior expansion of *gfi1a* expression within the trunk DA of these embryos (Figure S2D), although it is not expressed in the tail region, suggesting that these ectopic *runx1*-expressing cells may not be fully programmed HSC precursors. Earlier, at stage 26, some enhancement of *flk1* staining in the DLP was also observed (Figure 3E). Therefore, miR-142-3p enhances hematopoietic programming in the DLP and DA but may not be sufficient by itself to drive ectopic HSC formation. Taken together, the rescue and overexpression experiments endorse the view that miR-142-3p is a central player in the development of the hemangioblast precursors of the blood stem cell lineage in lateral plate mesoderm.

miR-142-3p Acts at the Top of the Regulatory Hierarchy Specifying the Blood and Endothelial Program in the DLP

The specification of the hemangioblastic precursors of definitive HSCs and endothelium is dependent upon a hierarchical network of transcription factors and signaling molecules (Ciau-Uitz et al., 2010; 2013; Liu et al., 2008; Walmsley et al., 2002; summarized in Figure 4A). To investigate where miR-142-3p is positioned in this regulatory hierarchy, we analyzed expression of the known members of the network in the miR-142-3p morphants (Figures 4B and 4C). The DLP network has an initiation phase from stages 20–22, in which Fli1 and Gata2 switch on *flk1* and *etv2* expression, followed by a lockdown/maintenance phase from stage 22 onward driven by Etv2 acting in a positive feedback loop to maintain *gata2* expression (Figure 4A; Ciau-Uitz et al., 2010, 2013; Liu et al., 2008). All of these TFs then collaborate with VegfA-Flk1 signaling to activate *scl* expression from stage 24, and Scl then activates a battery of downstream hemangiogenic genes involved in endothelial and hematopoietic differentiation (Ciau-Uitz et al., 2010, 2013). Recent work has unequivocally positioned Fli1 at the apex of the hierarchy regulating hemangioblast specification in *Xenopus.* A detailed time course analysis of gene expression showed that other factors, including *gata2*, *etv2*, *flk1*, and *scl*, are all downregulated in the DLP of Fli1 morphants, whereas *fli1* expression is unaffected by knockdown of these downstream factors (Ciau-Uitz et al., 2013).

Early in hemangioblast development at stage 22, during establishment of the hemangioblast program, there is a severe reduction in the expression of the most upstream regulators, *fli1*, *flk1*, and *etv2*, in the miR-142-3p morphants (Figure 4B). The full hemangioblast program is normally activated by stages 25–26. However, in miR-142-3p morphants, we discovered that all of

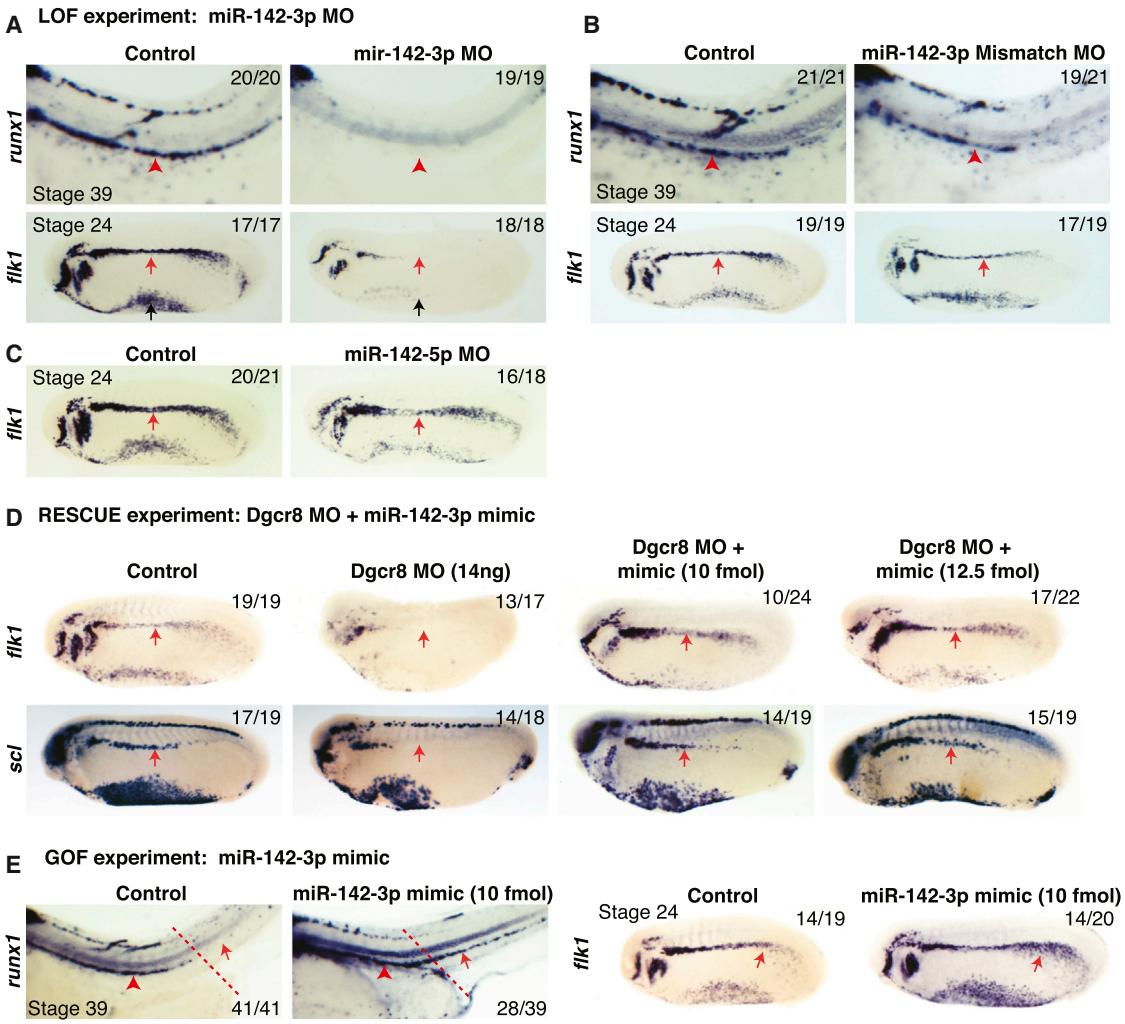

Figure 3. miR-142-3p Is Required for Definitive Hemangioblast Specification and Is Sufficient to Rescue Hemangioblast Programming in *dgcr8* Morphants

(A–C) LOF experiment: miR-142-3p is required for formation of hemogenic endothelium and for hemangioblast specification in the DLP. Embryos injected with 100 ng miR-142-3p MO (A), but not a mismatch control MO (B), phenocopy hematopoietic defects of Dgcr8 MO embryos. Top: WISH showing *runx1* expression in hemogenic endothelium (arrowheads) at stage 39. Bottom: WISH showing *flk1* expression in DLP hemangioblasts (red arrows) at stage 24. Endothelial cells in VBI are indicated by black arrows. (C) WISH showing *flk1* expression is normal in the DLP (arrows) at stage 24 in miR-142-5p (passenger strand) morphants.

(D) Rescue experiment: Coinjection of the miR-142-3p mimic is sufficient to restore hemangioblast formation in *dgcr8* morphants. Expression of *flk1* (top) and *scl* (bottom) in the DLP (arrows) analyzed by WISH at stages 24–25.

(E) GOF experiment: Enhanced hematopoietic programming by overexpression of miR-142-3p. Left: WISH of *runx1* at stage 39 shows that the hemogenic endothelium (arrowheads) is expanded posteriorly out of the trunk, into the postcloacal tail region of the DA (arrows) in the miR-142-3p mimic-injected embryos. Dashed line indicates the location of the cloaca. Right: WISH analysis of *flk1* expression in the DLP hemangioblasts (arrows) at stages 24–25.

The number of embryos represented by each panel, out of the number analyzed, is indicated in the top right corner. The anterior of the embryo is to the left and dorsal to the top in all images.

See also Figure S2.

the major TFs and signaling molecules acting in this genetic cascade (*fli1*, *gata2*, *flk1*, *etv2*, and *scl*) are severely downregulated in the DLP (Figure 3A [*flk1*]; Figure 4C). In contrast, hemangioblast programming is normal in embryos injected with either the control mismatch MO or the miR-142-5p (passenger strand) MO (Figures 3B, 3C, 4D, and 4E). Therefore, miR-142-3p is required for initiation of both the VEGF-dependent and VEGF-independent branches of the hemangioblast program and acts above Fli1, at the apex of the regulatory hierarchy governing initiation of the definitive hemangioblast program in the DLP mesoderm.

Furthermore, *dgcr8* morphants have similar defects in DLP hemangioblast specification as shown by reduced expression of *fli1* and *flk1* at stage 22 (Figure S1A) and *flk1*, *etv2*, and *scl* at stages 25–26 (Figures 1B, 3D, and S1A). The striking phenocopy of the *dgcr8* and miR-142-3p morphant phenotypes in the DLP, and the ability of the miR-142-3p mimic to rescue hemangioblast programming in *dgcr8* morphants, strongly

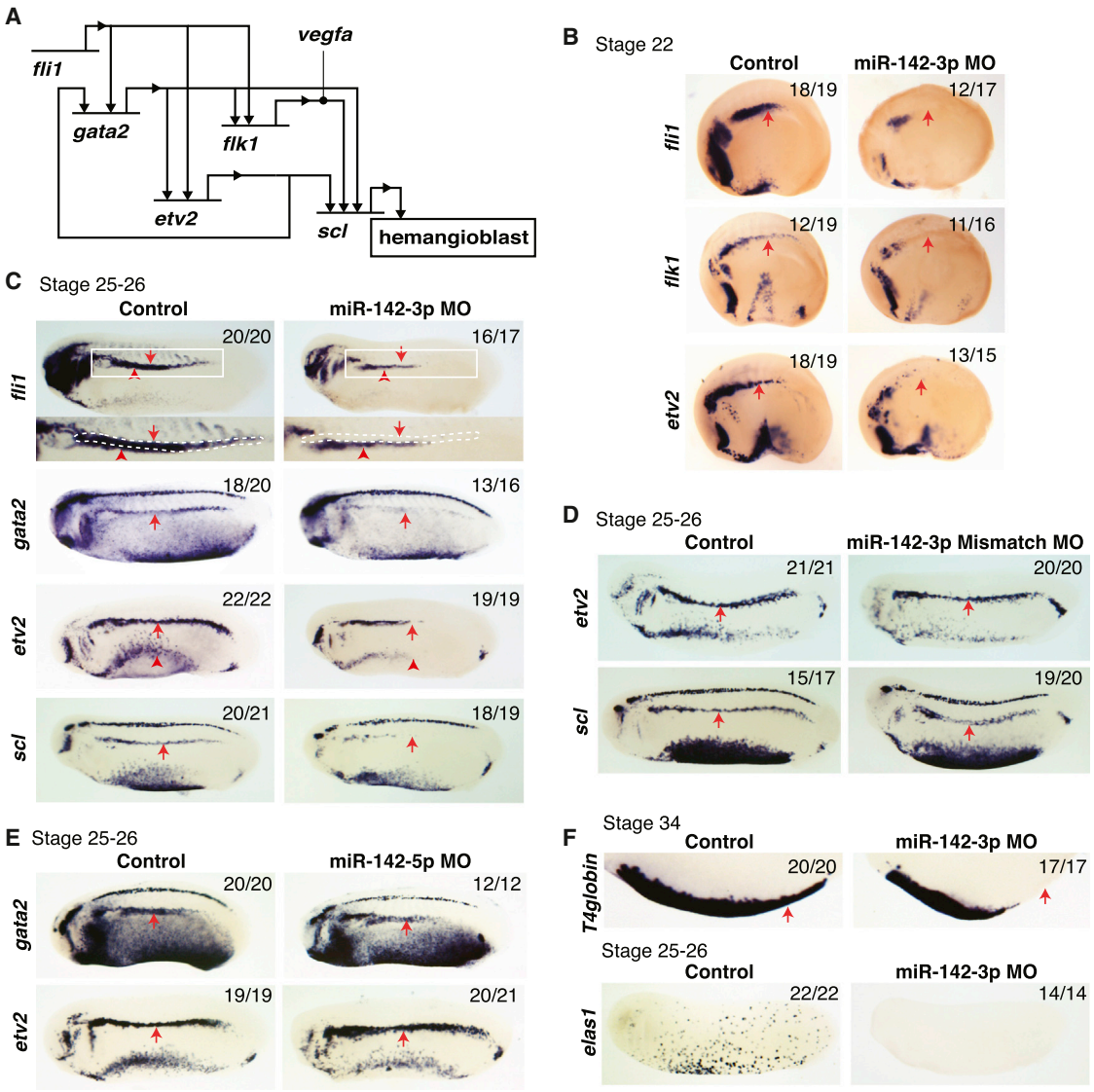

Figure 4. miR-142-3p Acts at the Top of the Regulatory Hierarchy Controlling Specification of HSC and Endothelial Cell Precursors in the DLP

(A) A provisional GRN controlling hemangioblast specification (Ciau-Uitz et al., 2010, 2013; Liu et al., 2008; Walmsley et al., 2002).

(B and C) Initiation of the hemangioblast program is defective in miR-142-3p MO embryos. (B) WISH showing that expression of early regulators, *fli1*, *flk1*, and *etv2*, is already highly reduced or absent in the DLP (arrows) in miR-142-3p morphants at stage 22 (initiation phase). (C) WISH analysis showing that the expression of hematopoietic *gata2* and *scl*) and endothelial (*fli1* and *etv2*) TFs in the DLP (arrows), are all dependent on miR-142-3p at stages 25–26. Boxed region of the *fli1* panels is magnified to show that the expression of *fli1* in the pronephric duct (arrowheads) abutting the DLP is unaffected in the morpants, whereas the expression of *fli1* in the DLP (white dashed outline and arrows) is abrogated by the miR-142-3p MO. Formation of endothelial cells in the VBI is also disrupted by the MO as shown by reduced *etv2* staining (arrowhead).

(D and E) Hemangioblast specification is normal in embryos injected with the control mismatch MO and miR-142-5p MO showing that the effect is specific to the miR-142-3p MO. (D) WISH analysis of *etv2* and *scl* expression in the DLP (arrows) of control mismatch morphants and (E) *gata2* and *etv2* expression in the DLP (arrows) of miR-142-5p morphants.

(F) Formation of primitive blood is defective in miR-142-3p morphants. Top: Erythroid differentiation is absent from the pVBI region as shown by WISH analysis of *αT4-globin* expression. Bottom: reduction in formation of myeloid cells as shown by WISH analysis of *elas1* expression.

The number of embryos represented by each panel, out of the number analyzed, is indicated in the top right corner. The anterior of the embryo is to the left and dorsal to the top in all images.

See also Figure S3.

supports the view that miR-142-3p is the major miRNA acting to initiate hematoendothelial fate in the DLP.

In addition, the formation of embryonic blood and the vitelline vessels in the VBI is also disrupted in miR-142-3p morphants. The VBI is lineally, spatially, and temporally separated from the definitive hematoendothelial lineage derived from the DLP (Ciau-Uitz et al., 2000). The ability to analyze the early specification of the two waves of hematopoiesis separately is an

important facet of the *Xenopus* model system. Our analysis revealed that miR-142-3p is required for the differentiation of myeloid cells from the anterior VBI and erythroid cells in the posterior VBI, suggesting that it plays a role in establishing both primitive and definitive waves of hematopoiesis (Figure 4F). In addition, the number of endothelial cells arising from the VBI is severely reduced in the miR-142-3p morphants (Figure 3A [*flk1*], black arrow; Figure 4C [*etv2*], red arrowhead). In agreement with these observations, *dgcr8* morphants exhibit similar defects in hematopoiesis and vasculogenesis in the VBI (Figures S1A and S1C). The defect in erythroid differentiation is consistent with the recent observations of Nishiyama and colleagues that zebrafish embryos depleted of miR-142-3p have reduced numbers of primitive erythrocytes (Nishiyama et al., 2012).

The specificity of miR-142-3p in promoting hemangioblast development is demonstrated by the observation that the pronephric duct, which derives from the same C3 blastomere of 32-cell stage embryos as the DLP (Ciau-Uitz et al., 2000) and develops directly adjacent to the DLP, is nonetheless specified normally (Figure S3A). It has also been recently suggested that in zebrafish, miR-142-3p depletion causes defects in cardiac function and somite morphogenesis later in development (Nishiyama et al., 2012). However, at the stages when the DLP mesoderm is being specified to a hematoendothelial fate, both cardiac tissue and somites are specified normally (Figures S3B and S3C). Therefore, miR-142-3p specifically promotes hemangioblast specification in lateral plate mesoderm and does not appear to have a more widespread role in mesoderm induction or patterning.

miR-142-3p Morphants Have Defective Vasculogenesis Arising from the Failure to Specify Definitive Hemangioblasts

The definitive hemangioblasts in the DLP are responsible for production of vascular endothelial cells of the DA and PCV, as well as hemogenic endothelium in the DA (summarized in Figure 1A). Hemogenic endothelium is not formed in miR-142-3p morphants as shown by complete ablation of the hematopoietic genes *runx1*, *spib*, and *gfi1a* in the trunk region of the DA (Figures 1B and 5A). We therefore also examined the expression of various endothelial markers in the miR-142-3p and *dgcr8* morphants. At stage 30, when hemangioblast-derived cells would normally be undergoing VEGFA-dependent migration to the midline to form the DA, effector genes downstream of Scl, including the VEGF receptors *flt1* and *flt4*, are downregulated in the miR-142-3p morphants, and there are reduced numbers of endothelial precursors migrating to the midline (Figure 5B). By stage 34 severe defects are seen in the formation of both the DA and the PCV as assessed by *CD31* and *AA4.1* expression (Figure 5C), and there are fewer cells expressing the arterial markers *dll4* and *cx37* in the midline of the morphants (Figure 5C). A disorganized vascular plexus is formed in the morphants, albeit a much reduced abnormal vascular network with no fully intact DA (Figures 5C and 5D). The *dgcr8* morphants have similar defects in vasculogenesis as shown by *AA4*.1 and *CD31* expression (Figure S1B). In contrast, the control mismatch MO had no effect on endothelial cell differentiation (Figures S4A and S4B).

There is no circulation apparent in the miR-142-3p or *dgcr8* morphants at stage 39 as a result of the severe vascular defects. Blood flow is required for HSC emergence in the DA (North et al., 2009) but is unlikely to explain the failure to express hematopoietic gene expression in the residual DA as failure to activate expression of *gata2* and *runx1* at stages 34 and 36, respectively, precedes the time that circulation normally commences in the embryos at stage 38 (Figures 5D and S1B).

In conclusion, the disruption of hemangioblast specification in the DLP in the miR-142-3p and *dgcr8* morphants results in a failure to form an intact DA that precludes the emergence of hemogenic endothelial cells and ultimately HSCs.

miR-142-3p Targets TGFβ Family Receptors in Order to Promote Hemangioblast Specification of Lateral Plate Mesoderm

We established that miR-142-3p is required to activate a cascade of TFs and signaling molecules in the DLP to promote definitive hemangioblast specification but miRNAs are generally negative genetic regulators. We therefore hypothesized that miR-142-3p must downregulate an inhibitor of the hemangioblast fate, forming a double-negative gate that controls entry into the hematoendothelial program. Using Targetscan (http://www.targetscan.org; Lewis et al., 2005), we identified three members of the TGFβ receptor superfamily, *tgfbr1*, *acvr2a*, and *acvr1b*, as predicted targets of miR-142-3p in *Xenopus* (Figure 6A). Both *tgfbr1* and *acvr2a* are conserved targets of miR-142-3p in mammals, as determined by all four major target prediction programs (Targetscan, Pictar, PITA, and Miranda) and have been validated by crosslinking and immunoprecipitation (CLIP) analyses, suggesting that miR-142-3p has a highly conserved role in regulating TGFβ family signaling (Chi et al., 2009; Gottwein et al., 2011; Yang et al., 2011). Furthermore, miR-142-3p has been linked with various components of the TGFβ signaling pathway, including *tgfbr1*, in a variety of settings (Carlsen et al., 2013; Danger et al., 2012; Kim et al., 2011; Lei et al., 2012; Wang et al., 2012b).

Many studies have suggested that TGFβ signaling has an inhibitory effect on blood development in vitro (Park et al., 2004; Poon et al., 2006; Sato et al., 2008; Wang et al., 2012a; Zhang et al., 2011), and therefore we asked whether miR-142-3p inhibits TGFβ signaling to promote the specification of definitive hemangioblasts. In situ hybridization analysis showed that the expression of the predicted miR-142-3p target, *tgfbr1*, is upregulated in the DLP region in miR-142-3p morphants (Figure 6B). Therefore, we hypothesized that elevated TGFβ signaling in the DLP of miR-142-3p morphants is responsible for the failure to initiate the hemangioblast program, and it may therefore be possible to rescue the effect of the MO by inhibiting TGFβ signaling (summarized in Figure 6C). To investigate this, we used both a pan-TGFβ/Activin/Nodal receptor type I inhibitor, SB431542 (Inman et al., 2002), and a specific Tgfbr1 inhibitor, LY2157299 (Bueno et al., 2008). Consistent with our hypothesis, both SB431542 and LY2157299 treatment from stages 16–17, prior to the start of the hemangioblast program in the DLP at stage 20, partially rescued *flk1* expression in the DLP of miR-142-3p morphants (Figure 6D). Therefore, our data suggest that miR-142-3p participates in a double-negative gate unlocking definitive hemangioblast programming in part by restricting inhibitory TGFβ signaling in lateral plate mesoderm (summarized in Figure 6E).

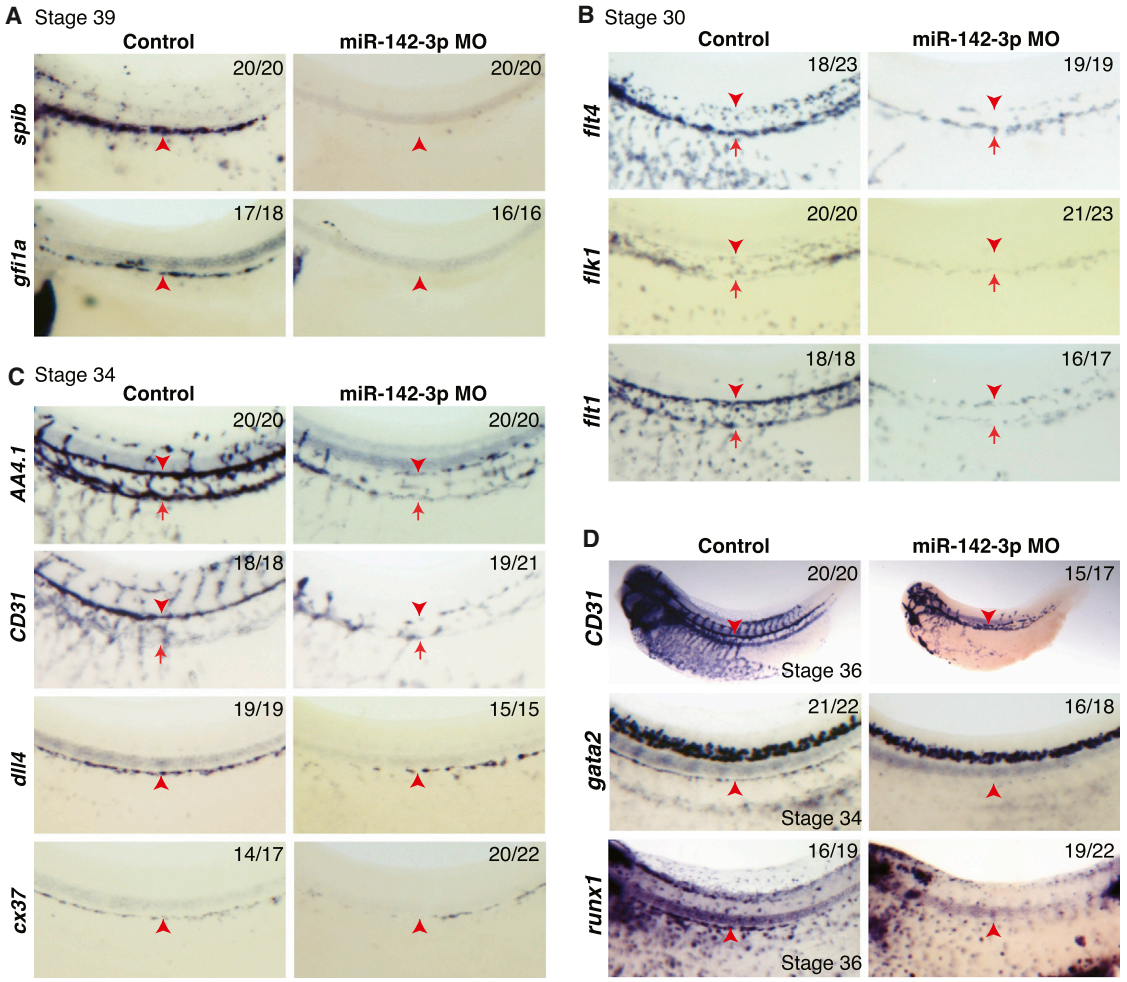

Figure 5. Vasculogenesis Defects in miR-142-3p Morphants

(A) Hemogenic endothelium is not formed in miR-142-3p morphants. In situ hybridization analysis of *gfi1a* and *spib* expression in the midline (arrowheads) at stage 39.

(B) Endothelial differentiation and migration defects in miR-142-3p morphants. WISH analysis of *flt4*, *flk1*, and *flt1* expression in DLP-derived angioblasts in the PCV (arrows) and in cells migrating to the midline to form the DA (arrowheads) at stage 30.

(C) Defective expression of endothelial markers in miR-142-3p morphants. WISH analysis of *AA4.1*, *CD31*, *dll4*, and *cx37* in the DA (arrowhead) and PCV (arrows) at stage 34.

(D) A residual DA is formed in miR-142-3p morphants (as shown by WISH analysis of *CD31*, arrowhead) but fails to switch on the hematopoietic TFs *gata2* at stage 34 or *runx1* at stage 36, as shown by WISH. Position of the DA is indicated by arrowheads.

The number of embryos represented by each panel, out of the number analyzed, is indicated in the top right corner. The anterior of the embryo is to the left and dorsal to the top in all images.

See also Figure S4.

DISCUSSION

We have identified an essential role for miRNA regulation in the gene regulatory network, controlling the specification of the hemangioblast precursors of the blood stem cell lineage from lateral plate mesoderm by knocking down the miRNA processing factor, Dgcr8. Using a morpholino to inhibit miR-142-3p, we demonstrated that this miRNA is required to initiate the specification of the definitive hemangioblasts. miR-142-3p morphants phenocopy closely the hematoendothelial defects in *dgcr8* morphants, and crucially, miR-142-3p is sufficient to rescue hemangioblast formation in Dgcr8-depleted embryos, suggesting that this is the primary miRNA governing the initial specification of the definitive hemangioblasts in lateral plate mesoderm. We have shown that miR-142-3p is the earliest known regulator of the hematoendothelial lineage, acting upstream of the hematopoietic TFs Fli1, Gata2, Etv2, and Scl, at the top of the hierarchy, specifying HSC and endothelial programming in the DLP and thereby tabling this miRNA as a master regulator of definitive hemangioblast specification. Our analysis of miR-142-3p has suggested the presence of a double-negative gate at the nexus of the GRN, controlling definitive hemangioblast specification, in which a repressor, miR-142-3p, activates the hematoendothelial program through downregulation of inhibitory factors, at least in part via modulation of TGFβ signaling. The double-negative gate may be a

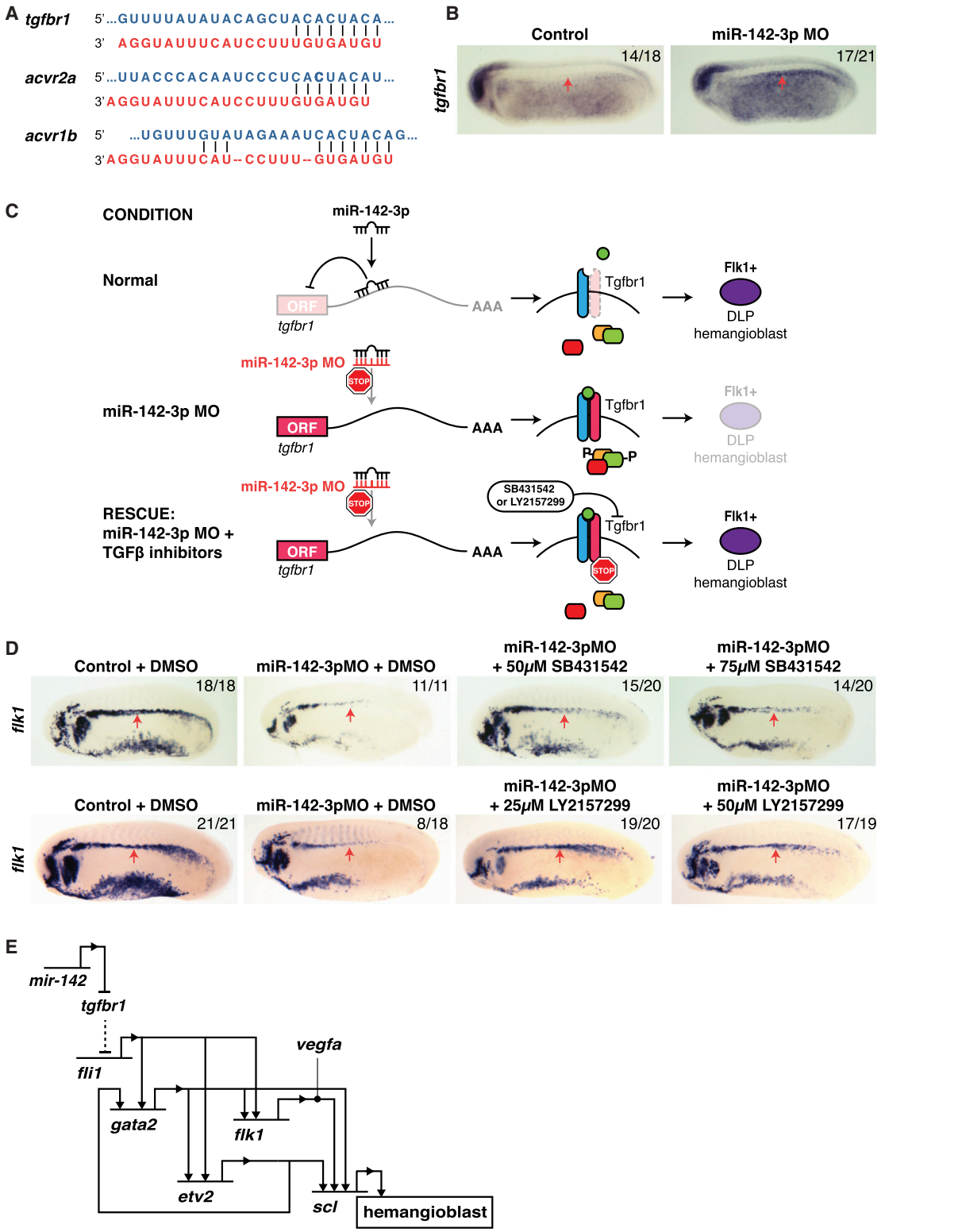

Figure 6. miR-142-3p Restricts Inhibitory TGFβ Signaling and Forms a Double-Negative Gate, Thereby Controlling the Initiation of the Hemangioblast Program

(A) Alignments of the target sites in *tgfbr1*, *acvr2a*, and *acvr1b* (blue) with miR-142-3p miRNA (red).

(B) WISH analysis at stages 25–26 showing that *tgfbr1* expression is elevated in the DLP region (arrows) in miR-142-3p morphants.

(C) Schematic diagram summarizing the rescue experiments performed with the TGFβ type I receptor kinase inhibitors, SB431542 and LY2157299, in miR-142-3p morphants.

(legend continued on next page)

general mechanism by which cell fates are unlocked during embryonic development (Davidson and Levine, 2008; Oliveri et al., 2008), and as obligate repressors, miRNAs are potentially important components of such motifs. These findings challenge the view that individual miRNAs simply fine-tune GRNs in which TFs are the major players (Hornstein and Shomron, 2006). Our data demonstrate that miR-142-3p is essential for the specification of the hemangioblastic precursors of the blood stem cell lineage and suggests that miRNAs can be instructive determinants of cell fate, as has been proposed for transcription factors (Graf and Enver, 2009).

miR-142-3p has been reported to have various roles in hematopoiesis and leukemia; however, we have discovered that it has an unanticipated function in the specification of the early hematoendothelial precursors of the blood stem cell lineage. Many of the TFs regulating HSC development in the embryo have evolved to have additional important roles in adult hematopoietic cells and are often mutated or dysregulated in leukemia. miR-142 was identified at the breakpoint of a translocation involving the MYC oncogene in an aggressive B cell leukemia (Gauwerky et al., 1989) and has recently been found to be mutated in 20% of diffuse large B cell lymphomas (Kwanhian et al., 2012). It will therefore be exciting to investigate whether epigenetic or genomic alterations in this miRNA are involved in driving hematological malignancies.

The identification of miR-142-3p as a master regulator of definitive hemangioblast specification suggests that miR-142-3p may facilitate HSC formation by enhancing the formation of hemangioblast precursors during in vitro hematopoietic differentiation of pluripotent stem cells. Furthermore, we have identified other candidate miRNAs that may regulate HSC programming through our analysis of miRNA expression in embryonic HSC precursors. It may therefore be possible to identify a set of miRNAs, including miR-142-3p, that are able to synergize with cell signaling pathways to drive HSC emergence from pluripotent stem cells for regenerative medicine, just as the miR-302/367 cluster efficiently reprograms somatic cells to a pluripotent state (Anokye-Danso et al., 2011).

EXPERIMENTAL PROCEDURES

Embryo Manipulation and Whole-Mount In Situ Hybridization

Embryos were obtained and cultured as described previously (Walmsley et al., 2005) and staged in accordance with the method outlined in Nieuwkoop and Faber (1967). Embryo microinjection and whole-mount in situ hybridization (WISH) were performed as previously described (Walmsley et al., 2005). Before photography, embryos were cleared in benzyl benzoate:benzyl alcohol (2:1). For details of probes used, see Table S1. All embryos were hybridized as whole mounts. All animal work was carried out according to UK Home Office regulations under the appropriate project license.

Morpholinos and miR-142-3p Mimic

Morpholinos (MOs) (GeneTools) and miR-142-3p mimic (Dharmacon, Thermo Fisher) were injected into both blastomeres of two-cell-stage *Xenopus laevis* embryos in the following amounts per embryo: Dgcr8 MO, 10–15 ng; miR-142-3p MO and control mismatch MO, 100 ng; miR-142-5p MO, 40 ng; miR-142-3p mimic, 10–12.5 fmol. MO sequences can be found in the Supplemental Experimental Procedures.

Preparation and FACS Sorting of Mouse Cells

For purification of the adult mouse HSC population, lineage negative cells were isolated from bone marrow of C57BL/6, 9-week-old mice as described previously (Rodrigues et al., 2005). Lineage depletion was performed using AutoMACS and goat anti-rat IgG MicroBeads (Miltenyi). We used the following lineage antibodies: purified rat anti-mouse CD45/B220, Gr-1, Mac1, CD3, CD4, CD8 (eBioscience), and Ter119 (BioLegend). We used the following antibodies for sorting HSCs: Sca1-PE (BD Pharmingen), c-Kit/CD117-APC, CD135/Flt3-biotinylated, and Streptavidin-PE-Cy7 (eBioscience). F(ab')2 fragment of goat anti-rat IgG (H^+L)-PE-Cy5 (Molecular Probes) secondary antibody was used to exclude Lin+ cells. Hoechst58 was used to exclude dead cells. Cell sorting was performed on a MoFlo (Beckman Coulter), and cells were collected in RNA later.

For isolation of mouse AGM populations (as described previously in Ruiz-Herguido et al., 2012), CD1 embryos were obtained at 11 days after detection of vaginal plug. The AGM region was dissected and digested in 0.1% collagenase (Sigma-Aldrich) in PBS supplemented with 10% FBS for 20 min at 37°C. Cells were stained for 30 min with the different antibodies. We used the following antibodies from Becton Dickinson : CD31-PE, CD45-PECy7, and CD117-APC. Ter119-FITC (BD) was used to exclude erythroid cells, and DAPI staining (Molecular Probes) was used to exclude dead cells. Cell sorting was performed on a FACS Aria (BD), and cells were collected in RNAlater solution.

miRNA Profiling and qRT-PCR

Megaplex primer pools were used to perform reverse transcription and preamplification of mouse RNA samples, and miRNA profiling was performed using Taqman Rodent miRNA Arrays (v2.0) on a 7900HT quantitative real-time PCR machine (Applied Biosystems). Individual Taqman miRNA assays were used to quantify relative expression of miR-142-3p and miR-142-5p in mouse and *Xenopus* RNA samples normalized to the endogenous control, U6 small nuclear RNA.

Bioinformatic and Statistical Analysis

miRNAs undetectable in all of the AGM populations analyzed were removed from the analysis, and mean ΔCt values were calculated for each remaining miRNA. Genesis software was used to perform clustering analysis. ΔCt values were mean centered and normalized by SD to calculate the *Z* score, and hierarchical clustering was performed using Pearson's correlation.

ChIP-seq peak data for ten hematopoietic TFs in mouse hematopoietic progenitors was obtained from a published data set (Wilson et al., 2010). The number of peaks was scored for each TF within ±5 kb of the mouse pre-miRNA loci obtained from miRBase (http://www.mirbase.org; Kozomara and Griffiths-Jones, 2011).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2013.06.023.

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(D) Treatment of miR-142-3p morphants with a pan-TGFβ type I receptor family inhibitor SB431542, (top row) or a Tgfbr1-specific inhibitor LY2157299, (bottom row), from stages 16–17 partially rescues the specification of hemangioblasts as assayed by WISH analysis of *flk1* expression in the DLP (arrows) at stage 25. The number of embryos represented by each panel, out of the number analyzed, is indicated in the top right corner.
(E) Circuit diagram representing the DLP hemangioblast GRN based on data presented here and in previous analyses (Ciau-Uitz et al., 2010, 2013; Liu et al., 2008; Walmsley et al., 2002). Dashed connector indicates that the mechanism by which TGFβ signaling impinges upon the GRN is not yet known.

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