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CHARACTERIZATION AND CONTROLLING THE SPECIFICATION OF DEFINITIVE HEMATOPOIESIS DURING THE DEVELOPMENT OF ZEBRAFISH

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ABSTRACT

Modern studies propose that, in the primary mesoderm, there is collaboration between the hematopoietic and cardiac lineages. Though, whether miRNAs can affect other lineages remains unidentified. Consequently, we have investigated whether hematopoietic miR-142-3p modulated the mesoderm formation. We account that knockdown of miR-142-3p, a hematopoietic-specific miRNA, in zebrafish occasioned in damage of hematopoiesis during embryonic development. Interestingly, we detected abnormal cardiac phenotypes and inadequacy of somitegenesis in KD-morphants. In the early developmental phase, a tiny heart, contractile dysfunction in the ventricle, cardiac arrhythmia and bradycardia were constantly observed. Histological examination exposed unadorned hypoplasia of the ventricle and disrupted muscle alignment. To determine the mechanism, we executed DNA microarray analysis. The results exposed that the expression of several mesodermal genes necessary for the development of cardiac and somatic mesoderm, such as no tail, T-box gene 16, mesoderm posterior a, one eye pinhead, and rho-associated, coiled-coil containing protein kinase (Rock2a), were augmented in miR-142-3p KD-morphants. In this paper, the characterization and controlling the specification of definitive hematopoiesis during the development of zebrafish has been elaborated.

Keywords: hematopoiesis, MicroRNA, mesoderm, cardiac development.

1. INTRODUCTION

A microRNA is a small non-coding RNA molecule (containing about 22 nucleotides) found in plants, animals and some viruses, that tasks in RNA silencing and post-transcriptional regulation of gene expression. While bulk of miRNAs are located inside the cell, some miRNAs, frequently recognized as circulating miRNA or extracellular miRNA, have also been found in extracellular environment, comprising numerous biological fluids and cell culture media. Mature miRNAs are engendered from long endogenous primary transcripts by the RNase III enzymes, Drosha and Dicer ensuing in 22-nt double-stranded RNAs. One strand of the duplex gets assembled into the RNA-induced silencing complex (RISC) coextensive with target identity and coupling. MiRNAs deliver an elegant mechanism that gives finely tuned control of protein levels by controlling translational efficacy and mRNA strength. These non-coding small RNA molecules are quickly becoming recognized as key regulators of varied cellular functions, including the specification and differentiation of hematopoietic cells. MiRNA can regulate the expression of genes involved in procedures such as development, differentiation, proliferation and apoptosis and play an imperative role in cancer. Furthermore, miRNA can regulate the expression of more than 30% of protein-coding genes and fascinatingly, more than 50% of miRNA genes are located in cancer-associated genomic regions, signifying that they possess a part in the pathogenesis of human cancers. The first miRNA (lin-4) was exposed in 1993 and since then, hundreds if not thousands of miRNAs have been identified and added to various databases. Accepting the function of miRNA stems from escalating their production, which commences with the transcription of primary transcripts called pri-miRNAs by RNA polymerase II. Pri-miRNAs range from hundreds to thousands of nucleotides in length and so must experience three cleavage steps before their activation to regulate genes (Rushworth). Processing begins in the nucleus using ribonuclease the Drosha and DGCR8 (Di-George syndrome critical region 8) [15] complex to custom an intermediate hairpin called a pre-miRNA of about 70-100 nucleotides long. The pre-miRNA is transported out of the nucleus by exportin-5 and taken to the cytoplasm to be converted to an 18-25 nucleotide, then mature double-stranded miRNA is treated using a ribonuclease named Dicer. Consequently, the miRNA double-strand is separated to form a mature, active miRNA using an effector complex called RNA-induced silencing complex (RISC). Following the establishment of this mature miRNA, the specific complementary region of the 3' un translated region (UTR) of a messenger RNA (mRNA) can be directed. Upon binding of the mRNA and miRNA, the RISC complex induces degradation of the double-stranded mRNA. Added possible mechanism that miRNAs use is the blocking of protein translation processes, thus resulting in the elimination of that specific protein that is being expressed.

Inspection of tumour-specific miRNA appearance profiles has exposed that miRNAs could be master regulators of many features of tumour biology and many studies have shown that miRNA [1] themselves can purpose as tumour suppressor genes or oncogenes, where gene repression or overexpression can have a analytical and prognostic significance. Up regulation of oncogenic miR-17 by cMyc was found to move cell cycle control mechanisms and to interrupt the apoptotic regulator E2F1. Following these key discoveries in 2005, several

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correlations between miRNA expression [13], with Weinberg's six hallmarks of cancer having been recognized. Despite the swell in epigenetic research in the last epoch, the characters of miRNA in numerous human cancers like leukaemia and lymphoma have not been obviously defined. Manipulation of miRNA regulation could be a fresh approach in achieving an understanding the regulatory mechanisms of miRNA in cancer [2, 3, 10]. Changes in the expression of a number of transcription factors have been related with various blood cancers, with increasing evidence supporting a role for the regulation of transcription factors by miRNAs. The cardiovascular system is the primary organ system that grows during embryogenesis, and is indispensable for embryo viability and survival. MiRNAs are also highly articulated in the cardiovascular system; though, their biological roles in the mammalian cardiovascular system have only been explained since 2005 [19]. Several studies have confirmed that miRNAs play significant roles not only in cardiovascular development, but also in cardiovascular disease [8].

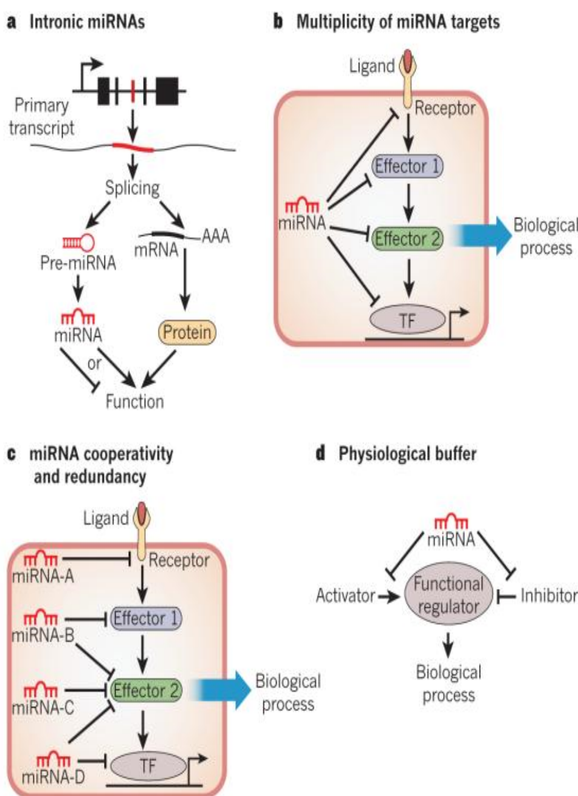

Figure-1. Concepts of miRNA function.

2. ROLE OF miRNAs IN CARDIOVASCULAR DEVELOPMENT

MicroRNAs are ~22-nucleotide single-stranded RNAs that constrain the expression of specific mRNA targets through Watson–Crick base pairing between the miRNA 'seed region' and sequences frequently situated in the 3′ untranslated regions (UTRs) [23]. The human genome is assessed to encode up to 1,000 miRNAs, which are moreover transcribed as standalone transcripts, regularly encoding several miRNAs, or produced by the processing of introns of protein-coding genes. The incorporation of miRNAs into introns of protein-coding genes helps to coordinate the expression of the miRNA with the mRNA encoded by that gene, without the requirement for a distinct set of *cis*-regulatory elements to drive expression of the miRNA (Figure-1). It is not infrequent for intronic miRNAs to modulate the identical biological procedures as the protein encoded by the host gene. The twin functions of such genes, encoding protein and miRNA, deliver sophisticated feedback and feedforward supervisory networks, explicit examples of which are emphasized throughout this review. The attractiveness of miRNAs in cardiac development has been well described in several studies. miR-1 is explicitly expressed in cardiac and skeletal muscle of embryonic mice, and this expression is organized by several key heart transcription issues such as SRF (SRF is a cardiac-enriched transcription factor responsible for the regulation of organized sarcomeres in the heart) and Mef2 (The myocyte enhancer factor-2 (MEF2) proteins are MADS-box transcription factors that are essential for differentiation of all muscle lineages).

Overexpression of miR-1 consequences in thin-walled ventricles, heart failure and developmental arrest at embryonic day 13.5, due to a noteworthy reduction in the number of cycling myocardial cells. In addition, overexpression of miR-1 decreased the level of Hand2 protein without altering its mRNA level, signifying that Hand2 is a target of miR-1 during heart development. The indispensable role of miRNAs in cardiovascular development is demonstrated meanderingly in Dicer-deficient mice that lose miRNAs: both heart and vessel development are extremely impaired. Additionally, targeted deletion of the muscle-specific miRNA miR-1-2 also associates miRNAs as important players in cardiovascular development. Dependable with the above animal studies, miRNAs also play an imperative role in Drosophila heart development.

3. THE ROLE OF MiRNA AND HUMAN BLOOD CANCERS

In 2004 Chen and contemporaries first recognized the connection between miRNAs and haematopoiesis regulation, determining that distinct haematopoietic cell types differentially stated miRNAs, given that the same miRNAs were not always expressed in all ancestries. Their results recommended that the exact miRNAs are induced during lineage differentiation and could influence haematopoietic lineage differentiation in mice, though differences in the expression pattern of the same miRNAs in humans have been described. Furthermore, mature miRNAs were found to control haematopoietic differentiation-associated mRNA on CD34+ cells, and particularly miR-155 represented an inhibitor of haematopoietic stem progenitor differentiation. It has also been described that members of the miR-30 family by targeting the transcription factor PRDM1, control the

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differentiation of lymphocytes to plasma cells. More than the deregulation of miRNAs, haematopoietic deletion of AGO2 or of Dicer resulted in disruption of erythropoiesis, with plain anemia [22], splenomegaly, and maturation arrest of erythroid precursors [16]. The possible use of miRNAs as prognostic markers in clinical practice has previously been demonstrated, as expression levels of miRNAs could forecast the time to first treatment in CLL patients, were associated with mutations of established molecular prognostic factors(Nana-Sinkam and Croce), and were also related with overall survival in patients with hepatocellular carcinoma, pancreatic cancer, colon adenocarcinoma, lung cancer, esophageal cancer, and melanoma. Likewise, miRNAs have been evaluated in the context of chemosensitivity to evaluate the individual chemoresponse in both *in vitro* and *in vivo* models.

The measurement of miRNAs levels in plasma or serum has concentrated them useful in the diagnosis of solid malignancies such colorectal cancer, lung, prostate, and kidney cancer. Nevertheless, it has not been clarified whether miRNA circulating levels are tumour-created or signify a systemic response, and it is not clear yet which is the best specimen among serum, plasma, or peripheral blood mononuclear cells, used for the miRNA signature detection [24]. The significant purposes of miRNAs in cancer make them striking therapeutic targets; consequently efforts should be made to recognize which miRNAs could be used to attain clinical benefits against cancer. There is undoubted evidence for a main role of miRNAs in cancer. This connection was first recommended in 2002 by Calin et al., with the detection that miR-15 and miR-16 were located on chromosome 13q14, a region regularly deleted in CLL. Upon inspecting the expression levels of these miRNAs, miR-15 and miR-16 were condensed or eliminated in 68% of all CLL cases tested. They also observed that the 13q14 deletion was commonly the only genetic irregularity in patients and hence the deletion of miR-15/16 may be a direct cause of CLL. Upon inspection of genomic locations of miRNAs, they stated that many miRNA-coding regions are situated in delicate regions of the genome that are regularly amplified or deleted in many cancers, disagreeing that gain or loss of miRNAs were nominated for in cancerous cells and underlie important tumourigenic steps. Global expression profiling discovered alterations of miRNA expression patterns first in CLL and then in other malignancies [18]. These readings reviewed by Munker et. al., displayed that the expression of several miRNAs (miR-17-5p, miR-20a, miR-21, miR-92, miR-106a and miR-155) was augmented in the majority of tumour types, arguing that these may be communal oncogenic miRNAs. It was also noted in these studies that miRNA expression patterns could discriminate tumours and tissue types, signifying that miRNA expression levels may be useful biomarkers for cancer. Such miRNA expression patterns were then found to be associated with deprived prognosis of CLL and lung cancer, proposing further demonstration of such potential [21]. Subsequent mechanistic studies confirmed that alteration of specific miRNAs could affect cell proliferation, apoptosis, tumour growth and angiogenesis in mouse models. Completely, the evidence is substantial that alterations of miRNAs occur during and contribute towards leukaemogenesis.

MiRNAs control elementary biological functions and are developing as key regulators of haematopoiesis. Previous study focused on the functional role of miR155 on megakaryocytic (MK) differentiation of human cord blood CD34+ haematopoietic progenitor cells (HPCs). MiR155, plentifully expressed in early HPCs, drops sharply during MK differentiation. Functional studies displayed that enforced expression of miR155 impairs proliferation and differentiation of MK cells. Furthermore, HPCs transfected with miRN155 exhibited a substantial reduction of their MK clonogenic capacity, signifying that down-modulation of this miRNA favors MK progenitor differentiation. These results display that the decline of miR155 is required for MK proliferation and differentiation at progenitors and precursors level and indicate that sustained expression of miR155 inhibits megakaryopoiesis. Hematopoiesis is regulated by miRNAs. These minor regulatory RNAs are master regulators of developmental processes which modulate expression of numerous target genes post-transcriptionally. Several miRNAs are up-regulated at specific stages during hematopoietic development and the functional relevance of miRNAs has been confirmed at many stages of lineage specification. Knock-out of specific miRNAs can produce dramatic phenotypes leading to severe hematopoietic defects. Furthermore, several studies demonstrated that specific miRNAs are differentially expressed in hematopoietic stem cells. Though, the emergent picture is tremendously complex - due to alterations between species, cell type dependent variation in miRNA expression and differential expression of diverse target genes which are involved in various regulatory networks. There is also indication that miRNAs play a role in cellular aging or in the inter-cellular crosstalk between hematopoietic cells and their microenvironment [4, 6]. The field is quickly evolving due to new summarizing tools and deep sequencing technology. The expression profiles of miRNAs are of diagnostic significance for classification of dissimilar diseases. Modern reports on generation of induced pluripotent stem cells with miRNAs have fueled hope that specific miRNAs and culture conditions facilitate directed differentiation or culture expansion of the hematopoietic stem cell pool. This current review condenses our current knowledge about miRNA expression in hematopoietic stem and progenitor cells and their role for the hematopoietic stem cell niche.

MiR-146a is a miRNA that is supposed to regulate physiological and pathophysiological pathways in hematopoietic cells. In this review, they emphasis on recent development in analyzing the functional roles of miR-146a in normal hematopoiesis and hematopoietic disease. They suggest that manipulation of miR-146a expression may signify a potential new therapy for several hematopoietic diseases, and may further help as a biomarker for diagnosis, prevention, and treatment of such disease. MiRNA control gene expression at the post-

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transcriptional level by debasing or translational repressing target messenger RNA (mRNA). Countless studies have addressed the role of miRNA in normal hematopoiesis, giving an explanatory key to the aberrant expression observed in human hematological diseases. Here, the advances of chief studies on the role of miRNA in normal hematopoiesis, and recognize the association of miRNA with the development, progression of myeloproliferative diseases, comprising miRNA and lymphopoiesis, miRNA and erythropoiesis, miRNA and megakaryopoiesis, miRNA and myelopoiesis. The past 5 years have seen an explosion of knowledge about miRNAs and their roles in hematopoiesis, cancer, and other diseases. In myeloid development, there is a rising gratitude for both the importance of particular miRNAs and the unique features of myelopoiesis that are being uncovered by experimental manipulation of miRNAs [12].

4. MEIS1 IN NORMAL HEMATOPOIESIS

Meis1 is one of the most extremely preserved transcription factors in hematopoiesis with over 90% amino acid sequence homology between zebrafish and other vertebrates [9, 11]. They exploited this high level of conservation and that of additional key regulators of hematopoiesis, e.g. scl, gata1 and gata2, and determined the outcome of morpholino (MO) giveaway of zebrafish meis1 on progenitor and hematopoietic stem cell development. In addition they considered the development of the vascular system where angiogenesis and remodeling procedures are accountable for the creation of a functional circulatory system and stem cell niches. In topical years, inquiries into the origin of Leukemia stem cells (LSCs) have exposed that these cells are amazingly similar to normal Hematopoietic stem cells (HSCs), with respect to their capability to self-renew, cell surface markers, and differentiation capacities. Consequently, it is imaginable and likely most credible that *Meis1* shows a pivotal role in normal HSC biology as well. Numerous additional lines of evidence indicate that this may be the case. First, *Meis1*[14], along with *Hox* genes, are co- expressed in the most primitive hematopoietic subpopulations and are down-regulated following differentiation. Second, Meis1-deficient mice die by embryonic day 14.5, giving with extensive hemorrhaging due to the absence of megakaryocytes. While ultimate myeloerythroid lineages are present in these embryos, the total numbers of colony-forming cells are expressively reduced. *Meis1*$^{-/-}$ fetal liver cells fail to radio protect fatally irradiated recipient mice and they participate poorly in repopulation assays even though they upkeep the formation of all hematopoietic lineages. It is conceivable that gene redundancy amongst the closely related *Meis* family members may recompense for the loss of *Meis1*, and thus may prevent the complete abrogation of HSC activity in these mice. Taken together, these readings provide robust support for the hypothesis that *Meis1* plays an important role in the HSC self-renewal/proliferation.

Over the years the zebrafish has confirmed its suitability as a model system for furthering our understanding of the genetic instruction of hematopoiesis in both normal and pathological states [17]. As in mammals, embryonic and definitive hematopoiesis take place in zebrafish in anatomically dissimilar locations and can be further distinguished on the basis of cell types produced. Primitive hematopoiesis yields primitive macrophages, which derive from cephalic mesoderm, and primitive erythrocytes from the intermediate cell mass (ICM). Whereas definitive hematopoiesis gives rise first to erythromyeloid progenitors in the posterior blood island and later to hematopoietic stem cells in the aorta-gonad-mesonephros (AGM) region, from the production site in the AGM, hematopoietic stem cells travel to the caudal hematopoietic tissue, where they enlarge and finally reach the pronephros and thymus, thus resolving in their final destination, the stem cell niches.

Since HOXA9 and MEIS1 play important developmental roles, are collaborating DNA binding proteins and leukemic oncoproteins, and are significant for normal hematopoiesis, the regulation of Meis1 by its partner protein is of interest. Loss of Hoxa9 produced down regulation of the Meis1 mRNA and protein, while forced HOXA9 expression up regulated Meis1 [5, 7]. Hoxa9 and Meis1 expression was associated in hematopoietic progenitors and acute leukemia. Meis1+/− Hoxa9−/− deficient mice, generated to test HOXA9 regulation of endogenous Meis1, were minor and had reduced bone marrow Meis1 mRNA and important defects in fluorescence-activated cell sorting-enumerated monocytes, mature and pre/pro-B cells, and functional B-cell progenitors. These data specify that HOXA9 modulates Meis1 during normal murine hematopoiesis. Recently; augmented evidence has shown that serum miRNA levels are a useful biomarker for the diagnosis, prognosis and therapeutic value of various diseases. Though, serum miRNA has not been explored in patients with systemic sclerosis (SSc), to our knowledge, Specify that serum levels of miR-142-3p may be raised specifically in patients with SSc, correlating with the sternness of this disease, and may be useful diagnostic markers for the presence of SSc and for the differentiation of SSc from SSD. RAC1 regulates a various array of cellular events including migration and invasion. MiRNAs have a significant role in the regulation of gene expression. They confirmed that miRNA-142-3p acted as a negative regulator of human RAC1. Overexpression of miR-142-3p diminished RAC1 mRNA and protein levels. Furthermore, the overexpression of miR-142-3p inhibited, while blocking of miR-142-3p increased colony formation, migration and invasion in hepatocellular carcinoma (HCC) cell lines (QGY-7703 and SMMC-7721).

5. MATERIALS AND METHODS

5.1. Zebrafish conservation and morpholino injection

Transgenic (Tg) zebrafish (Cmlc2: GFP and Gata1:dsRed) were kept under standard laboratory situations at 28°C. Morpholino (MO) antisense oligonucleotides were acquired from Gene Tools [dre-miR-142a-3p MO, TCCATAAAGTAGGAAACACTACA,dre-miR-142a-

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5pMO, AGTAGTGCTTTCTACTTTATG]. The general fertilized embryos were inoculated with the MO at the 1-4-cell stage.

5.2. Immunohistochemistry

Embryos were immovable for 1 hour at room temperature in presence of 4% paraformaldehyde, incubated for 1 hour in 10%, 20%, and then 30% sucrose/ phosphate-buffered saline (PBS), entrenched in OCT compound, and partitioned with a cryostat. Sections were stained with hematoxylin–eosin (H&E).

5.3. O-Dianisidine stain

Embryos were de-chorionated at 36 h post-fertilization (hpf) and stained for 15 min in 0.6 mg/mL O-dianisidine (D9143; Sigma), sodium acetate (0.01 M, pH 4.5), H_2O_2 (0.65%), and ethanol (40%). 2.4. Appearance of miR-142-3p in mouse tissues (real-time quantitative reverse transcription-polymerase chain reaction). Overall RNAs were isolated from dissimilar tissues of adult and embryo mice using mirVana (Ambion). The concentration and excellence of the isolated RNA were determined spectrophotometrically. After DNaseI treatment, 10 ng RNA was reverse-transcribed by the usage of the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and with each primer and probe set (hsa-miR-142-3p, 000464, U6 snRNA, 001973). Real-time polymerase chain reaction was done using TaqMan MicroRNA Assays according to the manufacturer's instructions.

5.4. DNA microarray analysis

Zebrafish genome-wide gene expression analysis was achieved using the Affymetrix GeneChip zebrafish genome array. RNA was extracted from 24 hpf embryos using an RNeasy mini kit (QIAGEN) conferring to the manufacturer's instructions. Microarray analysis was achieved using the standard protocol supplied with the Affymetrix GeneChip.

5.5. Statistical analysis

All experiments were performed at least three times. Data are assumed as the mean ± SD. Student's t-test was employed for statistical comparisons. $P < 0.05$ was considered noteworthy.

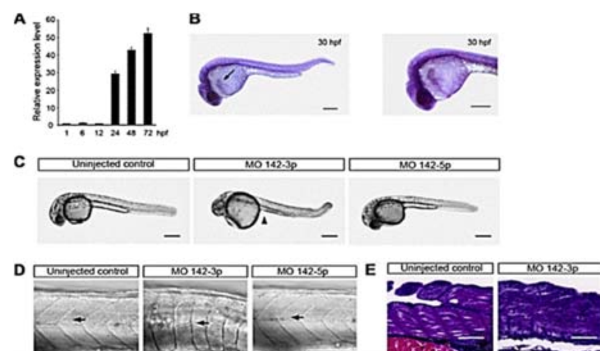

Figure-2. MiR-142-3p is extremely articulated in the yolk and inhibition of miR-142-3p consequences in disrupted somitogenesis. (A) Quantification of miR-142-3p at dissimilar embryonic phases by qRT-PCR. (B) Whole mount in situ hybridization (WISH) analysis of miR-142-3p at 30 h post fertilization (hpf). Countenance of miR-142-3p was detected over the yolk (arrow). Lateral views, anterior to the left. Original magnification _5 (left panel) and _8 (right panel). Scale bar, 300 lm. (C) Lateral views (anterior to the left) are revealed of uninjected control, miR-142-3p morpholino-injected zebrafish (MO142-3p) and miR-142-3p morpholino-injected zebrafish (MO142-5p) at 30 hpf. Bright field microscopy exposed reduced melanogenesis and inadequate yolk extension (arrowhead). Scale bar, 300 lm. (D) Lateral views of the trunk region of uninjected control, MO142-3p and MO142-5p embryos at 30 hpf. The somite boundary was uneven in MO142-3p embryos. Scale bar, 100 lm. (E) H&E-stained segments of the trunk at 72 hpf. Placement of muscle filaments is irregular in MO142-3p embryos. Scale bars, 100 lm.

6. RESULTS

6.1. miR-142-3p is highly enriched in all hematopoietic tissues

The mature sequence of miR-142 found in numerous species, comprising of human, mouse, and zebrafish, is extremely conserved (Figure-2A). The pre-miR-142 stem loop has two mature miRNAs, specifically miR-142-3p and miR-142-5p, in a hairpin structure (Fig. 2B). Northern blot analysis to describe miRNA localization in mouse tissues revealed that miR-142s were extremely uttered in all hematopoietic tissues. In the present study, to determine whether miR-142-3p was enriched in hematopoietic tissues in vivo, we have used quantitative (q) RT-PCR with RNA from tissues obtained from adult and embryonic mice. We found that miR-142-3p was extremely articulated in all hematopoietic tissues, comprising of the bone marrow, spleen, thymus and fetal liver. Higher expression on E12.5 in the fetal liver, an embryonic hematopoietic system, suggests that miR-142-3p may play an essential role in early hematopoietic development (Fig. 3A, B). In zebrafish, miR-142-3p expression was made in 24 hpf embryos and increased between 48 and 72 hpf (Fig. 2A). To inspect the anatomical localization of miR-142-3p, we estimated the miR-142-3p expression profile in zebrafish embryos by the usage of whole-mount in situ hybridization (WISH). At 30 hpf, miR-142-3p was observed in erythrocytes pooling over the yolk during fixation for WISH (Fig. 2B).

6.2. miR-142-3p modulates somitogenesis in vivo

The mature miRNA sequences of zebrafish miR-142-3p and miR-142-5p match those of their mammalian orthologs. Because temporal inhibition of miRNAs by antisense molecules is the method used to examine the loss of function of miRNA, we used it in the present study in zebrafish embryos. Microinjection into fertilized eggs of an antisense MO targeting miR-142-3p reduced miR-142-

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3p function during zebrafish development. At 6 hpf, MO-injected embryos did not display any differences compared with uninjected embryos. Nevertheless, at 30 hpf, gross morphological examination revealed that pigmentation of the skin seemed obscurely and yolk extension was inadequate (Figure-2C, arrowhead) in miR-142-3p MO-injected zebrafish (MO142-3p). Closer inspection revealed that somite formation was feeble globally (Figure-2D). To evaluate the defects in the MO-injected embryos further, we inspected histological sections. In wild-type embryos, regular alignment of muscle filaments is clearly seen. In contrast, in longitudinal H&E-stained sections from MO142-3p at 72 hpf, bundles of muscle filaments were disrupted (Figure-2E). We confirmed the specificity of the MO142-3p-induced phenotypes by injection of miR-142-5p MO (MO142-5p).

6.3. miR-142-3p is essential for hematopoiesis in vivo

The occurrence of circulating blood cells was importantly reduced at 48 hpf in MO142-3p (Figure-3A, B). We examined the percentage of gross phenocopies of hematopoietic defects at 30 hpf. Around 92% (n = 102/111) of MO142-3p embryos displayed markedly reduced circulating erythrocytes. We confirmed the effect of inhibition of miR-142-3p by the usage of Tg Gata1:dsRed reporter zebrafish, which directs the transgene in blood cells. Though circulation of blood occurred typically between 24 and 30 hpf, the presence of Tg gata1:dsRed-expressing blood cells in vessels was markedly reduced in MO142-3p embryos (Fig. 3C). In non-injected and MO142-5p embryos at 36 hpf, the appearance of erythroid cells in the vessels was evidenced by O-dianisidine staining. In distinction, MO142-3p embryos caused severe defect of erythrocytes indicated by abridged hemoglobinized cells throughout the embryo (Fig. 3D). We injected 0.2 mM and 1.0 mM morpholino, and the percentage of the reduction of erythroid cells was 29% (n = 7/24), 94% (n = 49/52), correspondingly. These data suggest that MO dose-dependently impairs hematopoiesis in zebrafish embryos (Figure-3E).

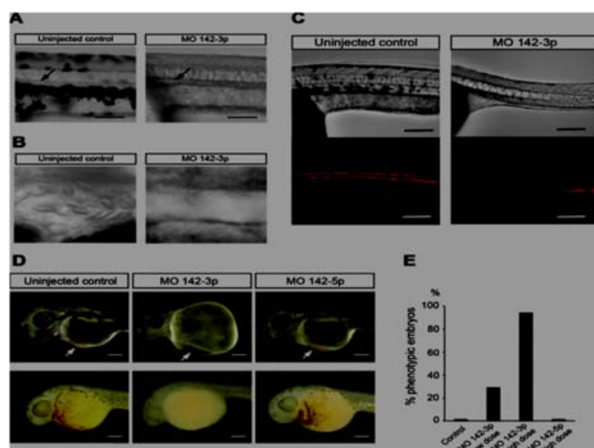

Figure-3. miR-142-3p is obligatory for the maturation of erythrocytes. (A) Reticence of miR-142-3p caused in a lessening of blood cells in vessels. Bright field images (48 hpf). Scale bar, 300 lm. (B) Advanced magnification view of the blood vessels enlightening a decrease of blood cells. (C) Lateral views of wild-type and miR-142-3p morpholino-injected transgenic (Tg; Gata1: dsRed) zebrafish at 30 hpf. There was a noteworthy lessening in blood cells (Gata1: dsRed) in the vessels in the morphants. Scale bar, 300 lm. (D) Inhibition of miR-142-5p does not reason for hematopoietic defects. There is a noteworthy lessening of blood cells in vessels in MO142-3p embryos. Reduced hemoglobin staining by O-dianisidine was obvious at 36 hpf. Normal hemoglobin expression was understood in wild-type (WT) and MO142-5p embryos. Scale bar, 300 lm. (E) There were a superior number of MO142-3p embryos with reduced circulating cells at 36 hpf.

6.4. miR-142-3p has an effect on cardiac function

Inhibition of miR-142-3p resulted in cardiac malformations. By means of a Tg Cmlc2:GFP reporter zebrafish, which articulates green fluorescent protein (GFP) in cardiomyocytes, we assessed the consequence of miR-142-3p knockdown on cardiac development. The MO142-3p-injected phenotype was first recognized at around 24 hpf as a tiny heart and bradycardia associated with wild-type embryos, in which the heart has accomplished tube formation and starts beating at this phase. At 48–72 hpf, the embryos with the cardiac phenotype displayed a looping irregularity and contractile dysfunction of the ventricle. Histological examination revealed that the ventricular wall appeared stripper in these embryos than in wild-type embryos. Nevertheless, the MO142-5p-injected phenotype showed very few cardiac anomalies. Also, MO142-3p-injected embryos showed cardiac arrhythmias, such as a 2:1 ratio of atrial: ventricular beating, and pericardial edema. The average heart rate of morphants was considerably slower than that of wild-type embryos. To enumerate the cardiac function, end-diastolic and end-systolic zones of wild-type and morphant ventricles were distinguished and the percentage fractional area change (%FAC) calculated. At 60 hpf, a reduction in %FAC was detected in MO142-3p-injected embryos. Laidback, these results demonstrate that miR-142-3p modulates normal cardiac formation and function [20].

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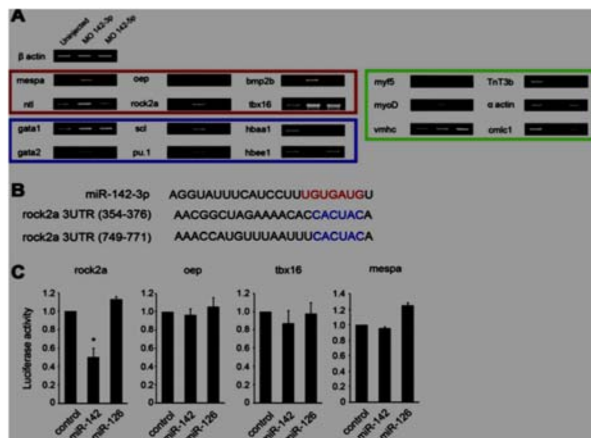

Figure-4. Identification of miR-142-3p targets. (A) Gene expression, as evaluated by reverse-transcription polymerase chain reaction (RT-PCR), at 24 hpf. (B) Sequence complementarities of a potential miR-142-3p-binding site in the rho-associated, coiled-coil containing protein kinase (Rock2a) 30-untranslated region (30-UTR). (C) Relative luciferase action of constructs comprising of the 30-UTR of the potential miR-142-3p binding site transfected into COS7 cells in the attendance of miR-142 or miR-126 (n = 3).

7. DISCUSSIONS

We used reverse genetic methods in the zebrafish to determine the purpose of miR-142-3p and confirmed that miR-142-3p controls hematopoiesis, cardiogenesis, and somitogenesis in vivo. Expression examination exposed that genes such as Scl/Tal1 and Gata2 were upregulated in MO142-3p embryos. Scl is a basic helix-loop-helix transcription factor and is stated from the 2- to 3-somite stage in the hemangioblast population, organized with Gata2. Knockdown of Scl in zebrafish caused in the disturbance of embryonic erythropoiesis and myelopoiesis. Gata2 is a zinc finger transcription factor that is mandatory for the proliferation and conservation of hematopoietic progenitor cells. In mammalian hematopoiesis, tenacious production of GATA2 maintains a stem cell phenotype and reductions in Gata2 expression or actions are mandatory for the differentiation of precursors to hematopoietic cells. Furthermore, gata1-positive cells were evidently reduced in MO142-3p embryos. Gata1 is a master transcription factor in erythrocyte maturation. In zebrafish, Gata1 is articulated from the 5-somite stage in the posterior lateral mesoderm along with Scl and Gata2. From the 12-somite stage, Gata1-positive cells of the ICM migrate anteriorly and then fright to express erythroid-specific genes and circulate. During erythroid differentiation, both GATA1 and GATA2 nucleate the binding of various protein complexes containing SCL following globin gene activation. The tenacious expression of Gata2 and Scl following knockdown of miR-142-3p may have impaired intact differentiation. On the basis of these findings, we think that miR-142-3p may disturb theadaptation from hematopoietic progenitor cells to mature erythrocytes that retain abundant hemoglobin. In the contemporary study, miR-142-3p was extremely expressed in all hematopoietic tissues, but not in other tissues, comprising the heart.

However, rock-bottom of miR-142-3p exaggerated cardiac and muscular development. Rendering to the microarray data, expression of Tbx16/Spadetail, a T-box transcription factor involved in mesoderm development, was augmented in MO142-3p embryos. The mutant phenotype of Spadetail displays disrupted primitive erythrocytes, as well as disrupted somitogenesis. In addition, Spadetail is mandatory for cardiac mesoderm formation via interaction with the Nodal-signaling pathway as Oep. Hence, a conceivable clarification for the cardiac and somatic phenotypes may be the persistent expression of transcriptional factors that, under normal circumstances, seem only transiently in the early mesoderm. MiR-142-3p may affect genes that play a significant role in the early stage of mesoderm formation, causing in disrupted hematopoiesis, cardiogenesis, and somitogenesis in MO142-3p embryos. As a result, the appearance of genes in each mature lineage could have been reduced.

Lately, the collaboration amongst hemangioblasts and cardiac progenitors in the anterior lateral plate mesoderm (ALPM) has attracted attention. Induction of the hematopoietic fate represses cardiac requirement and demarcates the volume of the heart field. In addition, scl is essential for superseding the latent cardiac developmental potential living within the rostral ALPM and thereby limiting heart size. In dissimilarity, cardiac precursors can provoke the hematopoietic lineage in embryonic stem cells. Over-expression of Nkx2.5 curbs hematopoietic differentiation. It has been projected that Nkx2.5 has a dual role in multipotent mesodermal progenitors of indorsing a cardiac fate and repressing a hematopoietic fate. Mammalian initial cardiac progenitors are recognized to express hematopoietic genes. The cardiac progenitors mined from crescent-stage embryos have plentiful hematopoietic genes. In addition, miR-142-3p is observed in E7.75 cardiac progenitors, as determined on the basis of microarray data. Based on this early expression pattern, we imagine that miR-142-3p is required in the early mesoderm and is an important regulator of the collaboration amongst both blood- and cardiac-inducing signals. Although it remains argumentative as to whether a common progenitor for cardiac and hematopoietic lineages exists, mesodermal cells perhaps have multilineage potential and can differentiate into hematopoietic and cardiac cells depending on the environment.

8. CONCLUSION AND FUTURE WORKS

Whether miRNAs can affect other lineages remains unidentified. Consequently, we have investigated whether hematopoietic miR-142-3p modulated the mesoderm formation. We account that knockdown of miR-142-3p, a hematopoietic-specific miRNA, in zebrafish occasioned in damage of hematopoiesis during embryonic development. Interestingly, we detected abnormal cardiac phenotypes and inadequacy of somitegenesis in KD-morphants. In the early

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developmental phase, a tiny heart, contractile dysfunction in the ventricle, cardiac arrhythmia and bradycardia were constantly observed. Histological examination exposed unadorned hypoplasia of the ventricle and dislocated muscle alignment. To regulate the mechanism, we executed DNA microarray analysis. The results exposed that the expression of several mesodermal genes necessary for the development of cardiac and somatic mesoderm, such as no tail, T-box gene 16, mesoderm posterior a, one eye pinhead, and rho-associated, coiled-coil containing protein kinase (Rock2a), were augmented in miR-142-3p KD-morphants. The character of MiR142-3p in the regulation of hematopoiesis during zebrafish development has been elaborated in this paper. As a future investigation, it would be interesting to elucidate how miRNAs, not mere mRNAs, control cell fate determination amongst hematopoietic and cardiac lineages from multipotent progenitors in mesoderm development.

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