Zebrafish microRNA miR-210-5p inhibits primitive myelopoiesis by silencing *foxj1b* and *slc3a2a* mRNAs downstream of *gata4/5/6* transcription factor genes

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Zebrafish *gata4/5/6* genes encode transcription factors that lie on the apex of the regulatory hierarchy in primitive myelopoiesis. However, little is known about the roles of microRNAs in *gata4/5/6*-regulated processes. Performing RNA-Seq deep sequencing analysis of the expression changes of microRNAs in *gata4/5/6*-knockdown embryos, we identified miR-210-5p as a regulator of zebrafish primitive myelopoiesis. Knocking down *gata4/5/6* (generating *gata5/6* morphants) significantly increased miR-210-5p expression, whereas *gata4/5/6* overexpression greatly reduced its expression. Consistent with inhibited primitive myelopoiesis in the *gata5/6* morphants, miR-210-5p overexpression repressed primitive myelopoiesis, indicated by reduced numbers of granulocytes and macrophages. Moreover, knocking out miR-210 partially rescued the defective primitive myelopoiesis in zebrafish *gata4/5/6*-knockdown embryos. Furthermore, we show that the restrictive role of miR-210-5p in zebrafish primitive myelopoiesis is due to impaired differentiation of hemangioblast into myeloid progenitor cells. By comparing the set of genes with reduced expression levels in the *gata5/6* morphants to the predicted target genes of miR-210-5p, we found that *foxj1b* and *slc3a2a*, encoding a forkhead box transcription factor and a solute carrier family 3 protein, respectively, are two direct downstream targets of miR-210-5p that mediate its inhibitory roles in zebrafish primitive myelopoiesis. In summary, our results reveal that miR-210-5p has an important role in the genetic network controlling zebrafish primitive myelopoiesis.

Myelopoiesis is a tightly regulated developmental process in which mature myeloid cells are generated. In vertebrates, myeloid cells arise from two successive waves during development: a primitive wave producing a limited number of myeloid cells and a definitive wave giving rise to all hematopoietic lineages including myeloid cells throughout lifetime. Zebrafish, unlike mammals, have a robust primitive myeloid pathway, which makes this model animal a powerful tool to study vertebrate myelopoiesis as well as human myeloid malignancies. In zebrafish, the primitive wave arising from both anterior lateral plate mesoderm (ALPM)[4] and posterior lateral plate mesoderm (PLPM), while a definitive wave occurs in the ventral wall of the dorsal aorta, which is analogous to the aorta-gonads-mesonephros region in mammals (1). Many studies have revealed the necessary role of a number of coding genes in primitive myelopoiesis, including *spi1b*, *irf8*, *gfi1ab*, *tal1*, and *etv2* (2–5). However, the roles of noncoding genes in zebrafish primitive myelopoiesis are still poorly understood.

Recent discoveries have implicated that microRNAs (miRNAs), a group of noncoding post-transcriptionally regulatory RNAs (~22 nt), are widely involved in murine hematopoiesis and zebrafish myelopoiesis (6–8). In murine myeloid progenitors, knocking out *Dicer1*, an essential miRNA processing RNase III enzyme, leads to abnormal myelopoiesis and depleted macrophages (9). Several miRNAs, such as miR-223, miR-146a, miR-21, and miR-196b have been intensively studied and shown to play important roles in murine and zebrafish definitive myelopoiesis (7, 10–12). These findings suggest an important role of miRNA during myelopoiesis in zebrafish, but essential players involved in primitive myelopoiesis are yet to be discovered.

Previously, others' and our works have proved that the transcriptional factors Gata4/5/6 lie on the apex of the regulatory hierarchy in zebrafish primitive myelopoiesis (13, 14). But whether there are miRNAs mediating the roles of Gata4/5/6 in the primitive myelopoiesis remains unknown. In the present work, we aim to study the miRNAs involved in the regulatory

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This article contains Figs. S1–S3 and Table S1.

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[4] The abbreviations used are: ALPM, anterior lateral plate mesoderm; PLPM, posterior lateral plate mesoderm; miRNA, microRNA; NC, negative control; nt, nucleotide(s); MO, morpholino; qRT, quantitative RT; sgRNA, single guide RNA; hpf, hour post fertilization.

network of zebrafish primitive myelopoiesis. We searched for *gata4/5/6*-regulated miRNAs by comparing their expression changes of *gata4/5/6*-knockdown embryos to those of control embryos. Our results revealed that miR-210-5p functions downstream of *gata4/5/6* and excess miR-210-5p plays an inhibitory role in zebrafish primitive myelopoiesis. In addition, the restrictive role of miR-210-5p was shown to be achieved by affecting the differentiation of hemangioblast into myeloid progenitor cells. Furthermore, we demonstrated that *foxj1b* and *slc3a2a* are two direct target genes of miRNA-210-5p to mediate its inhibitory roles in zebrafish primitive myelopoiesis.

Results

miR-210-5p works downstream of gata4, gata5, and gata6 to inhibit zebrafish primitive myelopoiesis

Previous works showed that *gata4/5/6* lie at the hierarchical apex of the regulatory network of the hemangioblast formation and primitive myelopoiesis in zebrafish (13, 14). To explore the roles of miRNAs in zebrafish primitive myelopoiesis, we employed deep sequencing to analyze the difference of miRNA expression profiles between *gata4/5/6*-knockdown embryos (*gata5/6* morphants) and control embryos. Because it is reported that morpholinos knocking down *gata5* and *gata6* also lead to the loss of *gata4* expression in zebrafish embryos (15), we used a combination microinjection of *gata5* and *gata6* morpholinos for knocking down all the three genes. With the Illumina Solexa platform, the deep sequencing for miRNAs generated 12.3 million reads from control embryos and 13.1 million reads from *gata5/6* morphants. Among them, about 7.16 and 6.83 million unique reads were annotated, respectively. We analyzed the expressions of 223 miRNAs and found the expression levels of 8 miRNAs exhibited a significant difference ($|\log FC| > 1$; $p < 0.01$) in *gata4/5/6*-knockdown embryos compared with controls (Fig. 1*A*). Among them, 5 were up-regulated and 3 were down-regulated, which was confirmed by quantitative RT-PCR (qRT-PCR) (data not shown). To test the function of these miRNAs during primitive myelopoiesis, we used miRNA mimics or inhibitors, which were chemically synthesized dsRNA oligos and could be used to mimic or inhibit the function of mature endogenous miRNAs *in vivo* (16), to achieve overexpression or inhibition of the 8 candidate miRNAs in WT embryos. The results revealed that the microinjection of miR-210-5p mimic led to the significant ($p < 0.01$) inhibition of primitive myelopoiesis with reduced numbers of granulocytes (*mpx*$^+$ cells), macrophages (*mfap4*$^+$ cells), as well as pan-leukocyte (*lcp1*$^+$ cells) as shown by whole mount *in situ* hybridization (Fig. 1, *B–H*) in the zebrafish embryos at 26 hpf. Consistently, microinjection of miR-210-5p mimic significantly ($p < 0.05$) reduced the expression levels of *mpx, mfap4*, and *lcp1* in the embryos at 26 hpf as demonstrated by qRT-RCR (Fig. 1*I*). However, the formation of both granulocytes and macrophages were not reduced together in the embryos microinjected with mimics or inhibitors of the other 7 miRNAs including miR-129-5p, miR-722, miR-7b, miR-30a, miR-125c, miR-738, and miR-153a, respectively (Fig. S1). These results suggest that miR-210-5p plays a restrictive role in zebrafish primitive myelopoiesis, and this miRNA was chosen for further study.

To confirm the restrictive role of miR-210-5p in zebrafish primitive myelopoiesis, we first tested whether microinjection of the miR-210-5p mimic into the zebrafish embryos did lead to the overexpression of the mature miR-210-5p. To do this, we performed whole mount *in situ* hybridization to examine the expression of miR-210-5p. The results showed that miR-210-5p was widely expressed in the embryos microinjected with mimic negative control (NC) at 14 hpf when primitive myeloid progenitors start to form (Fig. S2*A*) and the ones at 26 hpf when myeloid cells are formed (Fig. S2*B*), whereas the expression of miR-210-5p was significantly increased in the whole embryos at both 14 hpf (Fig. S2*C*) and 26 hpf (Fig. S2*D*) when the embryos were microinjected with the miR-210-5p mimic. To exclude the possibility that the increased expression of miR-210-5p was due to the denaturation of the double strand RNA (dsRNA) itself (miR-210-5p mimic) into the single strand RNA that is recognized by the LNA microRNA Detection Probes against miR-210-5p during whole mount *in situ* hybridization, we performed an *in vitro* assay by incubating the dsRNA with RNase A under the same conditions (without adding yeast tRNA) as the experiment of whole mount *in situ* hybridization, and the results showed that the dsRNA was still present after overnight incubation at 65 °C (Fig. S2*E*). The results support that the findings from the whole mount *in situ* hybridization represent the increase of mature miRNA-210-5p in the embryos microinjected with miRNA-210-5p mimic. To further confirm the results, we performed qRT-PCR examination to detect the mature miR-210-5p, and the results showed that the microinjection of miR-210-5p mimic caused significantly ($p < 0.01$) increased expression of mature miR-210-5p in the embryos at both 14 and 26 hpf (Fig. S2*F*). Taken together, our results verified that microinjection of miR-210-5p mimic did result in the increase of mature miR-210-5p in zebrafish embryos.

We next performed knocking-down *gata4/5/6* by microinjecting *gata5/6* MOs (14) or overexpressing *gata4/5/6* by microinjecting *gata4/6* mRNAs (13) into 1-cell stage zebrafish embryos and then examined the expression changes of miR-210-5p. Compared to its expression pattern in the embryos microinjected with control MO (Fig. 2, *A* and *B*), we found that expression of miR-210-5p was significantly increased in the *gata5/6* morphants (Fig. 2, *C* and *D*) but greatly reduced in the *gata4/5/6*-overexpressed embryos (Fig. 2, *E* and *F*) at 14 and 26 hpf, respectively. Consistently, the results from qRT-PCR confirmed the significantly ($p < 0.01$; $p < 0.05$) up-regulated expression of miR-210-5p in the *gata5/6* morphants compared with control embryos at both 14 and 26 hpf (Fig. 2*G*). The results were consistent with the deep sequencing data and supported that miR-210-5p functions downstream of *gata4/5/6* to negatively regulate zebrafish primitive myelopoiesis.

Knocking out miR-210 partially rescue the defective primitive myelopoiesis occurring in zebrafish gata4/5/6-knockdown embryos

To gain further insight into the regulatory mechanisms of miR-210-5p during zebrafish primitive myelopoiesis, we gener-

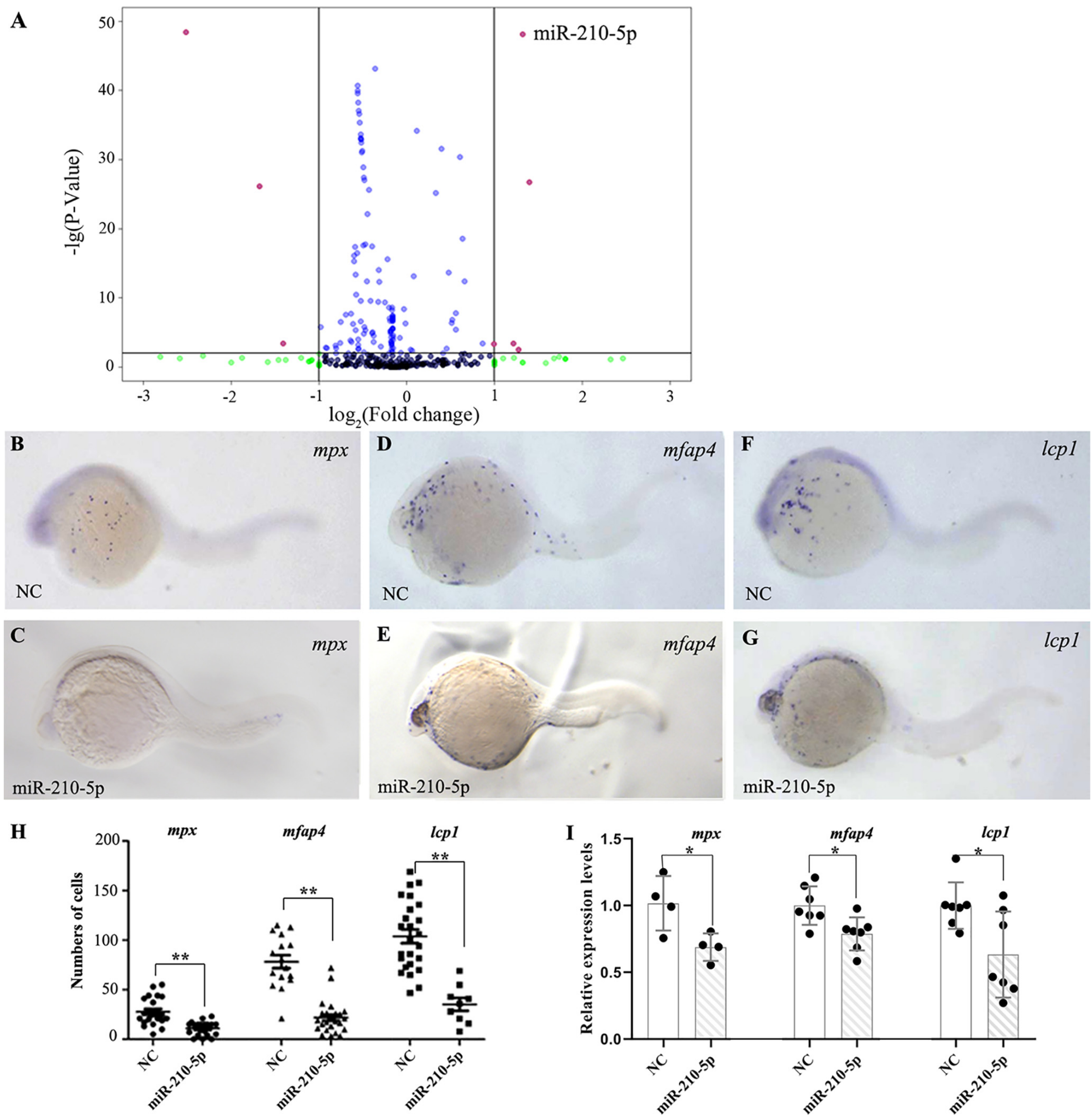

Figure 1. miR-210-5p functions downstream of *gata4/5/6* to regulate zebrafish primitive myelopoiesis. *A,* volcano plots showing the results from deep sequencing analysis for the expression changes of miRNAs in *gata4/5/6*-knockdown embryos. The miRNAs with significant expression changes (logFC>1 or LogFC<−1; $p < 0.01$) are marked in *red dots*. *B–G,* whole mount *in situ* hybridized embryos at 26 hpf showing the expression patterns of marker genes for primitive myelopoiesis including *mpx* (*B* and *C*), *mfap4* (*D* and *E*), and *lcp1* (*F* and *G*) in the embryos microinjected with NC (*B*, *D*, and *F*) and miR-210-5p mimic (*C*, *E*, and *G*), respectively. Embryos were viewed laterally, and positioned head left. The images in *B*, *D*, and *F* were reused in Fig. S1, *A–C* because these results were from one ISH experiment and shared the same controls. *H,* scatter plot showing the numbers of *mpx*, *mfap4*, and *lcp1* expression cells (shown in *y* axis) in the embryos microinjected with NC or miR-210-5p mimic (shown in *x* axis), respectively. *I,* qRT-PCR results showing the relative expression levels of *mpx*, *mfap4*, and *lcp1* (shown in *y* axis) in the 26 hpf embryos microinjected with NC or miR-210-5p mimic (shown in *x* axis), respectively. The gene expression levels in the NC group were all normalized as 1.0. The fold-changes of gene expression levels in the miR-210-5p mimic group were shown relative to the NC group, respectively. *NC,* mimic negative control; *miR-210-5p,* miR-210-5p mimic; **, $p < 0.01$; *, $p < 0.05$.

ated miR-210 knockout zebrafish using CRISPR/Cas9. Microinjecting a pair of sgRNAs and Cas9 mRNA into zebrafish embryos at 1-cell stage, we obtained zebrafish mutants carrying a mutated allele of miR-210 (*miR-210*$^{nju49/+}$). The mutated allele contains a deletion of the 107-bp genomic sequence encoding pre-miR-210 (Fig. 3, *A–C*). Analyses on the homozygous mutant of miR-210 (*miR-210*$^{nju49/nju49}$) revealed that the mutant embryos developed normally with no significant differences ($p > 0.05$) in expressions of the granulocyte marker gene *mpx* compared with WT embryos at 26 hpf (Fig. 3, *D–G*). Furthermore, the homozygous mutant zebrafish grew normally to adult with fertility. This result indicated that lack of miR-210

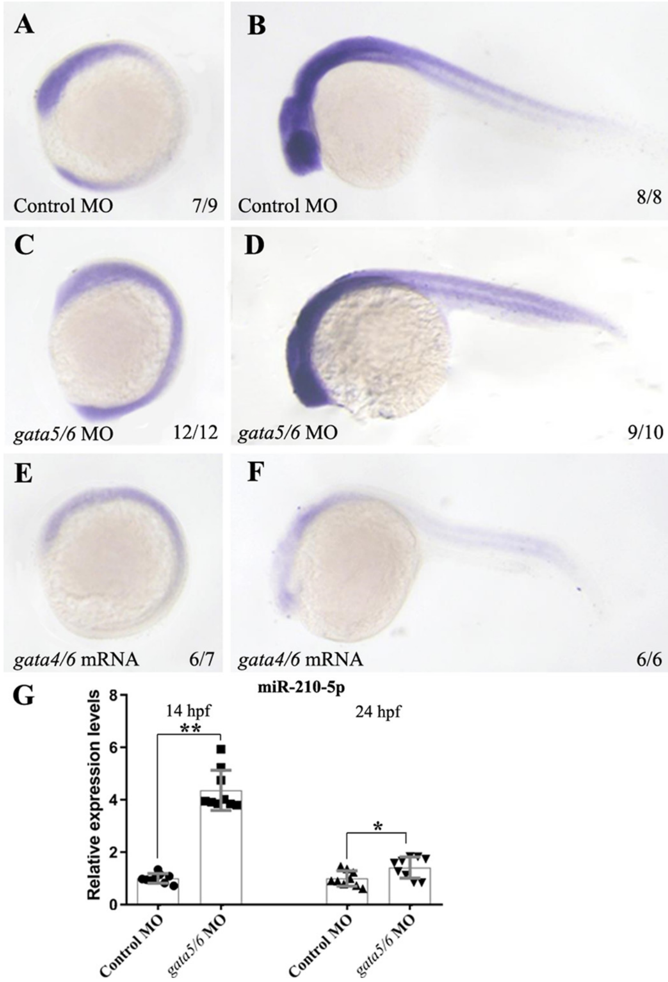

Figure 2. Gata4/5/6 inhibits the expression of miR-210-5p in zebrafish embryos at 14 and 26 hpf. WT embryos at 1-cell stage were microinjected with *gata5/6* MOs (*C* and *D*), *gata4/6* mRNAs (*E* and *F*), or control MO (*A* and *B*), respectively. When reaching 14 (*A*, *C*, and *E*) and 26 (*B*, *D*, and *F*) hpf, the microinjected embryos were fixed for *in situ* hybridization analysis, respectively. In the embryos microinjected with control MO, the miR-210-5p was found widely expressed at 14 hpf (*A*) or 26 hpf (*B*). The embryos microinjected with *gata5/6* MOs displayed significant increased expression of miR-210-5p (*C* and *D*), whereas the ones microinjected with *gata4/6* mRNAs exhibited significantly reduced expression of miR-210-5p (*E* and *F*) at 14 and 26 hpf, respectively. Embryos were positioned lateral and head left. *G*, results from qRT-PCR showing that the expression of mature miR-210-5p (shown in *y* axis) was significantly increased in *gata5/6* morphants compared with the embryos microinjected with control MO (shown in *y* axis). The expression level of miR-210-5p in the control MO group was normalized as 1.0 and the fold-change of miR-210-5p expression level in *gata5/6* morphant group was shown relative to the control MO group, respectively. **, $p < 0.01$; *, $p < 0.05$.

has no effect on zebrafish primitive myelopoiesis although excess miR-210-5p inhibits it.

To confirm that excess miR-210-5p plays a restrictive role in primitive myelopoiesis by working downstream of *gata4/5/6*, we knocked down *gata4/5/6* in miR-210 mutant embryos by microinjecting *gata5/6* MOs into the fertilized eggs derived from inbreeding of *miR-210*$^{nju49/+}$ zebrafish. Performing whole mount *in situ* hybridization or qRT-PCR analyses on the morphants, we found that both the number of *mpx*$^+$ cells and the expression levels of *mpx* decreased dramatically ($p < 0.01$) in WT embryos microinjected with *gata5/6* MOs compared with those with control MO (Fig. 3, *H, I,* and *L*), but these reduced *mpx*$^+$ cells were partially ($p < 0.01$) rescued in *miR-210*$^{-/-}$ embryos (Fig. 3, *I, K,* and *L*), whereas the number of *mpx*$^+$ cells and the expression levels of *mpx* had no change ($p > 0.05$) between *miR-210*$^{+/+}$ and *miR-210*$^{-/-}$ embryos microinjected with control MO (Fig. 3, *H, J,* and *L*). The results demonstrate that miR-210-5p regulates primitive myelopoiesis by acting downstream of *gata4/5/6,* and the incomplete rescue effect suggests that other factors besides miR-210-5p are involved in mediating the roles of *gata4/5/6* in regulating the formation of myeloid cells in zebrafish embryos.

miR-210-5p blocks the differentiation of hemangioblasts into myeloid progenitors

Zebrafish primitive myelopoiesis is initiated in the rostral end of ALPM to form anterior hemangioblasts marked by expressing the master regulators of *tal1* and *lmo2* between 3- and 5-somite stages (1, 5, 17). With development, a subset of the *tal1*$^+$ precursors acquires myeloid cell fates by expressing myeloid-specific transcription factor *spi1b* (18, 19). Now that overexpression of miR-210-5p led to inhibition of the primitive myelopoiesis, we therefore asked whether miR-210-5p plays a restrictive role in the formation of the myeloid precursors. To answer this question, we performed whole mount *in situ* hybridization to examine the expressions of key transcription factors including *tal1*, *lmo2*, and *spi1b* at 14 hpf when the myeloid precursors are differentiated from anterior hemangioblasts. The results from whole mount *in situ* hybridization showed that the expressions of *spi1b* were significantly restricted and reduced (Fig. 4, *A* and *B*), but the expressions of hemangioblast master regulatory genes including *tal1* and *lmo2* were expanded and increased in the rostral end of ALPM in the miR-210-5p overexpressing embryos at 14 hpf (Fig. 4, *C–F*). Consistently, qRT-PCR results confirmed the significant down-regulation of *spi1b* ($p < 0.01$) and the increased expressions of *tal1* and *lmo2* ($p < 0.05$) in the miR-210-5p overexpressing embryos at 14 hpf (Fig. 4*G*). These results suggest that miR-210-5p plays a restrictive role in the differentiation of the hemangioblast cells into myeloid precursors in the rostral end of ALPM in the zebrafish embryos at 14 hpf.

foxj1b and slc3a2a work as target genes of miR-210-5p to regulate the primitive myelopoiesis

To uncover the target genes of miR-210-5p to mediate its role in inhibiting zebrafish primitive myelopoiesis, we performed microarray analysis to identify differentially expressed genes in *gata4/5/6*-knockdown embryos (Fig. 5*A*). The downstream genes of *gata4/5/6* could potentially be regulated by miR-210-5p. By comparing the set of genes with reduced expression levels in *gata5/6* morphants to the predicted miR-210-5p target genes, which were obtained from miRanda database (http://www.microrna.org),[5] we found that 21 putative miR-210-5p downstream genes were down-regulated in *gata4/5/6*-knockdown embryos (Fig. 5, *B* and *C*). Among the candidate downstream targets, 4 genes including *foxj1b*, *rnasel3*, *slc3a2a*, and *stard3*, known to be involved in hematopoiesis or mesodermal development (20–23), were selected for further

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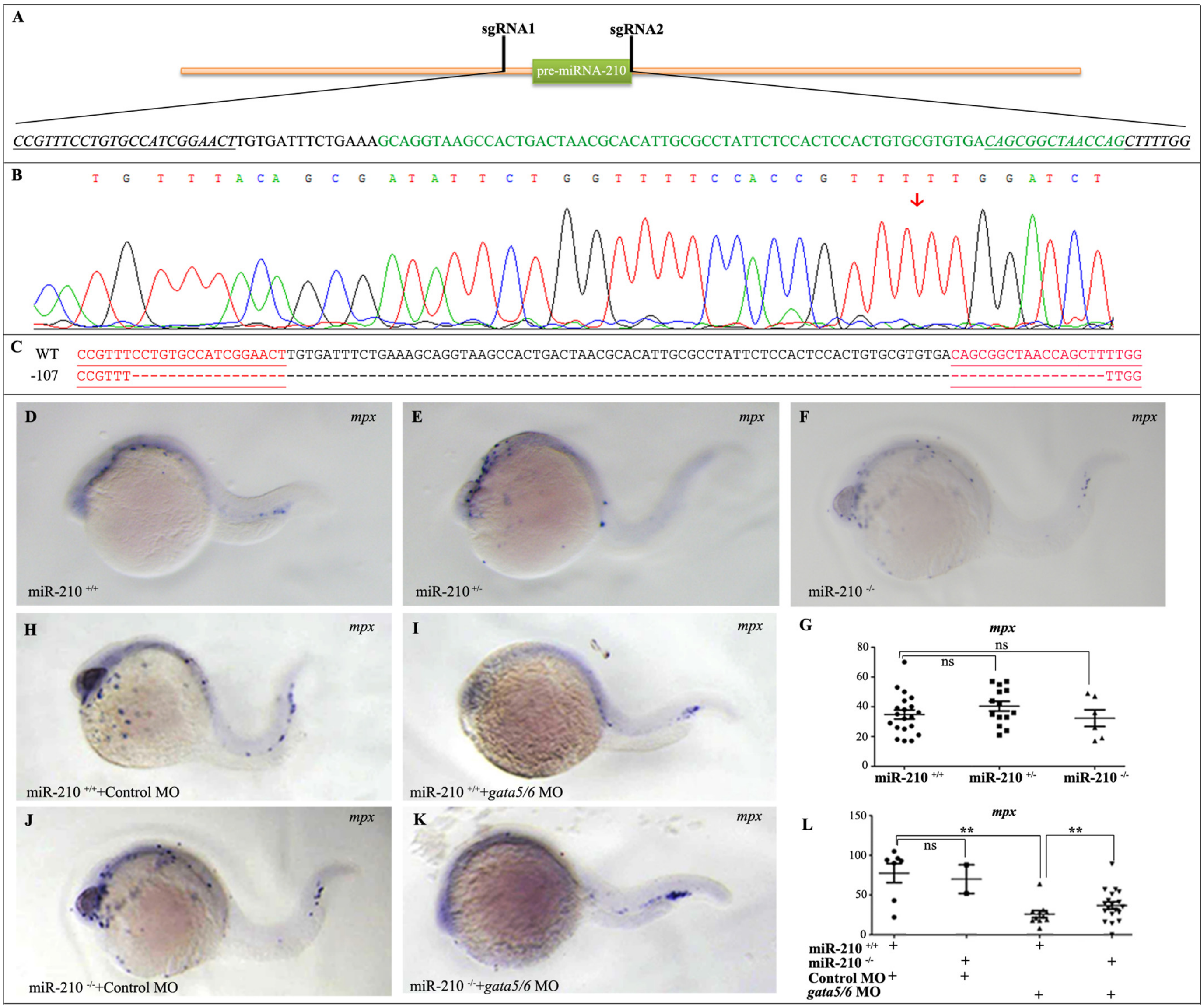

Figure 3. Knocking out *miR-210* partly rescues the primitive myelopoiesis in zebrafish embryos. *A*, schematic showing the recognition sites of two sgRNAs in the genomic sequence of pre-miRNA-210. sgRNA1 and sgRNA2 are designed to recognize the 5′ upstream and 3′ downstream of the miR-210 sequence (shown in *green*), respectively. sgRNA-recognition sites are *underlined* and *italic*. *B*, Sanger sequencing results showing an edit (mutated allele with a 107-bp deletion) was generated. *Red arrow* points to the location of deletion. *C*, DNA sequences showing the mutated allele (nju49) with 107 bp deletion. Sequences *underlined in red* show the recognition sites of two sgRNAs, respectively. *D–G*, knocking out *miR-210* did not affect the primitive myelopoiesis in zebrafish embryos. Whole mount *in situ* hybridized embryos showing that there was no significant difference in the number of mpx^+ cells between WT (*D*), heterozygous (*E*), and homozygous (*F*) embryos at 26 hpf. *G*, scatter plot showing the number of mpx^+ cells counted from the embryos typically shown in *D–F*. *H–K*, whole mount *in situ* hybridized embryos showing microinjection of control MO did not affect the formation of mpx^+ cells in miR-210 knockout embryos compared with their WT siblings (*H* and *J*), whereas knocking down *gata4/5/6* by microinjecting *gata5/6* MOs partially rescued the formation of mpx^+ cells in the miR-210-5p knockout embryos (*I* and *K*). *L*, scatter plot showing the number of mpx^+ cells counted from the embryos typically shown in *H–K*. Embryos are positioned laterally and head left. **, $p < 0.01$; *ns*, no significance ($p > 0.05$).

study. Performing qRT-RCR analyses on the embryos microinjected with the mimic of miR-210-5p, we found that the expressions of *foxj1b*, *slc3a2a*, and *stard3* were significantly ($p < 0.05$ or $p < 0.01$) reduced but the expression of *rnasel3* was not significantly changed ($p > 0.05$) in the embryos at 14 hpf (Fig. 5*D*). We therefore overexpressed *foxj1b*, *slc3a2a*, and *stard3* (3′ UTRs were all deleted from their mRNAs) and examined whether the expression of *spi1b* in miR-210-5p overexpressing embryos can be rescued at 14 hpf. The qRT-PCR results showed that overexpression of *foxj1b* or *slc3a2a*, but not *stard3*, significantly ($p < 0.01$) rescued the suppressed expression of *spi1b* (Fig. 5*E*). Consistently, whole mount *in situ* hybridization results showed that overexpression of *foxj1b* or *slc3a2a* significantly recovered the suppressed expression of *spi1b* in the 14 hpf embryos overexpressed with miR-210-5p (Fig. 5, *G–J*). To further confirm this conclusion, we tested whether the primitive myelopoiesis at 26 hpf in miR-210-5p-overexpressed embryos can be rescued by these two genes. After co-overexpressing miR-210-5p with *foxj1b* or *slc3a2a*, we examined the expressions of *spi1b*, *mpx*, *mfap4*, and *lcp1* in the embryos at 26 hpf. The qRT-PCR results revealed that overexpression of *foxj1b* or *slc3a2a* effectively ($p < 0.05$ or $p < 0.01$) recovered the suppressed primitive myelopoiesis at 26 hpf (Fig. 5*F*). Taken together, these results demonstrated that both *foxj1b*

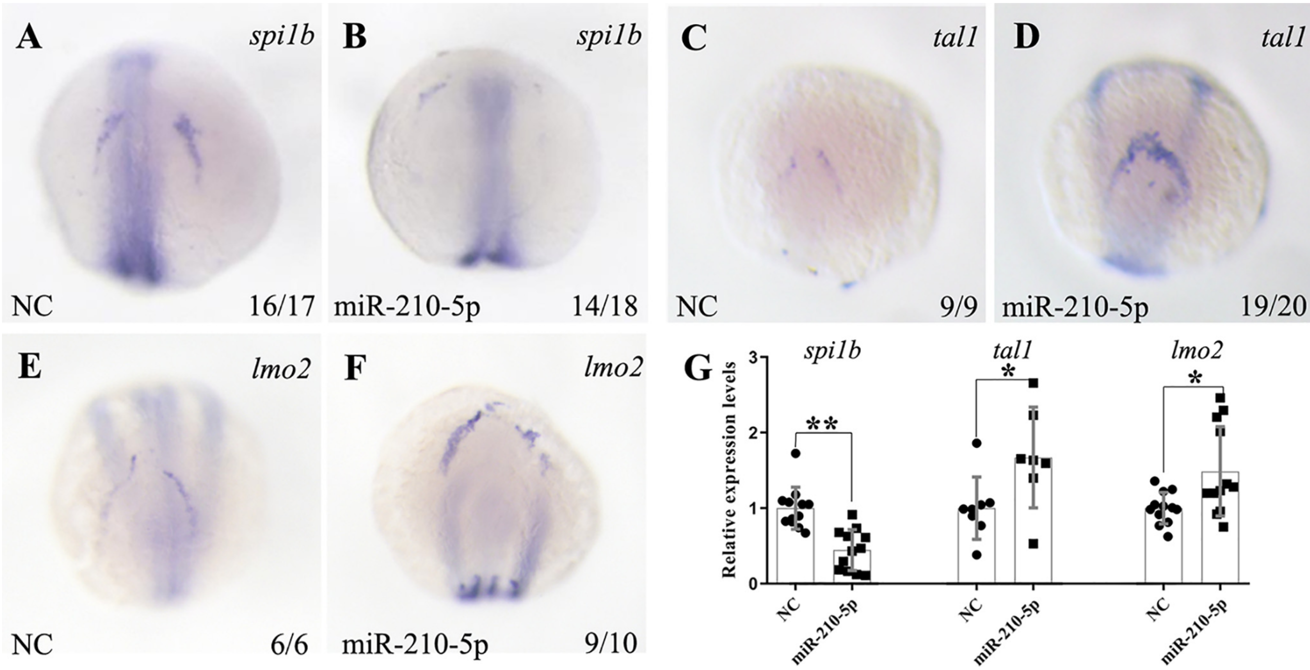

Figure 4. Overexpression of miR-210-5p inhibits the differentiation of anterior hemangioblast to the myeloid progenitors. *A–F*, whole mount *in situ* hybridization results showing the expressions of *spi1b*, *tal1*, and *lmo2* in the 14 hpf embryos microinjected with miR-210-5p mimic or NC. The embryos are positioned dorsally and anterior top. Compared with the embryos microinjected with NC (*A*), overexpression of miR-210-5p inhibited the expression of *spi1b* (*B*), whereas the expressions of *tal1* and *lmo2* were increased in the ALPM of miR-210-5p overexpressed embryos (*D* and *F*) compared with control embryos at 14 hpf (*C* and *E*). *G,* qRT-PCR results showing the reduced expressions of *spi1b* and increased expressions of *tal1* and *lmo2* in the miR-210-5p overexpressed embryos at 14 hpf. The gene expression levels in NC group was normalized as 1.0 and the fold-change of gene expression level in the embryos microinjected with miR-210-5p mimic was shown relative to the NC group (shown in *y* axis). *NC*, mimic negative control; *miR-210-5p*, miR-210-5p mimic; *, $p < 0.05$; **, $p < 0.01$.

and *slc3a2a* are downstream genes of miR-210-5p involved in mediating the roles of miR-210-5p to inhibit the primitive myelopoiesis.

To further support the conclusion, we examined the expressions of *foxj1b* and *slc3a2a* in the miR-210-5p knockout and *gata4/5/6*-knockdown embryos. The results from qRT-PCR showed that expressions of both *foxj1b* and *slc3a2a* were significantly ($p < 0.01$) up-regulated in *miR-210*$^{\mathrm{nju49}}$ embryos (Fig. S3, *A* and *B*). On the other hand, results from whole mount *in situ* hybridization revealed that the expressions of *foxj1b* and *slc3a2a* in *gata4/5/6*-knockdown embryos were significantly reduced (Fig. S3, *C, D, G*, and *H*). As expected, the expressions of *foxj1b* and *slc3a2a* were at least partially restored (Fig. S3, *C–J*) in the *gata4/5/6*-knockdown *miR-210*$^{\mathrm{nju49}}$ embryos.

To determine whether these two genes are direct targets of miR-210-5p, we analyzed the 3′ UTRs of the two genes for putative miR-210-5p targeting sites using an online tool (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid)[5] (45), which showed that there is one candidate target site of miR-210-5p in the 3′ UTRs of *foxj1b* or *slc3a2a* (Fig. 6*A*). To examine whether miR-210-5p regulates these genes through binding to the target sites, we performed dual-luciferase reporter assays on the expression of a firefly luciferase reporter gene fused with the WT 3′ UTR or mutated 3′ UTR of *foxj1b* and *slc3a2a*, respectively. The results showed the luciferase activities of the WT reporter of both *foxj1b* and *slc3a2a* were significantly ($p < 0.01$) repressed by miR-210-5p compared with the mutated reporters (Fig. 6, *B* and *C*). The results indicated that *foxj1b* and *slc3a2a* are direct target genes of miR-210-5p.

In summary, we demonstrate in this study that miR-210-5p works downstream of *gata4/5/6* to inhibit zebrafish primitive myelopoiesis by repressing the differentiation of anterior hemangioblast into myeloid progenitor cells, and *foxj1b* and *slc3a2a* act as two direct target genes of miR-210-5p to mediate its role in repressing zebrafish primitive myelopoiesis.

Discussion

MicroRNAs are an abundant and conserved class of small RNAs. They play important regulatory functions on gene activity. Advances in next-generation sequencing have permitted the discovery of many miRNAs involved in myelopoiesis, but the role of miRNAs in zebrafish primitive myelopoiesis remains elusive. For example, a recent attempt to screen for miRNAs involved in *spi1b* (*pu.1*)-dependent macrophage development in zebrafish has been reported by Ghani and colleagues (7). Their results revealed a *spi1b*-orchestrated miRNA program during myeloid differentiation. In this study, we searched for miRNAs regulating zebrafish primitive myelopoiesis using *gata4/5/6*-knockdown embryos. From the deep-sequencing data, we found 8 candidates and identified one miRNA, miR-210-5p, as an inhibitory regulator in zebrafish primitive myelopoiesis (Fig. 1). Unlike the research performed by Ghani and colleagues (7), our study focused on the *gata4/5/6*-dependent miRNAs that are involved in myeloid differentiation. As a result, our miRNA profile differs from the *spi1b*-regulated miRNAs, which potentially includes miRNAs lying genetically upstream of *spi1b* during myeloid development, that is, miR-210-5p.

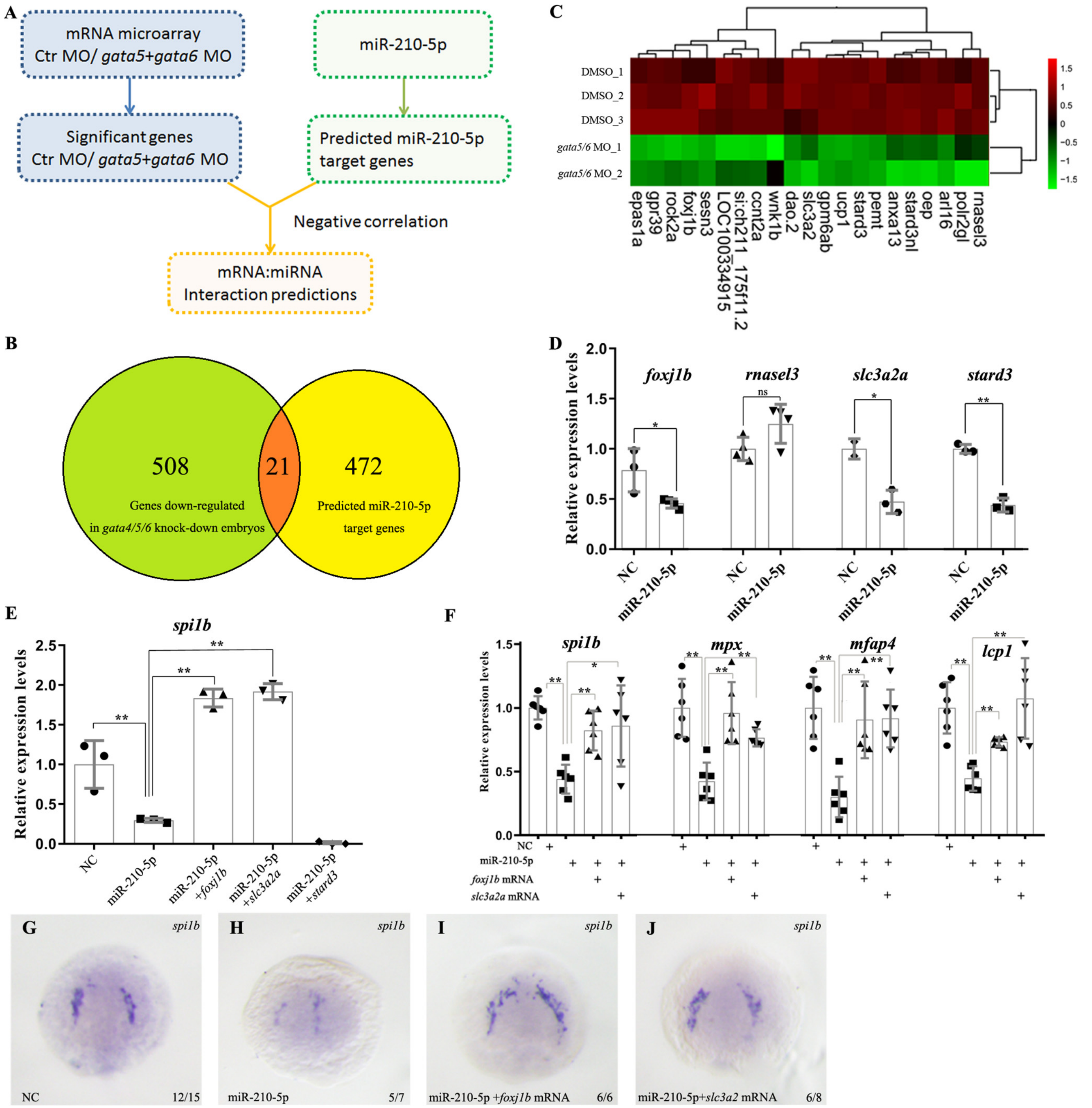

Figure 5. Both *foxj1b* and *slc3a2a* work downstream of miR-210-5p to mediate its role in inhibiting zebrafish primitive myelopoiesis. *A,* schematic showing the workflow to screen the candidate downstream genes of miR-210-5p to mediate its role in inhibiting zebrafish primitive myelopoiesis. The candidate genes were selected by the analysis of negative correlation between the set of miR-210-5p candidate target genes and the set of genes that reduced their expressions in the *gata4/5/6*-knockdown embryos at 14 hpf. *B,* Venn map showing the candidate target genes of miR-210-5p to mediate its roles in inhibiting zebrafish primitive myelopoiesis. *C,* heat map showing the clustering analysis of 21 candidate genes working downstream of miR-210-5p to inhibit the primitive myelopoiesis. The *horizontal lines* represent different treatment groups, and the *vertical columns* represent the expression levels of each gene. The expression abundance of each gene is represented by the color intensity. *Red* represents the high expression and *green* represents low expression of the gene in the embryos. The *upper* dendritic tree represents the expression profile similarity of each gene, and the *right* dendritic represents the degree of similarity of the expression profile in the different treatment groups. *D,* qRT-PCR results showing the expression changes of candidate target genes in the miR-210-5p overexpressed embryos at 14 hpf. The expression changes of *foxj1b*, *slc3a2a*, and *stard3*, except *rnasel3*, were similar to microarray results. *E,* overexpressions of *foxj1b* and *slc3a2a* but not *stard3* effectively rescued the expression of *spi1b* that was inhibited in the 14 hpf embryos overexpressed with miR-210-5p. *F,* overexpressions of *foxj1b* and *slc3a2a* effectively rescued the expressions of *spi1b*, *mpx*, *mfap4*, and *lcp1* in the zebrafish embryos at 14 and 26 hpf, respectively. *D–F*, gene expression levels in the NC group was normalized as 1.0 and the fold-change of the gene expression level in the embryos microinjected with miR-210-5p mimic was shown relative to the NC group (shown in *y* axis). *G–J,* whole mount *in situ* hybridization results showing the expressions of *spi1b* in the 14 hpf embryos microinjected with NC, miR-210-5p mimic alone, or miR-210-5p mimic together with *foxj1b* or *slc3a2a* mRNA, respectively. Compared with the embryos microinjected with NC (*G*), overexpression of miR-210-5p inhibited the expression of *spi1b* (*H*), whereas overexpressions of *foxj1b* or *slc3a2a* could effectively rescue the expression of *spi1b* suppressed by miR-210-5p (*I* and *J*). *NC*, mimic negative control; *miR-210-5p*, miR-210-5p mimic; *, $p < 0.05$; **, $p < 0.01$; *ns*: no significance ($p > 0.05$).

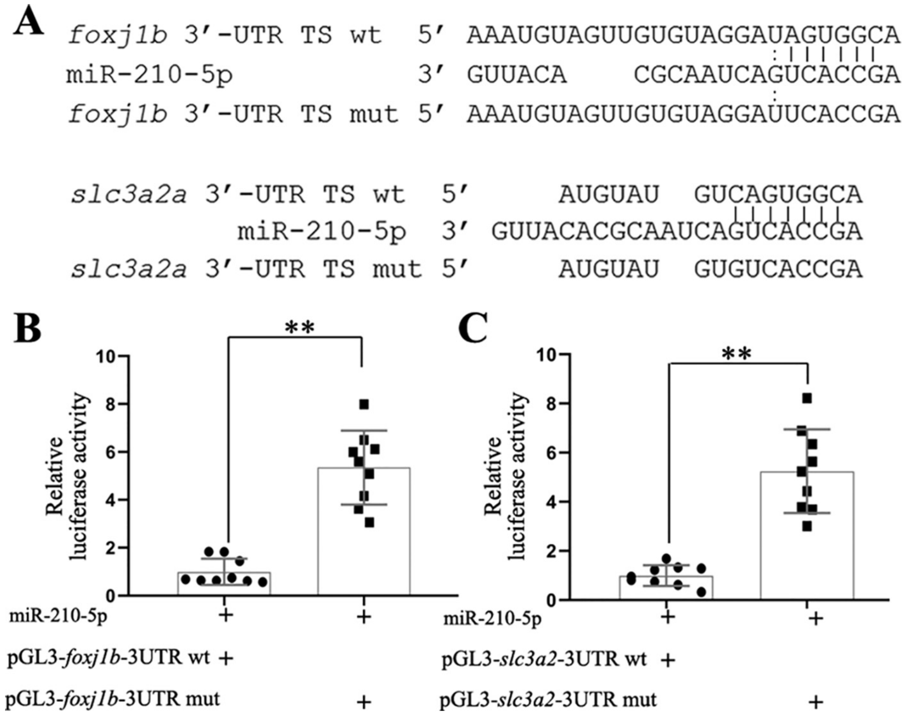

Figure 6. Dual-luciferase reporter assay shows that *foxj1b* and *slc3a2a* are direct targets of miR-210-5p. *A,* the alignment of miR-210-5p target sites (in the 3′ UTR) of *foxj1b* and *slc3a2a* with miR-210-5p, and with mutated target sites, respectively. *B* and *C,* dual-luciferase reporter assay showing the relative expression levels of firefly luciferase whose coding sequence fused with WT 3′ UTR of *foxj1b* or *slc3a2a* were down-regulated when miR-210-5p was overexpressed compared to those fused with mutant ones, respectively. The expression levels of luciferase reporter in the WT 3′ UTR group was normalized as 1.0 and the fold-change of the reporter expression level in the mutated 3′ UTR group was shown relative to the WT group (shown in *y* axis). Three independent assays were performed with 3 repeats in each group. *TS*, target site; *mut*, mutant; *miR-210-5p*, miR-210-5p mimic; **, $p < 0.01$.

Functions of miR-210 have been related to hypoxia and tumor-suppressing. The expression level of miR-210 is higher in different cells with hypoxia responses (24–26). In agreement with this, miR-210 expression is also elevated in animals subjected to transient focal ischemia and in a variety of human cancers (27–30). Based on these results, miR-210 was considered as a potential biomarker of hypoxia, breast cancer, and acute coronary syndrome (31–33). Interestingly, it is reported that the low-level of miR-210 expression is associated with poorer outcome for pediatric acute lymphoblastic leukemia treated with chemotherapeutic drugs in clinic (34). In another study, it is reported that high levels of miR-210 associate with poor prognosis in acute myeloid leukemia patients (35). The result indicates the important role of miR-210 in acute myeloid leukemia and myeloid differentiation. However, these published studies all focused on miR-210-3p, whereas the function of miR-210-5p remains elusive although it is also conserved in different vertebrates during evolution (data not shown). In this study, we demonstrate that zebrafish miR-210-5p is one of the key regulators in the common pathway of primitive myelopoiesis, working downstream of *gata4/5/6* (Fig. 2) and upstream of *spi1b* (Fig. 4) in the genetic hierarchy to block the differentiation of hemangioblasts into myeloid progenitors (Fig. 4).

In many cases, the biological functions of miRNAs can only be revealed in a context-specific manner (36). For example, the expression of miR-210 is intensively up-regulated in hypoxic states (37). Mice with deletion of miR-210 are viable at baseline and display no grossly abnormal phenotype. However, *miR-210*$^{-/-}$ mice are resistant to Fe-S-dependent pathophenotypes and pulmonary hypertension when exposed to hypoxia (38). Consistently, our results revealed that zebrafish carrying deleted miR-210 do not show any abnormal growth and development including myeloid development. However, in a *gata4/5/6*-deficient context, primitive myelopoiesis is partially recovered in *miR-210*$^{-/-}$ embryos. The results are in agreement with the experimental data that the expression of miR-210-5p is inhibited by the overexpressions of *gata4/5/6* (Fig. 3) and overexpression of miR-210-5p inhibits the primitive myelopoiesis in zebrafish embryos (Fig. 1). Therefore, our data suggest that the restrictive role of miR-210-5p is not inevitable during normal development, but it can further worsen the primitive myelopoiesis if the *gata4/5/6* signal is disturbed in zebrafish embryos.

miRNAs play important regulatory functions on gene activity mainly by interacting with the 3′ untranslated region (UTR) of target mRNAs. Previous study showed that *VPM1* is a direct and functional downstream target gene of miR-210 in hypoxia-induced tumor cell metastasis (30). In this study, we identified that *foxj1b* and *slc3a2a* are two direct target genes of miR-210-5p in zebrafish primitive myelopoiesis by comparing the set of genes with reduced expression levels in *gata5/6* morphants to that of the predicted miR-210-5p target genes from miRanda database[5] (http://www.microrna.org). Obviously, this screening strategy excluded the potential target genes whose mRNA translations were blocked but stableness was not affected when miR-210-5p binds to their 3′ UTRs. Additionally, we only tested 4 of the 21 candidates of miR-210-5p target genes to examine whether their reduced mRNA levels were due to the binding of miR-210-5p to their 3′ UTRs. Therefore, our results cannot rule out the possibilities of other potential direct targets of this miRNA working downstream of *gata4/5/6* to affect zebrafish primitive myelopoiesis. It is worth noting that we did not use the miR-210 knockout zebrafish as a model to screen the candidates of miR-210 target genes due to the reason that miR-210-5p knockout embryos exhibited normal primitive myelopoiesis in zebrafish.

Foxj1b is a transcription factor containing a forkhead box. It is expressed in the axial mesoderm and an area flanking the dorsal forerunner cells at 75% epiboly and in the PLPM and the precursor of the otic vesicle at the 7 somite-stage (39). Slc3a2a belongs to solute carrier family 3 (amino acid transporter heavy chain), member 2a. It is expressed in the yolk syncytial layer from 85% epiboly to 3-somite stage (40). To our knowledge, no previous research has reported that they were involved in primitive myelopoiesis in zebrafish. In this study, we found that the two genes were mainly expressed in the otic vesicle and yolk syncytial layer of embryos at 14 hpf (Fig. S3, *C* and *G*) but with no obvious expression in the ALPM where the primitive myelopoiesis occur. However, overexpression of *foxj1b* or *slc3a2a* greatly recovered the suppressed primitive myelopoiesis in embryos overexpressed with miR-210-5p (Fig. 5). Although it can be explained that the expression levels of *foxj1b* and *slc3a2a* in the ALPM might be too low to be detected by whole mount *in situ* hybridization, the mechanism underlying the two genes regulating the primitive myelopoiesis remains to be elucidated.

In summary, we report in this study that a *gata4/5/6*-regulated miRNA, miR-210-5p, is a restrictive regulator of zebrafish primitive myelopoiesis. It suppresses myeloid differentiation from hemangioblast through directly regulating the stableness of mRNAs of *foxj1b* and *slc3a2a*. Our results provide novel mechanistic insight into the role of miRNAs during zebrafish primitive myelopoiesis.

Experimental procedures

Ethics statement

Zebrafish were housed in the zebrafish room of the Model Animal Research Center, Nanjing University, in accordance with protocols approved by the IACUC of the Model Animal Research Center at Nanjing University. All methods were performed in accordance with the relevant guidelines and regulations.

Microinjection of morpholinos into zebrafish embryos

MOs were purchased from Gene Tools. The sequences of MOs are TGTTAAGATTTTTACCTATACTGGA (*gata5* MO), AGCTGTTATCACCCAGGTCCATCCA (*gata6* MO), and CCTCTTACCTCAGTTACAATTTATA (control MO) (13, 14). MOs were dissolved in nanopure water and microinjected into the embryos at 1-cell stages. The microinjection amount was 1 nl of solution containing 6.25 ng of *gata5* MO and 1.25 ng of *gata6* MO, or 7.5 ng of control MO per embryo (13).

Deep sequencing of miRNA transcripts

The embryos microinjected with control MO or *gata5/6* MO at 1-cell stage were incubated for 14 h and collected for miRNA deep sequencing. The miRNA sequencing and profiling was performed by Shanghai Biotechnology Corporation. Briefly, total RNA was isolated from 80 of the 14 hpf embryos, and its quantity was examined by electrophoresis using Agilent Bioanalyzer 2100 (Agilent Technologies, USA). The miRNA was isolated using mirVana miRNA Isolation Kit (Life Technologies) from the total RNA. The 3′- and 5′-adaptors were added to the miRNA sequentially. cDNA was then reverse transcribed from the miRNA and amplified using a PCR (98 °C 30 s, 11 × (98 °C 10 s, 60 °C 30 s, 72 °C 15 s) for 72 °C for 10 min, 4 °C). The cDNA was separated by 6% Novex TBE PAGE Gel for collecting the 147–157-nt long fragments. The cDNA library was constructed and the quantity was examined using a commercial kit (Qubit^{TM} dsDNA HS, Agilent 2100). The concentration of the library was no lower than 0.5 ng/μl and the size of cDNA was 140–160 bp. The cDNA library was sequenced using Illumina Solexa following version 8 multiplexed single-read sequencing recipe. Fastx (fastx_toolkit-0.0.13.2) was used to preprocess the original reads of the sequence. The sequence of the joint and the low mass sequence including the fuzzy base N, sequence length less than 18 nt, were removed. The CLC genomics_workbench5.5 was applied to match the preprocessed sequence to the Sanger miRBase database (version 19.0). No mismatch was allowed during the process.

Microinjection of miRNA mimics or inhibitors into zebrafish embryos

miRNA mimics, inhibitors, miRNA mimics negative control, and miRNA inhibitors negative control were purchased from Shanghai Genepharma Corporation (Shanghai, China). Their sequences are listed in Table S1. Mimics and inhibitors were dissolved in RNase-free nanopure water and microinjected into the embryos at 1-cell stage. The amount of microinjection is 1 nl containing 10 μM miRNA mimic or inhibitor per embryo.

Whole mount in situ hybridization on examining the expression patterns of coding genes and noncoding genes

Whole mount *in situ* hybridizations on detecting the expression patterns of coding genes were performed as described previously (13). The RNA probes detecting the expressions of *mpx*, *mfap4*, *lcp1*, *tal1*, *lmo2*, and *spi1b* were prepared as described previously (13). The number of cells expressing *mpx*, *mfap4*, or *lcp1* were counted manually under a dissecting microscope, and shown in a scatter plot, respectively. Whole mount *in situ* hybridizations on examining the expression patterns of miRNA were performed as described previously (41). The probe against miR-210 (miRCURY LNA microRNA Detection Probes) was purchased from a commercial company (Exiqon, Denmark). Photomicrographs were taken using a Olympus DP71 digital camera (Olympus, Japan) and further processed for brightness and contrast with Adobe Photoshop software.

Real-time RT-PCR assay

qRT-PCR was performed to examine the relative expression levels of the interested genes. For the coding genes in this study, we used ABI Stepone Plus (Applied Biosystems), as reported previously (13). Transcript levels of the examined coding genes were normalized to the *actb1* mRNA level according to standard procedures. For miR-210-5p, we extracted total RNA from at least 30 embryos in each group using Direct-zol RNA Mini-Prep (Zymol Research). qRT-PCR for the microRNAs was conducted using Mir-X miRNA First-strand synthesis and TB Green qRT-PCR Kit (Takara, Japan). The PCR was performed as follows: 95 °C for 10 s, 40× (95 °C of 5 s, 60 °C for 20 s), 95 °C for 15 s, 60 °C for 30 s, 95 °C for 15 s. The primers used are listed in Table S1.

In vitro synthesis of mRNA and microinjection of mRNAs into zebrafish embryos

mRNAs were *in vitro* synthesized, capped, and tailed using the method as described previously (13). The primers used for cloning the full-length coding sequences of *gata4* and *gata6* (13), *foxj1b* (GenBank accession number, NM_001008648.1), *slc3a2a* (GenBank accession number, NM_131601.2), and *stard3* (NM_131662.1) are listed in Table S1. About 1 nl of solution containing 50 pg of *gata4* and 25 pg of *gata6*, 200 pg of *foxj1b*, *slc3a2a,* or *stard3* were microinjected into 1-cell stage embryos, respectively. The Cas9 mRNA was synthesized using the template derived from the expression plasmid of pXT7-Cas9 (42).

Generation of zebrafish miR-210 knockout line

To generate miR-210 knockout zebrafish, we first designed a pair of CRISPRs using the online tool (CRISPR). sgRNAs were transcribed using MAXIscript T7 Kit (Ambion) from the templates prepared by PCR amplification from a template plasmid pT7-gRNA with gene-specific primers sgRNA1F or sgRNA2F and a universal reverse primer (Table S1) as reported previously (42). We then microinjected 1 nl of solution containing 150 pg of Cas9 mRNA, 50 pg of sgRNA1, and 50 pg of sgRNA2 into 1-cell zebrafish embryos. The embryos were raised to adults as founders. The F1 mutant zebrafish were screened using the method as described before (43). Briefly, F1 generation zebrafish were genotyped by the PCR method using the genomic template prepared from the caudal fins clipped from zebrafish older than 6 weeks with a commercial kit (YSY, China). The primers used for amplifying the genomic sequences of the miR-210 were AATGTTCAGCTGCAGAACGG and GATCACACAACGCACCCATT. The PCR conditions were 95 °C for 2 min, 35 × (94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s), and a final extension of 5 min at 72 °C. F1 mutant zebrafish carrying the miR-210 knockout allele were selected and inbred to get miR-210 null progeny (F2).

Microarray analysis on the mRNAs isolated from gata4/5/6-knockdown embryos

The embryos microinjected with control MO or *gata5/6* MOs at 1-cell stage were collected at 14 hpf for analyzing the gene expression changes affected by knocking down *gata4/5/6*. The microarray analysis was performed by Shanghai Biotechnology Corp. using zebrafish gene expression microarray 4*44K (Agilent). Total RNA extraction and purification, RNA amplification and labeling, hybridizations, as well as data acquisition by LC Sciences (USA) were used with strict adherence to Agilent's standardized protocols. Genes down-regulated in *gata5/6* morphants (LogFC< -0.585, $p < 0.05$) were further analyzed using hierarchical clustering.

Dual-luciferase reporter assay

The 3′ UTR cDNAs of *foxj1b* and *slc3a2a* were first cloned into pGEM-T vector (Promega) by RT-PCR using the primer pairs of *foxj1b*-3utr-F and *foxj1b*-3utr-R, and *slc3a2a*-3utr-F and *slc3a2a*-3utr-R (Table S1), respectively. They were then recombined into pGL3-promoter Luciferase (Promega) using a One-Step Cloning Kit (Vazyme, China) with primer pairs of *foxj1b*-3UTR-F and *foxj1b*-3UTR-R, and *slc3a2a*-3UTR-F and *slc3a2a*-3UTR-R, respectively. The mutant 3′ UTR cDNAs of *foxj1b* and *slc3a2a* were first cloned into pUC57 vector. They were then recombined into pGL3-promoter luciferase (Promega) using One-Step Cloning Kit (Vazyme, China) with primer pairs of F-*foxj1b*-3UTR-MT and R-*foxj1b*-3UTR-MT, and F-*slc3a2a*-3UTR-MT and R-*slc3a2a*-3UTR-MT, respectively. 100 pg of reporter expression plasmids (pGL3-*foxj1b*-3UTR-wt or pGL3-*foxj1b*-3UTR-mut) or 50 pg of reporter expression plasmids (pGL3-*slc3a2a*-3UTR-wt or pGL3-*slc3a2a*-3UTR-mut), 1 pg of *Renilla* luciferase expression plasmid, and 20 pmol of miRNA mimic were microinjected together into zebrafish embryos at 1-cell stage. Dual-luciferase reporter assays were performed as described previously (44).

Statistical analysis

Data are presented as mean ± S.E. Statistical significance was determined using the unpaired two-tailed *t* test. A value of $p <$ 0.05 (*) was considered statistically significant, and $p < 0.01$ (**) were considered statistically very significant.

Author contributions—W. J., D. L., N. L., P. H., J. Y., and Q. Z. data curation; W. J., D. L., N. L., Z. D., J. L., X. D., P. H., J. Y., and Q. Z. formal analysis; W. J., D. L., N. L., M. L., Z. D., J. L., X. D., Y. Y., and Q. Z. investigation; W. J., D. L., N. L., M. L., Z. D., J. L., X. D., Y. Y., and Q. Z. methodology; W. J., D. L., N. L., and Q. Z. writing-original draft; Q. Z. and D. L. conceptualization; Q. Z. supervision; Q. Z. and D. L. funding acquisition; Q. Z. project administration; Q. Z. writing-review and editing.

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