MicroRNA miR-24 inhibits erythropoiesis by targeting activin type I receptor ALK4

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Abstract

MicroRNAs have been suggested to modulate a variety of cellular events. Here we report that miR-24 regulates erythroid differentiation by influencing the expression of human activin type I receptor ALK4 (hALK4). Ectopic expression of miR-24 reduces the mRNA and protein levels of hALK4 by targeting the 3’-untranslated region of hALK4 mRNA and interferes with activin-induced Smad2 phosphorylation and reporter expression. Furthermore, miR-24 represses the activin-mediated accumulation of hemoglobin, an erythroid differentiation marker, in erythroleukemic K562 cells and decreases erythroid colony-forming and burst-forming units of CD34+ hematopoietic progenitor cells. ALK4 expression is inversely correlated with miR-24 expression during the early stages of erythroid differentiation, and the forced expression of miR-24 leads to a delay of activin-induced maturation of hematopoietic progenitor cells in liquid culture. Thus, our findings define a regulation mode of miR-24 on erythropoiesis by impeding ALK4 expression.
**Introduction**

Erythropoiesis is the complex process during which a fraction of primitive multipotent hematopoietic stem cells become committed to the red cell lineage, undergoing erythroid progenitors (burst-forming unit erythroid, BFU-E and colony-forming unit erythroid, CFU-E), normoblasts, erythroblasts, reticulocytes, and ultimately differentiating to mature erythrocytes. This process is regulated by various factors such as erythropoietin, testosterone, estrogen, interleukin-3, granulocyte-macrophage colony-stimulating factor and interleukin-9.

Activin, a member of the transforming growth factor-β (TGF-β) superfamily, plays an important role in modulating proliferation and differentiation of erythroid progenitors. In cooperation with erythropoietin, activin promotes erythroid differentiation by increasing the number of hemoglobin-synthesizing colonies derived from the progenitors CFU-E and enhances the formation of BFU-E. Activin, like other TGF-β superfamily members, signals through two types of transmembrane receptors that have intrinsic serine/threonine kinase activity. It binds to type II activin receptor, leading to the recruitment, phosphorylation and activation of type I activin receptor (ALK4, also known as ActRIB). The activated ALK4 transiently interacts with and then phosphorylates Smad2 and Smad3 that, upon phosphorylation, form a heterocomplex with Co-Smad (Smad4), and the resulting Smad complex is accumulated in the nucleus, binds to the promoter of the target genes and regulates their expression.

MicroRNAs (miRNAs) are 21–25-nucleotide small regulatory RNAs that modulate gene expression by targeting mRNA for degradation or blocking translation via base-pairing
to complementary sites in the 3’-untranslated region (3’-UTR) of the target mRNAs. Hundreds of miRNAs have been identified and they participate in a diverse collection of regulatory events. Investigation of the miRNA expression profile in the course of hematopoietic development suggests their potential regulatory roles in hematopoietic differentiation. For example, ectopic expression of miR-181 in hematopoietic progenitor cells increased the proportion of B-lineage cells, while miR-142s and miR-223 promote the differentiation to T-lineage but not to B-lineage or myeloid cells. MiR-155 transgenic mice exhibited pre-B cell proliferation and B cell malignancy. Overexpression of miR-150 in hematopoietic stem cells specifically impaired the formation of mature B cells by blocking the transition from the pro-B to the pre-B stage. Although many miRNAs have been implicated in hematopoiesis, few of their targets have been identified. MiR-221 and miR-222, both of which are clustered on the X chromosome and were suggested to inhibit normal erythropoiesis and erythroleukemic cell growth, down-modulated kit receptor expression.

In this report, we showed that miR-24 could decrease human ALK4 (hALK4) expression at the mRNA and the protein levels through binding to the 3’-UTR of hALK4 mRNA, and interfere with activin signaling. Furthermore, we report that miR-24 inhibits erythroid differentiation of K562 cells, erythroid colony formation and maturation of human CD34+ hematopoietic progenitor cells.

**Materials and Methods**

**Cell culture and reagents**

HEK293 cells were maintained in DMEM medium with 10% fetal bovine serum (FBS).
K562 cells were grown in RPMI-1640 DMEM medium supplemented with 10% FBS. All the growth factors and anti-human ALK4 antibody (AF222) were purchased from R&D Systems, anti-p-Smad2 antibody from Upstate Biotech and anti-β-actin antibody from Santa Cruz Biotech.

**Constructs**

To generate a luciferase reporter to evaluate miRNA activity, the CMV promoter was subcloned into pGL3-basic vector. About 3000bp fragment encompassing the entire hALK4 3’-UTR was cloned from HeLa genomic DNA and inserted downstream of the firefly luciferase’s open reading frame. About 500bp miRNA transcripts, which included the miRNA hairpin precursor and its native flanking sequence, were amplified by PCR from the HeLa cell genomic DNA and subcloned into pcDNA3.1(+). All the sequences were confirmed by DNA sequencing.

**Oligonucleotide sequences**

2'-O-methyl oligoribonucleotides:

- anti-24 (5’-CUGUUCUCUGGAACUGAGCCA-3’);
- anti-24M (5’-CUGUUCUCUGGAACUGCUUUA-3’);
- anti-GFP (5’-AAGGCAAGCUGACCCUGAAGUU-3’).

Northern probes:

- miR-30d (5’-CTTCCAGTCGGGATGTTTACA-3’);
- miR-30a-3p (5’-GCTGCAAACATCCGACTGAAAG-3’);
- miR-24 (5’-CTGTTCCTGCTGAACTGAGCCA-3’);
- miR-205 (5’-CAGACTCCGGTGGAATGAGCCA-3’);
- miR-189 (5’-ACTGATATCAGCTCAGTAGCCAC-3’);
- U6 (5’- AACGCTTCACGAATTTGCGT-3’).
PCR primers: hALK4 3′-UTR (forward, 5′-TCTAGACTGCAGCATCAAGAAGACCCT-3′; reverse, 5′-GCGTCTAGAGCAGAAATTGGACAGGCTCA-3′); miR-30d transcript (forward, 5′-TACAGATCTAGCAGCTAAGTCTGGGAAG-3′; reverse, 5′-TATGTCGACTTTGACTTGGAACCACCTTT-3′); miR-30a-3p transcript (forward, 5′-GACAGATCTAAATTCTATGCATACACAGAGC-3′; reverse, 5′-GACGAGATCTAGACCTTATTTGTTGTTGTTGTTTGAACATCTCA-3′); miR-24 transcript (forward, 5′-GACAAGATCTTTTGCAAGCTTGCAAGAAGACGTGT-3′; reverse, 5′-GAAGTCGACTCCAATCTGCCCCATACACCCG-3′). RT-PCR primers: hALK4 (forward, 5′-GCTCAGTCTCTCTGTATT-3′; reverse, 5′-TAAATGTACAGAAATGG-3′); GAPDH (forward, 5′-CATCACTGCCACCCAGAAGA-3′, reverse, 5′-GCTGTAGCCAAATCTCGTTT-3′). Stem-loop RT-PCR primers: miR-30d (RT primer, 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTTCCA-3′; forward, 5′-CGACATTGTAAACATCCCCGAC-3′; reverse, 5′-GTGCAGGGTCCGAGGT-3′); miR-30a-3p (RT primer, 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCTGCA-3′; forward, CGACGACTTTCAATCGGTATGTT; reverse, 5′-GTGCAGGGTGAGGTGTA-3′); miR-24 (RT primer, 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCTGCA-3′; forward, 5′-GCAGATTGGTCTCAGTGTACAGGCAACCTGTTC-3′; reverse, 5′-GTGCAGGGTGAGGTGTA-3′); miR-205 (RT primer, 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCTGCA-3′; forward, 5′-GCAGATTGGTCTCAGTGTACAGGCAACCTGTTC-3′; reverse, 5′-GTGCAGGGTGAGGTGTA-3′).
5’-GTCGTATCCAGTCAGGGTCCGAGGTATTCGCACTGGATACGACCAGACT-3’; forward, CAGAAGTCCTTCATTCCACCGG; reverse, 5’-GTGCAGGGTCCGAGGT-3’;
U6 (RT and reverse primer, 5’-GTGCAGGGTCCGAGGT-3’; forward primer, 5’-CTCGCTTCGGCAGCACA-3’).

**RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)**

RNA was isolated using Trizol reagent (Bio Basic Inc). RNA was reverse-transcribed using ReverTra Ace-α (Toyobo). To detect the mRNA of hALK4, PCR was performed at 94°C for 15s, 51°C for 30s and 68°C for 90s, 30 cycles. GAPDH expression was used as an internal control. To detect mature miR-24 expression, stem-loop RT-PCR was performed as described. Total RNA (2µg) was reverse-transcribed and PCR was performed at 94°C for 15s, 55°C for 30s and 68°C for 20s, 40 cycles.

**Northern blot analysis**

Total RNA (30µg) was separated on 15% denaturing polyacrylamide gel, electrotransferred to nylon membrane (Hybond) and hybridized with UltraHyb-Oligo buffer (Ambion). Oligonucleotides complementary to mature miRNAs were end-labeled with T4 kinase (Takara). The blot was washed 3×10 min at room temperature in 3× SSC, 0.5% SDS and then 15 min in 1× SSC, 0.5% SDS and processed for autoradiography.

**Isolation of CD34⁺ hematopoietic progenitor cells (HPCs)**

Umbilical cord blood was collected from normal full-term pregnancies with consent as approved by the Research Ethics Committee of Qilu Hospital, Jinan, China. Mononuclear cells were isolated using Ficoll-Hypaque (1.077±0.001 kg/L, Sigma). CD34⁺ cells were isolated by positive selection using the miniMACS immunomagnetic isolation system.
(Miltenyi Biotec GmbH, Glodbach, Germany) according to the manufacturer’s instructions. The isolation system yielded about 90% CD34-positive cells.

**Cell Transfection**

HEK293 cells were transfected with calcium phosphate precipitation, and K562 cells with TransFast transfection reagent (Promega) or electroporation. CD34+ HPCs were cultured in a serum-free medium for 48 hrs, and then were transfected by electroporation as previously described with the following modifications: the cells in Opti-MEM containing 10% FBS were mixed with desired amount of plasmid DNA together with pEGFP-N1 or oligoribonucleotides with FITC-nonspecific oligoribonucleotide. The electroporation was performed with GenePulser Xcell (Bio-Rad) at 220V, 1600 µF. The transfection efficiency for K562 is obtained over 70%, and for CD34+ cells over 40% by electroporation. Electroporated HPCs were allowed to recover for 24 hr with fresh medium and then selected by FACS sorting.

**Luciferase assay**

HEK293 cells or K562 cells were transfected with the indicated constructs or oligoribonucleotides, together with the internal control pRenilla-TK vector (20ng). Cells were stimulated with activin A (25ng/ml) or TGF-β1 (1.25ng/ml) for 16 hr before harvest for luciferase assay. Each experiment was performed in triplicate and the data represent the mean ± standard deviation (S.D.) of three independent experiments after normalized to Renilla activity.

**Benzidine staining**

Cells (1.5×10^5) were treated with or without 25ng/ml activin A. Three days later, cells
were harvested and analyzed by benzidine staining.  

**Colony-forming cell (CFC) assay**

CFC assay was performed in triplicate using Human Methylcellulose Complete Media (R&D Systems) according to the manufacturer’s instructions. HPCs were cultured in 35mm plates with the medium containing 1.3% methylcellulose, 25% FBS, 2% bovine serum albumin, 2mM L-glutamine, 0.05mM 2-mercaptoethanol, and 50ng/ml SCF, 10ng/ml IL-3, 3U/ml EPO, 10ng/ml GM-CSF. Activin A (25ng/ml) was added as indicated. To enumerate CFU-E derived colonies, the dishes were examined for hemoglobinized and compact colonies with eight or more cells on day 7 of culture. BFU-E was scored on day 14 and identified as large aggregates of 64 or more hemoglobinized cells or as clusters of three or more subcolonies with eight or more hemoglobinized cells per subcolony.

**Erythroid differentiation of HPCs and Wright-Giemsa staining**

Purified CD34+ HPCs were cultured in Iscove's modified Dulbecco's medium supplemented with 30% fetal calf serum, 2mM glutamine, 1mg/ml deionized bovine serum albumin (Sigma), 50pM 2-mercaptoethanol (Merck), 100ng/ml rhIL-3, 100ng/ml rhSCF and 6 units/ml rhEPO, and in the presence or the absence of activin A (25ng/ml) as indicated. The cells were harvested at the indicated time. Wright-Giemsa staining was performed on cytospin preparations of cells with modified Wright-Giemsa solution (Sigma, WG16) according to the manufacturer’s instructions.

**Imaging processing and statistics**

The cell images were visualized using a Leica DMIL microscope (Leica, Heidelberg, Germany) equipped with a Leica DC200 camera. All the images were processed with
Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA). Images were obtained using 0.12
numeric aperture Planapo objective lens. Student’s t tests were performed to assess the
significance of treatments vs. controls. Asterisks in the figures represent P values of less
than 0.05 to indicate statistical significance.

Results

MiR-24 targets the 3’-UTR of hALK4 mRNA

To identify miRNAs that would regulate the expression of hALK4, we used a
computational method based on the conservativeness across multiple species of the potential
binding sites in the 3’-UTR of hALK4 mRNA and identified four potential miRNAs:
miR-30a-3p, miR-30d, miR-24 and miR-205. To experimentally verify the computational
prediction, we constructed a firefly luciferase reporter that contained the full length 3’-UTR
of hALK4 mRNA and whose expression was driven by the CMV promoter (Fig. 1A). To
express these miRNAs, about 500bp miRNA transcripts that cover the miRNA hairpin
precursor and its native flanking sequence were generated, and the expression of these
miRNAs were confirmed by stem-loop RT-PCR (Fig. 1B) and by Northern blotting (data not
shown). The reporter assay showed that only miR-24 was able to significantly repress
luciferase expression (Fig. 1B). Furthermore, the inhibitory effect of miR-24 on reporter
expression is dose-dependent, but increasing concentrations of miR-205 had no effect on
luciferase activity (Fig. 1C), indicating miR-24 specifically targets the hALK4 3’-UTR.

There are two human miR-24 genes, encoding miR-24-1 and miR-24-2. 22,23  Mir-24-1,
mir-23b and mir-27b are encoded by a miRNA cluster on 9q22.1, while mir-24-2, mir-23a and mir-27a are encoded by another homologous miR cluster on 19p13.2. The construct we had generated contained miR-24-1 transcript and excluded mir-23b and mir-27b precursors. However, the human homologue of mouse miR-189 appears to originate from the 5' arm of the same precursor of miR-24-1 (Fig. 1D), and a recent study suggested miR-189 regulates the expression of the Tourette’s Syndrome candidate gene SLITRK1.

To examine which miRNA was generated from our miRNA expression construct, we used the probes complementary to miR-189 or miR-24 for Northern analysis and found that the miR-24 probe revealed a 500-nucleotide and a 22-nucleotide band in the miR-24 expressing cells but not in the cells expressing empty vector or control miRNA (con-miR) (Fig. 1E), corresponding to the miRNA transcript and the mature miRNA, respectively. However, the miR-189 probe uncovered only the 500-nucleotide miRNA transcript. This suggests that only miR-24 can be expressed in our experimental system and therefore is responsible for the inhibitory effect on reporter expression.

**MiR-24 interferes with activin signaling via down-regulating hALK4 expression**

MiRNAs modulate gene expression either by targeting mRNAs for degradation or by repressing translation. To investigate how miR-24 modulates hALK4 expression, we examined the effects of miR-24 on the mRNA and protein levels of hALK4 in HEK293 cells, which are highly responsive to activin and TGF-β stimulation and have low miR-24 expression (Fig. 1B). We observed that ectopic expression of miR-24 led to a 50% decrease of the hALK4 mRNA level in HEK293 cells (Fig. 2A). Consistently, the
endogenous hALK4 protein level was also apparently reduced by miR-24 (Fig. 2B).

To explore whether miR-24 would interfere with activin signaling, Smad2 phosphorylation was determined. As shown in Fig. 2C, ectopic expression of miR-24 suppressed activin-induced Smad2 phosphorylation. We analyzed the effect of miR-24 on the transcriptional responses of activin A or TGF-β using the activin/TGF-β-responsive reporters ARE-luciferase, which is Smad2/Smad4-specific, and CAGA12-luciferase, which is Smad3/Smad4-specific. MiR-24 specifically decreased the activin A- but not TGF-β-induced expression of both reporters (Fig. 2D and Fig. 2E). Furthermore, miR-24 did not interfere with the expression of CAGA-luciferase mediated by the constitutively active ALK4 (caALK4-T206D) which did not carry the 3’-UTR and was exogenously expressed with the CMV promoter (Fig. 2F).

Taken together, these results suggest that miR-24 specifically interferes with activin signaling by targeting the 3’-UTR of hALK4 mRNA and reducing hALK4 expression.

Repression of hALK4 by miR-24 is via a conserved binding site in the 3’-UTR of hALK4 mRNA

MiRNAs execute their functions by forming miRNA-mRNA duplex through binding to respective target sites. The binding sites of miRNAs in their target genes are usually evolutionarily conserved and perfectly matched to the 5’ end of the miRNA. We found two potential binding sites of miR-24 in the 3’-UTR of hALK4 mRNA. One lies at 445 to 467bp downstream of the stop codon, and the other at 985 to 1011bp (Fig. 3A). Both sites are conserved in several mammals. To confirm that repression of hALK4 by miR-24 is via
the predicted miR-24 binding sites, we mutated these sites and introduced the mutant sequences into the 3’ end of the luciferase, yielding M1 and M2 mutants, respectively. Reporter assays revealed that M2 mutant was resistant to the miR-24-mediated repression, while miR-24 had the similar inhibitory effect on M1 mutant compared with wild-type 3’-UTR (Fig. 3B). These data suggested that the sequence at 985 to 1011bp is the major miR-24 binding site and further confirmed that miR-24 directly targets the 3’-UTR of hALK4 mRNA to execute its inhibitory function on activin signaling.

**MiR-24 antagonizes activin A-mediated erythroid differentiation of K562 cells**

Expression profiling studies indicate that most miRNAs are under the control of developmental or tissue-specific signaling. MiR-24 is highly expressed in lung and heart, low in kidney, spleen and thymus, and not detectable in brain and liver (our unpublished data). It is well known that activin plays a critical role in promotion of hematopoiesis through its receptors. To investigate whether miR-24 functions in hematopoietic cells by regulating hALK4 expression, we first determined miR-24 expression in human hematopoietic cells. The results of stem-loop RT-PCR showed that miR-24 was expressed in K562 cells at a detectable level and highly expressed in HPCs, and the ectopic expression level of miR-24 was evidently higher than the endogenous miR-24 (Fig. 4A).

Activin A can induce human erythroleukemia K562 cells to undergo erythroid differentiation and cause the accumulation of erythroid marker hemoglobin (Hb). To explore the role of miR-24 in erythroid differentiation, we first examined its activity in modulating activin signaling in K562 cells by reporter assay. As shown in Fig. 4B, miR-24
attenuated the activin A-induced expression of CAGA-luciferase in K562 cells. 2’-O-methyl antisense oligoribonucleotides have been used to interfere with miRNA activity\textsuperscript{30,31}. To study the function of endogenous miR-24, we synthesized several such oligoribonucleotides. The miR-24 inhibitor anti-24 oligoribonucleotide enhanced the activin-mediated reporter expression while anti-GFP or anti-24M that harbors 4 base substitution from anti-24 had no effect (Fig. 4C). These data suggest that miR-24 regulates activin signaling in K562 cells at the endogenous level.

To assess the effect of miR-24 on activin-mediated erythroid differentiation, we examined activin A-mediated Hb accumulation in K562 cells by benzidine staining. As shown previously\textsuperscript{3,7,21}, activin A increased Hb production (Fig. 4D). Overexpression of miR-24 but not control miRNA significantly inhibited the activin A-dependent Hb accumulation, as the benzidine-stained Hb-positive cells dropped from 45% to 25% (Fig. 4D and 4E). Furthermore, knockdown of endogenous miR-24 by anti-24 oligoribonucleotide further enhanced the activin A-promoted Hb accumulation, whereas anti-24M had no effect (Fig. 4F). The miR-24 expression was unchanged by activin treatment in 3 days (data not shown). Consistent with the inhibitory effect of miR-24 on Hb accumulation, miR-24 specifically reduced the endogenous hALK4 protein and Smad2 phosphorylation (Fig. 4G). Collectively, these results suggest that miR-24 interferes with activin signaling by targeting ALK4 expression and thus attenuates erythroid differentiation of K562 cells.

**MiR-24 attenuates erythroid differentiation and promotes proliferation of HPCs**

To investigate the effect of miR-24 on activin A-mediated hematopoietic development,
primary CD34+ hematopoietic progenitor cells (HPCs) isolated from human umbilical cord blood were transfected with indicated plasmids and pEGFP-N1, or anti-24 oligoribonucleotide and FICT-labeled nonspecific oligoribonucleotide. Fluorescence-positive cells were selected by FACS and planted in methylcellulose-based media for colony-forming cell assay or in liquid erythroid differentiation media in the presence or absence of activin A. The number of CFU-E, which represents the more mature erythroid progenitors, was counted one week later after planting, while BFU-E, which represents the primitive erythroid progenitors, was counted until two weeks. Ectopic expression of miR-24 led to about 30% decrease in the colony formation of both CFU-E and BFU-E (Fig. 5A and 5B). When exogenous hALK4 (lacking the 3’-UTR) was co-expressed, miR-24 had no significant effect on colony formation (a slightly decrease of colony formation could be due to the inhibitory of miRNA on endogenous hALK4 expression). Ectopic expression of miR-24 also mildly repressed the forming of CFU-GEMM (colony forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte), but had no influence on CFU-M (colony forming unit-macrophage) formation (data not shown). CFU-G (colony forming unit-granulocyte) and CFU-GM (colony forming unit-granulocyte, macrophage) were rarely detected in our experimental system. These data suggest that miR-24 modulates erythroid differentiation but has no effect on macrophage development.

As miR-24 is highly expressed in HPCs (Fig. 4A and 5E), we attempted to investigate whether endogenous miR-24 plays a physiological role in erythroid differentiation of HPCs. CD34+ HPCs were transfected with anti-24 or anti-24M oligoribonucleotides, and then
subjected to colony-forming assay. As shown in Fig. 5C and 5D, miR-24 knockdown specifically increased the number of both CFU-E and BFU-E. The effect of anit-24 in the absence of exogenously added activin A could be through the autocrine activity of activin secreted by these cells. These results strongly indicated that miR-24 inhibits HPC differentiation along the erythroid lineage both at the early BFU-E stage and the late CFU-E stage.

The miRNA expression profile in hematopoietic lineages showed that miRNAs are differentially expressed during the course of hematopoietic development. To further assess the physiological function of miR-24 in erythropoiesis, we examined the expression levels of miR-24 and hALK4 during erythroid differentiation of HPCs. Stem-loop RT-PCR analysis revealed that mature miR-24 was abundant in HPCs, but its expression markedly declined after day 3 culture in liquid erythroid differentiation condition (Fig. 5E). Accordingly, hALK4 expression increased prominently at days 5 and 7, which was inversely correlated to miR-24 expression. These data suggest that miRNA-24 may be important to maintain hematopoietic cells at early stem-progenitor stages and block their erythroid differentiation through restraining hALK4 expression.

To analyze effects on erythroid differentiation, we placed CD34+ HPCs transfected with control, miR-24 or anti-24 oligoribonucleotide in liquid erythroid differentiation media in the presence or the absence of activin. Cell differentiation and proliferation were assessed over 15 days, during which CD34+ HPCs can complete their development to mature erythrocytes. We harvested the cells at indicated time points and determined their differentiation state by Wright-Giemsa staining. Activin treatment accelerated the differentiation of HPCs to
erythrocytes, and the promoting effect of activin became evident after 5 days incubation (Fig. 5F). Consistent with the results of colony formation assay, overexpression of miR-24 delayed the activin-induced erythroid differentiation of HPCs, while miR-24 knockdown had an opposite effect (Fig. 5F). Most of the cells had already been differentiated to red cells after day 9 (data not shown). These results indicate that HPCs are more sensitive to activin stimulation at the late stages of erythroid differentiation when miR-24 level is low and ALK4 expression is accordingly high.

In agreement with the previous studies, activin treatment profoundly inhibited HPC proliferation (Fig. 5G). MiR-24 knockdown enhanced the inhibitory effect of activin on cell growth, while ectopic miR-24 expression attenuated the anti-proliferative effect of activin (Fig. 5G). Together with the above data, these results suggest that miR-24 inhibits erythroid differentiation and promotes proliferation of HPCs by modulating activin signaling.

**Discussion**

In this study, we showed that miR-24 down-regulated hALK4 by targeting the 3′-UTR of its mRNA, reduced activin-mediated Smad2 phosphorylation and attenuated the transcriptional responses of activin. The inhibitory effect of miR-24 on activin signaling is specific as miR-24 did not affect the reporter expression induced by TGF-β although both activin and TGF-β share the common Smad pathway and have some of common targets. Furthermore, miR-24 specifically targets on the 3′-UTR of hALK4 mRNA as this miRNA has no effect on the transcriptional response of the constitutively active form of ALK4 as
evaluated with reporter expression.

MiR-24 has been implicated to regulate cell growth and differentiation. Antisense inhibition of miR-24 promotes HeLa cell proliferation, but attenuates A549 cell growth. MiR-24 was recently reported to downregulate the expression of human dihydrofolate reductase (DHFR) by targeting the 3’-UTR of DHFR mRNA. A naturally occurring single nucleotide polymorphism (829C/T) in the DHFR gene, near the miR-24 binding site, results in DHFR overexpression and methotrexate resistance in the chemotherapeutic treatment to several malignancies. In addition, miR-24 expression is upregulated during 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced monocytic differentiation of human leukemia HL-60 cells, implying it may function in hematopoietic differentiation. Here, we showed that miR-24 modulated erythropoiesis by influencing activin signaling in K562 cells and primary CD34+ HPCs. Overexpression of miR-24 antagonized activin A-mediated hemoglobin accumulation in K562 cells and caused about 30% decrease in the colony formation of both CFU-E and BFU-E of HPCs. We further demonstrated that the function of miR-24 in erythropoiesis is physiologically relevant as inhibition of the endogenous miR-24 activity by antisense oligoribonucleotide specifically enhanced hemoglobin accumulation and the expansion of CFU-E and BFU-E. Although we cannot exclude the possibilities that other miRNAs may cooperate with miR-24 to block erythropoiesis through repressing hALK4 expression and that miR-24 may have other targets during erythroid differentiation at this moment, our data strongly suggested that miR-24 hampers erythropoiesis by disturbing activin signaling and ALK4 is its major target as exogenous expression of ALK4 was able to rescue the inhibitory effect of miR-24 on
erythroid differentiation of HPCs. In agreement with our conclusion that miR-24 modulates erythropoiesis, recent bioinformatic and functional analyses indicated that multiple miRNAs, including miR-24, might maintain hematopoietic cells at an early stem-progenitor stage and block their differentiation to more mature cells 34.

Other TGF-β family members such as BMP2 and BMP4 have also been implicated in the regulation of hematopoiesis 35,36. BMP2 was suggested to act at the early stages of erythroid differentiation while activin A functions on a more differentiated population when in vitro erythroid differentiation model of CD34+ progenitor cells from peripheral blood and bone marrow was used 37. In our liquid erythroid differentiation system of CD34+ HPCs, we found that miR-24 not only inhibited activin-induced erythroid differentiation, but also rescued activin-induced cell growth arrest. More interestingly, the expression of endogenous miR-24 gradually and markedly declined after 3 days’ differentiation. Accordingly, hALK4 expression was evidently increased at days 5 and 7, which was inversely correlated to miR-24 expression. Furthermore, the effect of activin to accelerate the erythroid differentiation of HPCs was evident after 5 days incubation. These data suggest that miR-24 may be important to maintain hematopoietic cells at early stem-progenitor stages and block their erythroid differentiation through restraining hALK4 expression, and the decrease at day 14 of BFU-E derived colonies is due to an effect on the early stage of erythroid differentiation. The stimulatory activity of activin is prominent at late stages (after 5 days in our in vitro liquid differentiation system) when miR-24 level is low and ALK4 expression is accordingly high. At this moment, we are unable to pinpoint the specific differentiation stage when the activin action starts to be critical as the isolated
CD34+ HPCs are not homogenous and the \textit{in vitro} differentiation systems do not allow synchronized differentiation. In summery, our findings for the first time suggest that miR-24 modulates erythropoiesis by attenuating activin signaling.

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**Author contributions**

Qiang Wang has performed most of the experiments and manuscript writing. Zheng Huang and Huiling Xue have performed bioinformatics analysis. Chengcheng Jin has made some constructs. Xiu-Li Ju has provided umbilical cord blood and helped with isolation of CD34+ cells. Jing-Dong J. Han has guided bioinformatics analysis and provided helps in data analysis and discussion. Ye-Guang Chen has been involved in experiment design, data analysis and manuscript writing.

COMPETING INTERESTS STATEMENT: The authors declare that they have no competing financial interests.
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Fig. 1  **MiR-24 inhibits reporter activity through the 3’-UTR of hALK4 mRNA.**  (A) Schematic representation of the luciferase reporter constructs. The CMV promoter and the full length 3’-UTR of hALK4 mRNA were inserted to pGL3-basic vector as indicated. (B) HEK293 cells were cotransfected with the hALK4-3’-UTR luciferase reporter (0.3µg) and miRNA (1µg) as indicated. At 48 hr post-transfection, cells were harvested for luciferase assay (up panel). The expression of the four miRNAs was detected by stem-loop RT-PCR with U6 as loading control. The asterisk indicates a significant difference between miR-24 and the control (P<0.05). (C) HEK293 cells were cotransfected with the hALK4-3’-UTR luciferase reporter and increasing amounts of miR-24 or miR-205 (1µg). (D) Sequence of human miR-24/miR-189 stem-loop. (E) Detection of the miR-189 and miR-24 expression by Northern blot analysis in HEK293 cells transfected with the miR-24/miR-189 precursor vector. Con-miR: a vector expressing a nonspecific small RNA molecule (5’-AGCGGACTAAGTCCATTGCTT-3’) as a negative control miRNA. Reporter assay was performed in triplicate and the data represent the mean ± S.D. of three independent experiments after normalized to Renilla activity.

Fig. 2  **MiR-24 specifically interferes with activin signaling.**  (A, B) RT–PCR (A) and Western blot analysis (B) of hALK4 expression in HEK293 cells transfected with various miRNA vectors as indicated. Quantitation of hALK4 mRNA levels after normalization to GAPDH and hALK4 protein levels to β-actin from three independent experiments were shown. (C) HEK293 cells transfected with indicated vectors were treated with activin A (25ng/ml) for 1 hr and then harvested for anti-phospho-Smad2 Western blot analysis.
Smad2 expression was examined with anti-Smad2 immunoblotting to verify protein expression. (D, E) HEK293 cells were cotransfected with ARE-luciferase and FoxH1 (D) or CAGA₁₂-luciferase (E) along with indicated miRNA vectors, and treated with activin A (25ng/ml) or TGF-β (1.25 ng/ml) for 16 hr before harvested for luciferase assay. (F) HEK293 cells were transfected with CAGA₁₂-luciferase and active ALK4 (caALK4) with indicated miRNA vectors. Luciferase assay was performed at 48 hr post-transfection. Reporter assay was performed in triplicate and the data represent the mean ± S.D. of three independent experiments after normalized to Renilla activity.

Fig. 3  There is a miR-24 binding site in the 3’-UTR of hALK4 mRNA.  (A) Sequence alignment between miR-24 and its two putative binding sites in the 3’-UTR of ALK4 mRNA from different species. One lies in 445 to 467bp downstream of the stop codon of the 3’-UTR of hALK4 mRNA, and the other in 985 to 1011bp.  (B) HEK293 cells were transfected with luciferase reporter containing the hALK4 3’-UTR with wild-type or mutated target sites (shown in A, M1 and M2) along with empty or miR-24 vector. Luciferase assay was performed at 48 hr post-transfection. Reporter assay was performed in triplicate and the data represent the mean ± S.D. of three independent experiments after normalized to Renilla activity. The asterisk indicates a significant difference between miR-24 and control vector (P<0.05).

Fig. 4  MiR-24 reduces activin A-induced hemoglobin accumulation in K562 cells.  (A) The expression of miR-24 in K562 cells and HPCs was assessed by stem-loop RT-PCR with
U6 as loading control. Murine lung miR-24 was a positive control. (B, C) K562 cells were cotransfected with CAGA12-luciferase along with miRNA vectors (B) or anti-miRNA oligoribonucleotides (C) as indicated. After treated with activin A (25ng/ml) for 16 hr, the cells were harvested for luciferase assay. Reporter assay was performed in triplicate and the data represent the mean ± S.D. of three independent experiments after normalized to Renilla activity. (D-G) K562 cells were transfected with the indicated constructs or anti-miRNA oligoribonucleotides. The transfected cells were placed in the absence or presence 25ng/ml activin A for 3 days and processed for benzidine staining. The results are expressed in percentages of benzidine-positive (blue) cells compared to the total cell number. The values represent the means ± S.D of three separate experiments. A representative field (original magnification X 100) (D) and quantitation of three independent experiments (E and F) were shown. The asterisk indicates a significant difference between miR-24 and control vector or between anti-24 and anti-24M oligoribonucleotides (P<0.05). (G) K562 cells were cotransfected with indicated miRNA plasmids and treated with activin A for 1 hr before the cells were harvested for Western blotting to reveal the protein levels of hALK4 (up panel), phospho-Smad2 (3rd panel) or total Smad2 (4th panel).

Fig. 5 MiR-24 inhibits activin A-induced erythroid differentiation of CD34⁺ hematopoietic progenitor cells. (A-D) CD34⁺ HPCs were transfected by electroporation at 220V, 1600µF (see Materials and Methods) with GFP and miRNA plasmids, the plasmid expressing ALK4 that lacks the 3’-UTR or anti-miRNA oligoribonucleotides as indicated. Then GFP-positive cells were sorted and placed for colony-forming cell assays in semisolid
media. CFU-E was scored at day 7 (A, C) of culture and BFU-E at day 14 (B, D). The data represent the mean ± S.D. of three independent experiments. The asterisks indicate a significant difference between control miRNA and miR-24 or between anti-24 and its mutant control (* indicates P<0.05; ** indicates P<0.01). (E) CD34+ HPCs were maintained in liquid erythroid differentiation culture condition for 7 days. MiR-24 expression at the indicated times was detected by stem-loop RT-PCR, and endogenous ALK4 expression detected by RT-PCR. (F, G) CD34+ HPCs were transfected with GFP and miRNA vectors or anti-miRNA oligoribonucleotides as indicated. Then GFP-positive cells were sorted and cultured in liquid differentiation media in the absence or presence of activin A. The differentiation state of the cells was determined by Wright-Giemsa staining of cytospin preparations, and cell numbers at different differentiation stages (F) or total cell numbers (G) were counted at the indicated times. For panel F, about three hundred cells were counted from 3 different fields.
Figure 1

A

B

C

D

E

Homo sapiens miR-24/miR-189 stem-loop:

miR-189 5' cuoa gu cu cuaguca guagu u u

miR-24 3' gaug ca gga aauugauu ggauca u u

a a c -c cacau
Figure 3

A

putative binding site: 445 ~ 467 bp

miR-24: 3′ GAC--AAG-G-ACGACUUUGACUCG GU 5′

hALK4 3′UTR: 5′ CT--CTCACAGGCAGCTCTGAGCCG 3′

Human ALK4 3′UTR: CT----CTCAAGGCAGCTCTGAGCCG
Chimpanzee ALK4 3′UTR: CT----CTCAAGGCAGCTCTGAGCCG
Mouse ALK4 3′UTR: TT----CTCAAGGCAGCTCTGAGCCG
Rat ALK4 3′UTR: TT----CTCAAGGCAGCTCTGAGCCG
Dog ALK4 3′UTR: CT----CTCAAGGCAGCTCTGAGCCG
Consensus CT CTC CAGGCAGCTCTGAGCC

hALK4 3′UTR mutants: M1: CT CTC ACAGGCAGCTCT GCTAAG

putative binding site: 985 ~ 1011 bp

miR-24: 3′GA –CA –AGGAC -G—a -C –UUGA CU CGGU 5′

hALK4 3′UTR: 5′TTAGTGTCAGCCGTGGGAAAATGAGCCA 3′

Human ALK4 3′UTR: TTAGTGTCAGCCGTGGGAAAATGAGCCA
Chimpanzee ALK4 3′UTR: TTAGTGTCAGCCGTGGGAAAATGAGCCA
Mouse ALK4 3′UTR: TTAGTGTCAGCCGTGGGAAAATGAGCCA
Rat ALK4 3′UTR: TTAGTGTCAGCCGTGGGAAAATGAGCCA
Dog ALK4 3′UTR: TTAGTGTCAGCCGTGGGAAAATGAGCCA
Consensus TTAGTGTCAGCCGTGGGAAAATGAGCCA

hALK4 3′UTR mutants: M2: TTAGTGTCAGCCGTGGGAAAATGCTAAC

B

![Bar chart](image)

- Relative Luciferase Activity
- **Vector**
- **miR-24**

WT M1 M2
Figure 5

A

Number of CFU-E

- Activin
- + Activin

ALK4 - - - + +
Con-miR - + - - +
mIR-24 - - + - -

B

Number of BFU-E

- Activin
- + Activin

ALK4 - - - + +
Con-miR - + - - +
mIR-24 - - + - -

C

Number of CFU-E

- Activin
- + Activin

Anti-24M -
Anti-24 -

D

Number of BFU-E

- Activin
- + Activin

Anti-24M -
Anti-24 -

E

0d 1d 3d 5d 7d
miR-24
U6
hALK4
GAPDH

F

Percentage of differentiated cells

3d

A B C D

5d

A B C D

7d

A B C D

- Activin
+ Activin
miR-24+activin
Anti-24+activin

G

Cell number \( \times 10^5 \) /ml

0d 1d 3d 5d 7d 9d 12d 15d

- activin
+ activin
miR-24+activin
anti-24+activin
MicroRNA miR-24 inhibits erythropoiesis by targeting activin type I receptor ALK4

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