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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 erythroleukemia 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. (Blood. 2008;111:588-595)

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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.1 This process is regulated by various factors such as erythropoietin, testosterone, estrogen, interleukin-3, granulocyte-macrophage colony-stimulating factor, and interleukin-9.2

Activin, a member of the transforming growth factor-β (TGF-β) superfamily, plays an important role in modulating proliferation and differentiation of erythroid progenitors.2-4 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.3,5-7 Activin, like other TGF-β superfamily members, signals through 2 types of transmembrane receptors that have intrinsic serine/threonine kinase activity.8,9 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, which, 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.10,11

MicroRNAs (miRNAs) are 21- to 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 miRNAs.12,13 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.14,15 For example, ectopic expression of miR-181 in hematopoietic progenitor cells increased the proportion of B-lineage cells, whereas miR-142s and miR-223 promote the differentiation to T-lineage but not to B-lineage or myeloid cells.15 MiR-155 transgenic mice exhibited pre-B cell proliferation and B cell malignancy.16 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.17 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.18

In this study, we showed that miR-24 could decrease human ALK4 (hALK4) expression at the mRNA and the protein levels through binding to the 3'-untranslated region (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.

Methods

Cell culture and reagents

Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS).
K562 cells were grown in RPMI-1640 medium/DMEM supplemented with 10% FBS. All the growth factors and anti-human ALK4 antibody (AF222) were purchased from R&D Systems (Minneapolis, MN), anti-p-Smad2 antibody from Upstate Biotechnology (Charlottesville, VA) and anti-β-actin antibody from Santa Cruz Biotechnology (Santa Cruz, CA).

** Constructs**
To generate a luciferase reporter to evaluate miRNA activity, the CMV Construct was cloned from HeLa genomic DNA and inserted downstream of the firefly luciferase’s open reading frame. miRNA transcripts of approximately 30–60 nt, were amplified by polymerase chain reaction (PCR) from the luciferase’s open reading frame. miRNA transcripts of approximately 30a-3p (RT primer, 5’-GAAGCAGTGGTCACTTCAC-3’; reverse, 5’- ATGCATACACAGAGC-3’); miR-30a-3p (RT primer, 5’-GGGTCCGAGGT-3’; reverse, 5’- CAATGGCTCAGTTCAGCAG-3’); miR-205 (RT primer, 5’-GCAGATGGCTCAGTTCAGCAG-3’; reverse, 5’- GCAGATGTAAA-3’); miR-189 (5’-UACGCTTCACGAATTTGCGT-3’). Stem-loop RT-PCR primers: miR-205 (forward, 5’-CAGGAAGTTGCAAGCAGGAGT-3’; reverse, 5’- TAATGTACAGCAGAAATTGG-3’); miR-24 (RT primer, 5’-GCGTCTAGAGCAGAAATTGGACAGGCTCA-3’; forward primer, 5’-CGACGACTTTCA-3’); miR-189 (RT primer, 5’-GCGTCTAGAGCAGAAATTGGACAGGCTCA-3’; forward, 5’-GTGCA-3’); miR-520 (3’-UTR (forward, 5’-CCAGACT-3’; forward primer, 5’-CGACGACTTTCA-3’); reverse, 5’- GTGCA-3’); miR-205 (RT primer, 5’-GCGTCTAGAGCAGAAATTGGACAGGCTCA-3’; forward, 5’- GTGCA-3’); miR-520 (3’-UTR (forward, 5’-CCAGACT-3’; forward primer, 5’-CGACGACTTTCA-3’); reverse, 5’- GTGCA-3’). All the sequences were confirmed by DNA sequencing.

**Oligonucleotide sequences**
2’-O-methyl oligoribonucleotides: anti-24 (5’-CUGU/CUCUGCUAGCUCUCUUA-3’); anti-green fluorescent protein (GFP) (5’-AGGCAGCUACCGCUCCUCUC-3’); Northern probes: miR-30d (5’-CCTCCAGTGGAGAATTGCACTTCAC-3’); miR-30a-3p (5’-CCTGCAAACATCGAATGGAGG-3’); miR-24 (5’-CTGTCCTGCTGAATGGACAGCA-3’); miR-205 (5’-CAACTGGTGGAATAGAAG-3’); miR-189 (5’-ACTGATATCGTACGTTAGGAC-3’); U6 (5’-AACGCTTCAGCAGATTGTGG-3’). PCR primers: hALK4 3’-UTR (forward, 5’-CTCGACTGCGCAGCA-3’; reverse, 5’-TGGTACGTAGTGGTGCAGGGT-3’); miR-30d transcript (forward, 5’-TACAAGCTCTACGCTTCTTCTTCTTT-3’; reverse, 5’-ATATGCGACCACCATGCTTC-3’); miR-30a-3p transcript (forward, 5’-GACAGATCTAAATTCTGGTGAACCACTT-3’; reverse, 5’-ATATGCGACCACCATGCTTC-3’); miR-24 transcript (forward, 5’-CAGAGATCAAACAGGCTTCTTCAGTTGTTAG-3’; reverse, 5’-TAATGTACAGCAGAAATTGG-3’); miR-205 transcript (forward, 5’-GCAAGATTTGGCAGAAGCAGG-3’; reverse, 5’-GAAGTGCACCCGCTTCTTTCTTCC-3’; reverse, 5’-GAAGTGCACCCGCTTCTTTCTTCC-3’; reverse, 5’-GAAGTGCACCCGCTTCTTTCTTCC-3’; reverse, 5’-GAAGTGCACCCGCTTCTTTCTTCC-3’; reverse, 5’-GAAGTGCACCCGCTTCTTTCTTCC-3’; reverse, 5’-GAAGTGCACCCGCTTCTTTCTTCC-3’; reverse, 5’-GAAGTGCACCCGCTTCTTTCTTCC-3’; reverse, 5’-GAAGTGCACCCGCTTCTTTCTTCC-3’; reverse, 5’-GAAGTGCACCCGCTTCTTTCTTCC-3’; reverse, 5’-GAAGTGCACCCGCTTCTTTCTTCC-3’; reverse, 5’-GAAGTGCACCCGCTTCTTTCTTCC-3’; reverse, 5’-GAAGTGCACCCGCTTCTTTCTTCC-3’; reverse, 5’-GAAGTGCACCCGCTTCTTTCTTCC-3’; reverse, 5’-GAAGTGCACCCGCTTCTTTCTTCC-3’; reverse, 5’-GAAGTGCACCCGCTTCTTTCTTCC-3’; reverse, 5’-GAAGTGCACCCGCTTCTTTCTTCC-3’; reverse, 5’-GAAGTGCACCCGCTTCTTTCTTCC-3’; reverse, 5’-GAAGTGCACCCGCTTCTTTCTTCC-3’; reverse, 5’-GAAGTGCACCCGCTTCTTTCTTCC-3’; reverse, 5’-GAAGTGCACCCGCTTCTTTCTTCC-3’; reverse, 5’-GAAGTGCACCCGCTTCTTTCTTCC-3’. All the sequences were confirmed by DNA sequencing.

**RNA isolation and reverse transcription-PCR**
RNA was reverse-transcribed using ReverTra Ace-o (Toyobo Engineering, Osaka, Japan). To detect the mRNA of hALK4, PCR was performed at 94°C for 15 seconds, 51°C for 30 seconds, and 68°C for 90 seconds, 30 cycles. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as an internal control. To detect mature miR-24 expression, stem-loop reverse transcriptase (RT)-PCR was performed as described previously.29 Total RNA (2 μg) was reverse-transcribed and PCR was performed at 94°C for 15 seconds, 55°C for 30 seconds, and 68°C for 20 seconds, 40 cycles.

**Northern blot analysis**
Total RNA (30 μg) was separated on 15% denaturing polyacrylamide gels, electrotransferred to nylon membrane (Hybond), and hybridized with UltraHyb-Oligo buffer (Ambion, Austin, TX). Oligonucleotides complementary to mature miRNAs were end-labeled with T4 kinase (Takara, Kyoto, Japan). The blot was washed 3 times for 10 minutes each at room temperature in 3× SSC and 0.5% sodium dodecyl sulfate (SDS), and then 15 minutes in 1× SSC and 0.5% SDS and processed for autoradiography.

**Isolation of CD34+ hematopoietic progenitor cells**
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, Gladbach, Germany) according to the manufacturer’s instructions. The isolation system yielded approximately 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+ hematopoietic progenitor cells (HPCs) were cultured in a serum-free medium for 48 hours, and then were transfected by electroporation as described previously20 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 fluorescein isothiocyanate (FITC)-nonspecific oligoribonucleotide. The electroporation was performed with GenePulsor Xcell (Bio-Rad Laboratories, Hercules, CA) at 220 V, 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 hours with fresh medium and then selected by fluorescence-activated cell sorting (FACS).

**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 (25 ng/mL) or TGF-β1 (1.25 ng/mL) for 16 hours before harvest for luciferase assay. Each experiment was performed in triplicate and the data represent the mean plus or minus standard deviation (SD) of 3 independent experiments after normalized to Renilla reniformis activity.

**Benzidine staining**
Cells (1.5 x 10^5) were treated with or without 25 ng/mL activin A. Three days later, cells were harvested and analyzed by benzidine staining.21

**Colony-forming cell assay**
Colony-forming cell (CFC) assay was performed in triplicate using human methycellulose complete media (R&D Systems) according to the manufacturer’s instructions. HPCs were cultured in 35-mm plates with the medium containing 1.3% methycellulose, 25% FBS, 2% bovine serum albumin, 2 mmol/L-L-glutamine, 0.05 mmol/L 2-mercaptoethanol, 50 ng/mL stem cell factor (SCF), 10 ng/mL interleukin-3 (IL-3), 3 U/mL erythropoietin (EPO), and 10 ng/mL granulocyte macrophage–colony-stimulating factor. Activin A (25 ng/mL) was added as indicated in Figure 5. To enumerate CFU-E-derived colonies, the dishes were examined for hemoglobinized and compact colonies with 8 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 3 or more subcolonies with 8 or more hemoglobinized cells per subcolony.

**Erythroid differentiation of HPCs and Wright-Giemsa staining**
Purified CD34+ HPCs were cultured in Iscove’s modified DMEM supplemented with 30% fetal calf serum, 2 mmol/L glutamine, 1 mg/mL deionized...
bovine serum albumin (Sigma), 50 pmol/L 2-mercaptoethanol (Merck, Whitehouse Station, NJ), 100 ng/mL recombinant human (rh) IL-3, 100 ng/mL rhSCF, and 6 units/mL rhEPO, and in the presence or the absence of activin A (25 ng/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, Mountain View, CA). Images were obtained using 0.12 numeric aperture Planapo objective lens. Student t tests were performed to assess the significance of treatments vs controls. Asterisks in the figures represent P values of less than .05 to indicate statistical significance.

Results

MiR-24 targeted 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 4 potential miRNAs: miR-30a-3p, miR-30d, miR-24, and miR-205. To experimentally verify the significance of treatments vs controls. Asterisks in the figures represent P values of less than .05 to indicate statistical significance.

Figure 1. MiR-24 inhibited 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 (0.3 μg) and miRNA (1 μg) as indicated. At 48 hours after transfection, cells were harvested for luciferase assay (top panel). The expression of the 4 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 < .05). (C) HEK293 cells were cotransfected with the hALK4-3'-UTR luciferase reporter and increasing amounts of miR-24 or miR-205 (1 μg). (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 nongenomic small RNA molecule (5'-AGCGGACTAAAGTCCT-GCTT-3') as a negative control miRNA. Reporter assay was performed in triplicate, and the data represent the mean plus or minus SD of 3 independent experiments after normalized to R Renilla activity.

 MiR-24 interfered with activin signaling via down-regulation of hALK4 expression

MiRNAs modulate gene expression either by targeting miRNAs for degradation or by repressing translation. 12 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 (Figure 1B). We observed that ectopic expression of miR-24 led to a 50% decrease of the hALK4 mRNA level in HEK293 cells (Figure 2A). Consistently, the endogenous hALK4 protein level was also apparently reduced by miR-24 (Figure 2B).

To explore whether miR-24 would interfere with activin signaling, Smad2 phosphorylation was determined. As shown in Figure 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/transfoming growth factor-β (TGF-β)-responsive reporters activin response element (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 (Figure 2D,E). 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 (Figure 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 occurred 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 2 potential binding sites of miR-24 in the 3’-UTR of hALK4 mRNA. One lies at 445–467 bp downstream of the stop codon, and the other at 985-1011 bp (Figure 3A). Both sites are conserved in several mammals. To confirm that repression of hALK4 by miR-24 occurs 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, whereas miR-24 had the similar inhibitory effect on M1 mutant compared with wild-type 3’-UTR (Figure 3B). These data suggested that the sequence at 985-1011 bp 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 antagonized 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. 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 (Figure 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
sense oligoribonucleotides have been used to interfere with miRNA (asterisk indicates a significant difference between miR-24 and control vector). Consistent with the inhibitory effect of miR-24 on Hb accumulation, miR-24 specifically reduced the endogenous hALK4 protein and Smad2 phosphorylation (Figure 4G). Taken together, these results suggest that miR-24 interferes with activin signaling by targeting ALK4 expression and thus attenuates erythroid differentiation of K562 cells.

**MiR-24 attenuated erythroid differentiation and promoted proliferation of HPCs**

To investigate the effect of miR-24 on activin A-mediated hematopoietic development, primary CD34+ HPCs isolated from human umbilical cord blood were transfected with indicated plasmids and pEGFP-N1, or anti-24 oligoribonucleotide and FITC-labeled non-specific 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-Es, which represents the more mature erythroid progenitors, was counted 1 week later after planting, whereas BFU-E, which represents the primitive erythroid progenitors, was counted until 2 weeks. Ectopic expression of miR-24 led to approximately 30% decrease in the colony formation of both CFU-E and BFU-E (Figure 5A,B). When exogenous hALK4 lacking the 3′-UTR was coexpressed, 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-granulocyte/erythrocyte/macrophage/megakaryocyte, but had no influence on CFU-macrophage formation (data not shown). CFU-granulocyte and CFU-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.

Because miR-24 is highly expressed in HPCs (Figures 4A, 5E), we attempted to investigate whether endogenous miR-24 plays a physiologic 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 Figure 5C,D, 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 inhibited HPC differentiation along the erythroid lineage at both 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 physiologic 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 (Figure 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 absence of activin. Cell differentiation and proliferation were assessed over 15 days, during which CD34+ HPCs could...
complete their development into mature erythrocytes. We harvested the cells at indicated time points and determined their differentiation state by Wright-Giemsa staining (Figure 5F). Activin treatment accelerated the differentiation of HPCs to erythrocytes, and the promoting effect of activin became evident after 5 days of incubation (Figure 5F). Consistent with the results of colony formation assay, overexpression of miR-24 delayed the activin-induced erythroid differentiation of HPCs, whereas miR-24 knockdown had an opposite effect (Figure 5F). Most of the cells had already been differentiated to red cells after day 9 (data not shown). These results indicate that HPCs were more sensitive to activin stimulation at the late stages of erythroid differentiation when miR-24 level was low and ALK4 expression was accordingly high.

In agreement with the previous studies, activin treatment profoundly inhibited HPC proliferation (Figure 5G). MiR-24 knockdown enhanced the inhibitory effect of activin on cell growth, whereas ectopic miR-24 expression attenuated the antiproliferative effect of activin (Figure 5F). 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 in that 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 common targets.9-11 Furthermore, miR-24 specifically targets on the 3′-UTR of hALK4 mRNA because this miRNA has no effect on the transcriptional response of the constitutively active form of ALK4 as evaluated by reporter expression.

MiR-24 has been implicated in the regulation of cell growth and differentiation. Antisense inhibition of miR-24 promotes HeLa cell proliferation but attenuates A549 cell growth.30 MiR-24 was recently reported to down-regulate the expression of human dihydrofolate reductase (DHFR) by targeting the 3′-UTR of DHFR mRNA.32 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 up-regulated during 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced monocytic differentiation of human leukemia HL-60 cells,33 implying that 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 primary CD34⁺ HPCs. We further demonstrated that the function of miR-24 in erythropoiesis is physiologically relevant.
in that inhibition of the endogenous miR-24 activity by anti-sense 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 by repressing hALK4 expression and that miR-24 may have other targets during erythroid differentiation at this moment, our data strongly suggested that miR-24 hampered erythropoiesis by disturbing activin signaling and that ALK4 was its major target, because 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, whereas 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 because the isolated CD34+ HPCs are not homogenous and the in vitro differentiation systems do not allow synchronized differentiation. In summary, our findings suggest for the first time that miR-24 modulates erythropoiesis by attenuating activin signaling.

Acknowledgments

We are grateful to Dr Dong Li and Dr Guo-Yun Wang (Qilu Hospital of Shandong University, Jinan, China) for collection of umbilical cord blood.

This work was supported by grants from the National Natural Science Foundation of China (30430360, 30500883, 30428002) and 973 Program (2004CB720002, 2006CB943401, 2006CB910700).

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