

MicroRNA-181a* Targets Nanog in a Subpopulation of CD34⁺ Cells Isolated From Peripheral Blood

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Exploiting the properties of stem cells by microRNA (miRNA) profiling offers an attractive approach to identify new regulators of stem cell fate. Although numerous miRNA have been screened from hematopoietic stem cells (HSC), the targets corresponding to many of these miRNA have not yet been fully elucidated. By miRNA profiling in a subpopulation of CD34⁺ cells isolated from peripheral blood, we have identified eight clusters of miRNA that were differentially expressed. Further analysis of one of the clusters by bioinformatics revealed that a miRNA, miR-181a*, which is highly expressed in the adherent CD34⁺ cells, affects the expression levels of Nanog, a stem cell surrogate marker. We show specifically by reporter assay and mutational analysis that miR-181a* targets a seedless 3' compensatory site in the 3'UTR of Nanog and affects gene expression. We demonstrate that inhibiting miR-181a* upregulates the Nanog expression level, in addition to an increase in alkaline phosphatase activity. Our studies suggest that miR-181a* may be important in controlling the expression level of Nanog in a subpopulation of CD34⁺ cells.

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Subject Category: siRNAs, shRNAs, and miRNAs

INTRODUCTION

In recent years, studies in embryonic and somatic stem cells have provided basic insight into the molecular and cellular properties of stem cells.¹ It is recognized that pluripotent stem cells such as embryonic stem cells (ESC) can replenish differentiated cell types and achieve long-term tissue reconstitution. Similar to ESC, adult stem cells also have the capacity for self-renewal and multilineage differentiation.² Adult stem cells constitute ~1–2% of the total cell population within a specific tissue and are essential for maintaining homeostasis. These cells are normally quiescent and are held in an undifferentiated state within their niche until they receive a signal to self-renew or differentiate.³ They reside in various tissue types including the bone marrow, brain, digestive system, skin, retina, muscles, pancreas, and liver.⁴

Hematopoietic stem cells (HSC) are the most widely studied and characterized adult stem cells.⁵ They play an essential role in sustaining the formation of the blood and the immune system.^{6,7} These adult stem cells reside in the bone marrow along with mesenchymal stem cells (MSC).^{5,8} It was previously thought that adult stem cells were lineage restricted; however, recent studies have shown that HSC and mesenchymal stem cells have enormous plasticity.^{9,10} These characteristics have made them attractive for developing

stem cell-based therapies. Moreover, they offer several advantages including ease of manipulation, lack of serious ethical issues and, in the autologous setting, the absence of immunogenicity.

MicroRNAs (miRNAs) are short single-stranded RNA molecules of about 17–25 nucleotides that control gene expression post-transcriptionally in many different cellular processes either by blocking translation or by inducing mRNA degradation through sequence complementation.^{11–14} Although numerous signaling pathways, transcription factors and epigenetic changes are essential cellular regulators in determining stem cell fate, recent studies have shown that miRNAs are also implicated in coordinating the necessary changes in gene expression.¹⁵ Typically, miRNAs are capable of controlling the fate of cells in a time and tissue specific manner through regulation of cellular differentiation, developmental patterning, and morphogenesis. Therefore, manipulation of miRNAs could potentially be a useful approach to developing strategies for stem cell therapy.

In this study, we investigated the role of miRNAs in an adherent subpopulation of CD34⁺ cells isolated from granulocyte colony-stimulating factor mobilized peripheral blood cells. We identified several clusters of miRNAs that were differentially expressed in these stem cells by miRNA profiling. Further analysis of one of the clusters revealed a miRNA that targets

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the stem cell gene Nanog. We show specifically that miR-181a* targets the 3'UTR of Nanog. Our data provide evidence that miR-181a* targets Nanog in a subpopulation of CD34⁺ cells.

RESULTS

miRNA expression in CD34⁺ progenitor cells

We performed miRNA profiling on a subpopulation of adherent CD34⁺ cells mobilized peripheral blood cells.¹⁶ Using a 466 miRNA chip, we compared the miRNA signatures of adherent and nonadherent CD34⁺ cells (Figure 1a). These two subpopulations of CD34⁺ cells have previously been established as having distinct morphology, immunophenotype, and gene expression profile.¹⁶ Moreover, the adherent CD34⁺ cells display greater plasticity than the nonadherent population as they have the potential to express determinants specific to liver, pancreas, heart, muscle, and nerve cell differentiation.¹⁶ Hierarchical clustering revealed eight unique miRNA clusters that were differentially expressed in the adherent and nonadherent populations. This finding is not entirely surprising since the biological roles of miRNAs are known to vary according to their expression in distinct cell populations in normal tissues.^{17,18}

Interestingly, there were several miRNAs that have not been previously characterized for human in cluster 8 (Table 1). We were particularly interested in the miRNAs in cluster 8 because among the miRNAs in this cluster, miR-181a* (miR-181a-3p) was highly upregulated and studies have also shown that the miR-181 family are associated with regulation of CD34⁺ cells. However, the mRNA targets are largely unknown.^{18,19} We did identify some conserved regions within the mature human miR-181 family of sequences which consist of miR-181a, miR-181b, miR-181c, and miR-181d (Figure 1b). We have taken the first step toward better understanding the role of miR-181a* in CD34⁺ cells.

We first determined whether miR-181a* affected the alkaline phosphatase activity in these cells. Higher alkaline phosphatase activity is generally associated with stem cells that are in a less differentiated state.²⁰ We found increasing levels of alkaline phosphatase activity in cells transfected with miR-181a* inhibitor when compared to transfection with miR-181a* mimic (Figure 1c). The data raises the possibility that these stem cell population could be maintained in a less differentiated state by inhibiting miR-181a* expression.

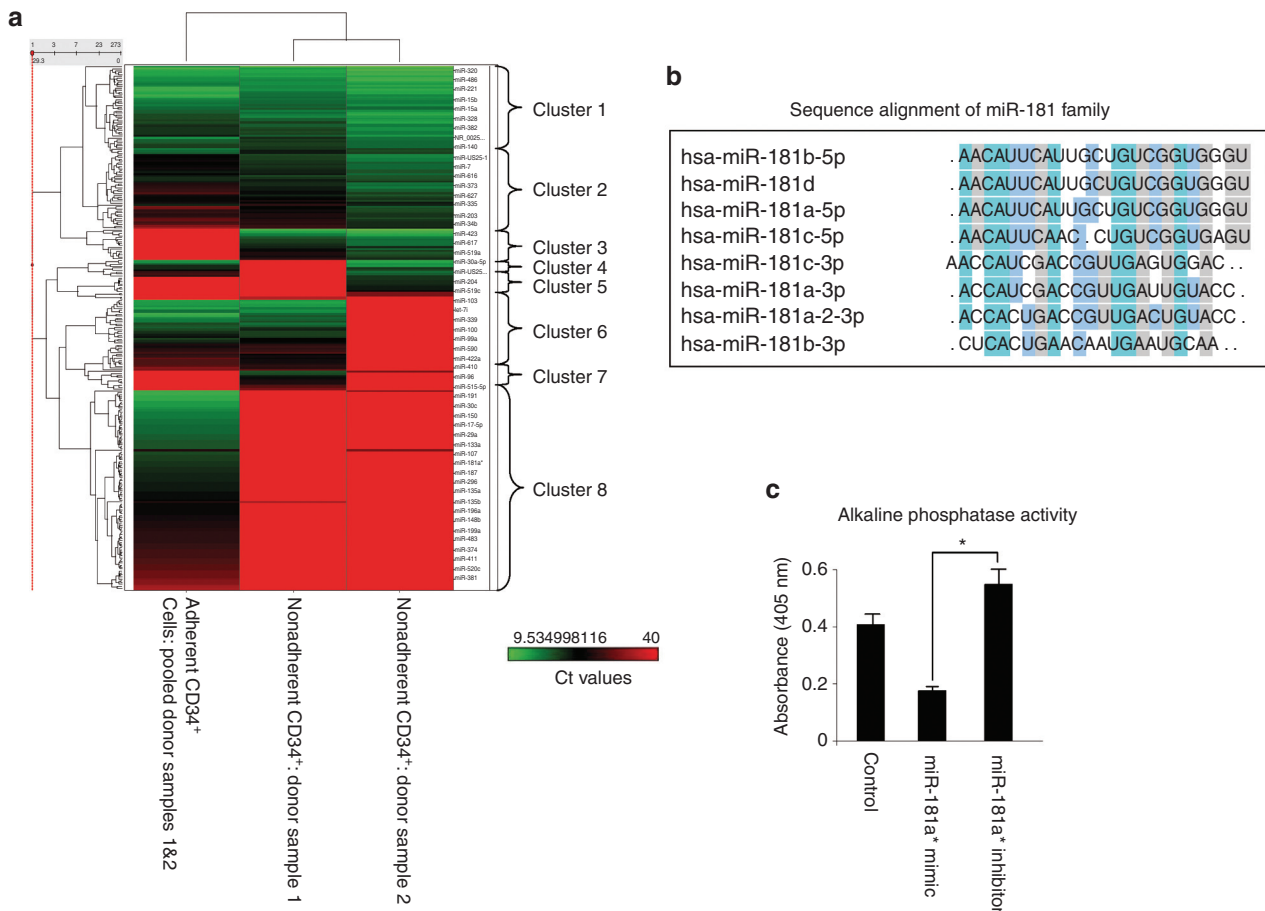


Figure 1 MicroRNA profile of CD34⁺ stem cells. (a) Unsupervised hierarchical clustering heat map of microRNA (miRNA) expression using expression levels (Ct value) of 192 miRNAs, $P < 10^{-5}$. Higher Ct values correspond to a lower expression level (red color on the heat map). Eight distinct miRNA clusters were identified. (b) A multiple sequence alignment of human miR-181 family by ClustalW. The miR-181a* is the same as miR-181a-3p. (c) Alkaline phosphatase activity was performed 72 hours post-transfection. Bar represents mean \pm SD ($n = 3$). Student's t-test, * $P < 0.01$.

miRNA-181a* targets Nanog 3'UTR

We next applied an in-house genomic-bioinformatics database to identify and validate potential candidate target messages for miR-181a*. Being important factors in stem cell maintenance, the genomic loci of Nanog, STAT3, Hic1, Sox2, HoxB4, and Pou5f1 (Oct4) were scanned for putative target sites that was complementary to the miR-181a* sequence (5'-accaucgaccguuauuguacc-3'). Specifically, the annotated 3' UTR sequences of these genes were scanned for seed sites with perfect reverse-complementarity to miRNA-181a* seed sequence (nucleotides 2–7 from the 5' end) and sites with strong overall complementarity to miRNA-181a*. Of the putative target genes, we discovered that miR-181a* formed the most stable base pairing at the Nanog 3'UTR (Figure 2a). Since Nanog is an important regulator of stem cell maintenance and self-renewal,²¹ we determined whether miR-181a* targeted Nanog transcripts. Using endpoint reverse transcription-PCR (RT-PCR) (Figure 2b) and quantitative

RT-PCR (Figure 2c) in transfected CD34⁺ stem cells, we found that inhibiting miR-181a* significantly upregulates transcript levels of Nanog. It has been reported that there exists a sensitive feedback loop that maintains the differentiation of stem cells through the control of essential stem cell genes such as Nanog. This loop enables where by environmental or molecular cues to influence the expression levels of genes, leading to a binary decision to switch downstream differentiation genes on or off.²² The data raises the possibility that miR-181a* may be involved in directly regulating Nanog expression in CD34⁺ stem cells.

Target validation of miR-181a* to the Nanog 3'UTR via 3'-compensatory site

To gain more insight into the functional interaction between miR-181a* and Nanog mRNA, we further interrogated the entire sequence alignment between the two RNA molecules. We discovered a high degree of base pairing at the 3' region (7 nucleotides) and a weak base pairing at the 5' seed site of miR-181a* (Figure 3a, top panel). In general most miRNA target sites have a strong base pairing at the 5' seed site (7–8 nucleotides).¹¹ However, the 5' seed site rule is not always the case and studies have shown that the 3' region of miRNA also play an important role in targeting mRNA but are rare events.¹¹ These sites at the 3' region of miRNAs are called 3'-supplementary and 3'-compensatory sites.^{11,23,24} In addition to the 3' sites, a recent report has identified another site called the centre site²⁵ suggesting that miRNA targeting is more complex and diverse. In our case, because the base pairing at the 5' seed site of miR-181a* was weak, we speculated that the strong complementarity pairing at the 3' region would compensate and may follow the 3'-compensatory site rule similar to one of the earlier miRNA that was discovered such as let-7^{11,26} (Figure 3a, bottom panel). Both miR-181a* and let-7 miRNAs, in this setting show a very similar overall structural identity in the way they bind to their targets. To determine whether the 3' region of miR-181a* was important, we generated, using RNAhybrid modeling,^{26,27} the most energy stable complementarity base pairing between miR-181a* and Nanog 3' UTR mRNA (Figure 3b, top panel) as well as determining the best mutations that would disrupt the 3' region base pairing. This was generated by replacing two bases at the 3'UTR of Nanog (Figure 3b, bottom panel).

To test this modeling we generated a dual-luciferase reporter construct for miR-181a* and Nanog 3' UTR mRNA. We inserted, upstream of the firefly luciferase gene, either the wild-type 3'UTR of Nanog or a mutant variant containing the two point mutations predicted to abolish miR-181a* targeting (Figure 3b, bottom panel). Transfecting the wild-type Nanog 3'UTR reporter construct by itself or with the miR-181a* mimic into CD34⁺ stem cells significantly reduced expression of luciferase (Figure 3c). Transfecting miR-181a* inhibitor caused a significant reversal in suppression of luciferase expression indicating that binding of miR-181a* to the 3'UTR of Nanog was suppressed thus allowing expression of the luciferase reporter. By contrast, transfection of the mutant Nanog 3'UTR reporter alone or in combination with miR-181a* mimic or inhibitor had no repressive effect on expression of the luciferase reporter (Figure 3c). This data therefore suggests that miR-181a* directly targets the 3'UTR region of Nanog.

Table 1 miRNAs from cluster 8 targets and functions in human stem cells

miRNA	Putative target(s)	Known function(s)	Refs.
miR-191	SOX4	Helps the transition of epithelial cells into mesenchymal cells	41
miR-30c	PAI-1, ALK2	Enhances the differentiation of osteocytes and adipocytes; Controls mesenchymal and hematopoietic cell lineages progression	42,43
miR-150	NOTCH3	Mediates T-cell development	44
miR-17-5p	E2F1, Bim	Regulates stem cell differentiation and mediates embryonic development	45,46
miR-29a	CDC24, TPM1, FZD5	Plays an important role in stem cell differentiation; Regulates early hematopoiesis	47,48
miR-135b	IBSP, Osterix	Modulates osteoblastic differentiation.	49
miR-196a	HOXC8	Plays a vital role in differentiation and proliferation of human adipose tissue-derived mesenchymal stem cells	50
miR-199a	Aggrecan, SOX9, FABP4	Regulates pluripotent stem cells (iPSCs) production; Mediates hMSC as well as chondrocyte differentiation	51,52
miR-148b	Unknown	Differentiation of hMSC	53
miR-374	Unknown	Plays a role in neuronal progenitors transdifferentiated	54
miR-520c	Unknown	Hepatocyte differentiation	55
miR-133a	Unknown	Unknown	
miR-483	Unknown	Unknown	
miR-411	Unknown	Unknown	
miR-381	Unknown	Unknown	
miR-515-5p	Unknown	Unknown	
miR-107	Unknown	Unknown	
miR-187	Unknown	Unknown	
miR-296	Unknown	Unknown	
miR-135a	Unknown	Unknown	
miR-181a*	Unknown	Unknown	

Abbreviations: hMSC, human mesenchymal stem cells; miRNA, microRNA.

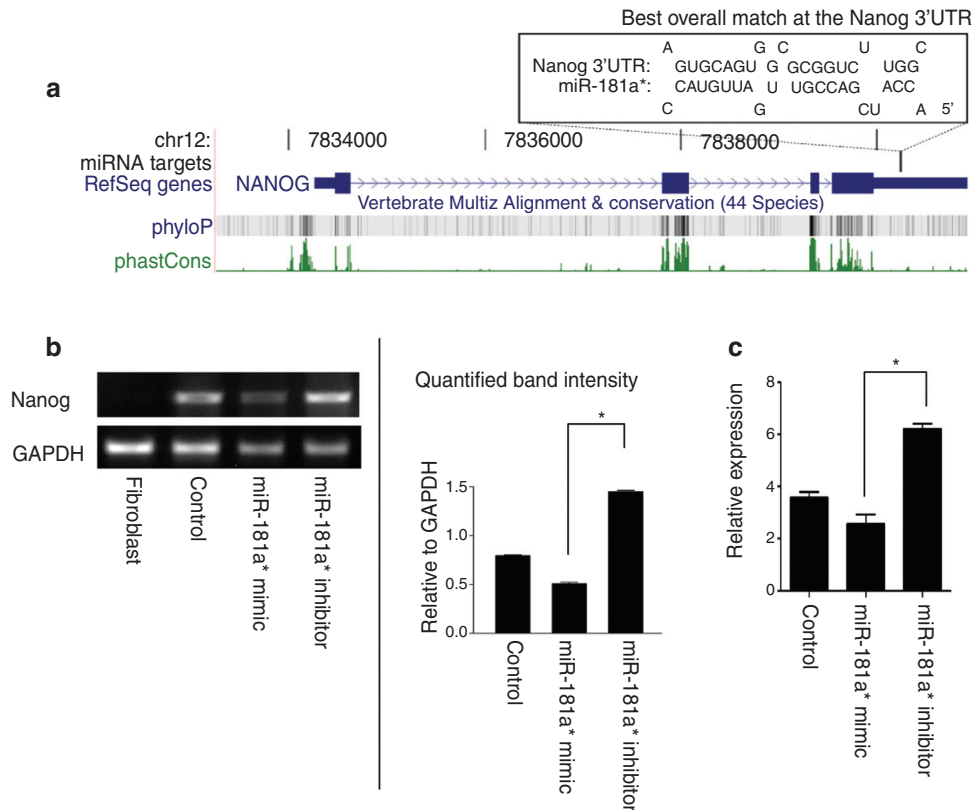


Figure 2 Identification of miR-181a* target gene. (a) Nanog genomic locus (hg18) and complementarity-binding site for miR-181a*. (b) mRNA expression level of Nanog by endpoint reverse transcription-PCR (RT-PCR) analysis. The panel shows quantified gel band intensity. Statistical analysis, * $P < 0.001$. (c) mRNA expression level of Nanog by quantitative RT-PCR normalized with GAPDH and calibrated with expression profile of Nanog in differentiated fibroblasts. Bar represents mean \pm SD ($n = 4$). Statistical analysis, * $P < 0.0005$.

This is the first attempt to characterize miR-181a* in CD34⁺ cells and we have identified Nanog as its potential target.

DISCUSSION

Understanding the cellular and molecular mechanisms responsible for the multi-pluripotency of stem cells will be critical for their practical use in therapy. MicroRNAs have been shown to be important and necessary for proper stem cell regulation.^{18,28–30} In this study, we generated a miRNA profile from a subpopulation of adherent CD34⁺ cells isolated from G- colony-stimulating factor mobilized peripheral blood and demonstrated a marked difference in expression patterns for a small cluster of miRNA. Using bioinformatics, we identified Nanog as a potential target for miR-181a*. Interestingly, there have been works published regarding other potential targets for the miR-181 family. For example, miR-181b has been shown to be an important regulator of nuclear factor- κ B signaling in the vascular endothelium by targeting importin- α 3.³¹ In another cell type, miR-181b has been shown to target Tcl1 oncogene in chronic lymphocytic leukemia.³² Similar to miR-181b, miR-181a also has several targets such as Prox1 in lymphatic endothelial cells, C2H2 zinc-finger proteins, and multiple phosphatases such as SHP2, PTPN22, DUSP5, and DUSP6.^{33–36}

Although miR-181a*'s binding site within the 3'UTR of Nanog displayed a weak canonical 5' miRNA seed pairing, it also showed strong complementarity at the miRNA 3' end and

at the central Ago2 catalytic site 10–11 nucleotides from the 5' miRNA site.²⁷ This binding pattern is similar with the "3' compensatory site model previously identified with let7-miRNA."^{11,23} Importantly, by introducing two point mutations to disrupt the 3' and central complementary sites, we abolished miR-181a* regulation of a reporter construct containing the 3'UTR of Nanog. Our studies highlight a new stem cell-related target for the miR-181 family and show that miR-181a* directly targets Nanog.

MATERIALS AND METHODS

Isolation and growth of a CD34⁺ stem cell population. The hematopoietic blood samples were obtained with informed patient consent and approved by the Hammersmith Hospital Research Ethics Committee. Samples of granulocyte-colony stimulating factor mobilized peripheral blood progenitor cells were processed by leukapheresis at the Stem Cell Laboratory at the Hammersmith Hospital. Briefly, human mobilized peripheral blood samples were diluted in a ratio of 1:4 in Hanks'-buffered saline solution (Gibco, Paisley, UK), the mononuclear cells were separated by centrifugation over a Lymphoprep (Dundee, Scotland) density gradient at 1,800 rpm for 30 minutes. The mononuclear cell fraction at the interface was aspirated and washed twice with Hanks'-buffered saline solution, and finally with MACS buffer (phosphate-buffered saline solution) at pH 7.2 supplemented with 0.5% bovine serum albumin and 2 mmol/l EDTA). CD34⁺ cells were isolated using a CD34⁺

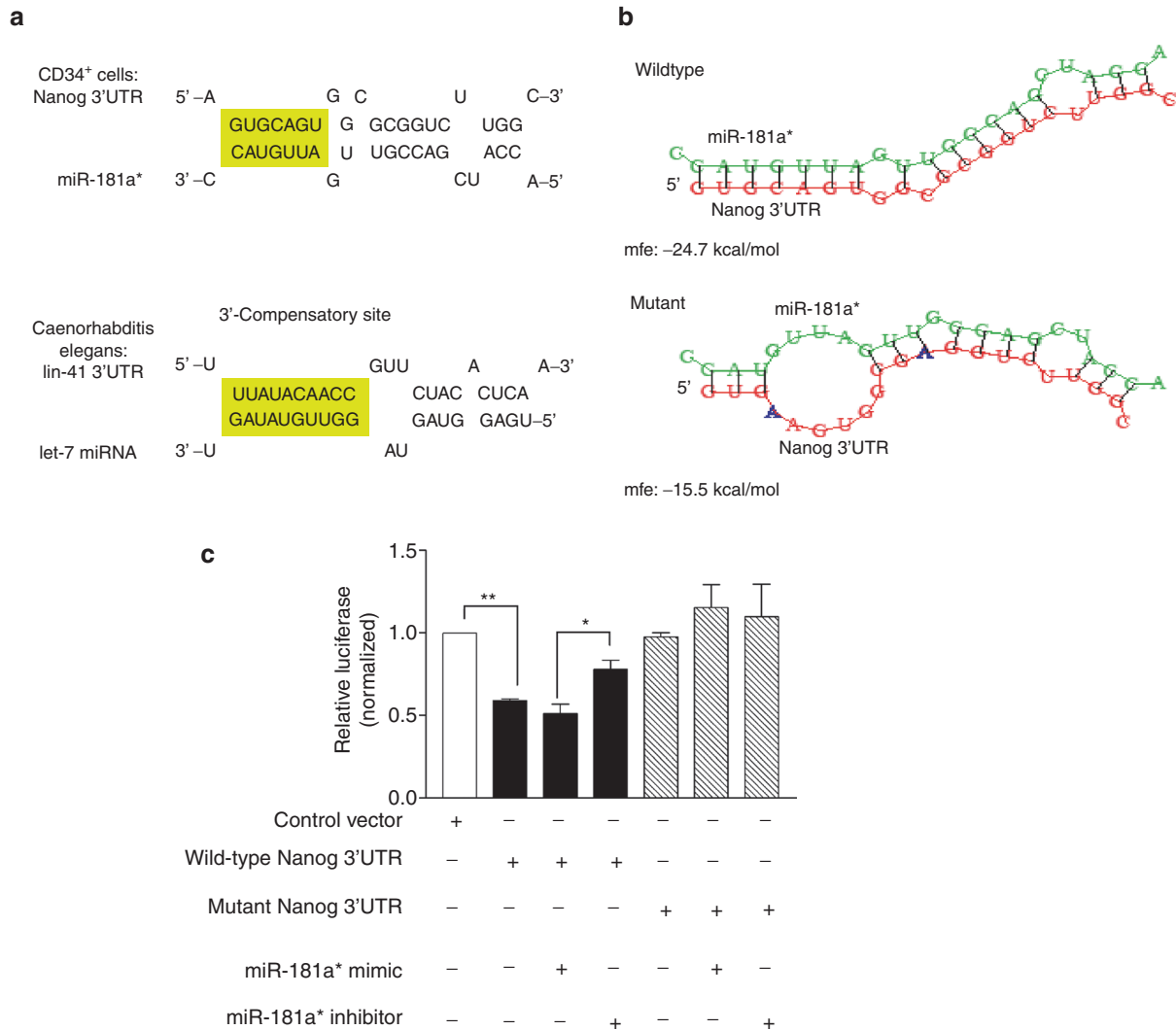


Figure 3 Functional analysis of miR-181a* and Nanog 3' UTR region. (a, Top panel) a schematic of the base pairing between Nanog 3'UTR and miR-181a*. (a, Bottom panel) an example of compensatory pairing as seen with let-7 microRNA (miRNA).¹¹ The high complementarity-binding site at the 3' region is indicated by the yellow highlights. (b, Top panel) in silico hybridization between miR-181a* and Nanog 3' UTR region. RNAhybrid modeling demonstrating the most energy stable complementarity base pairing between miR-181a* and Nanog 3' UTR mRNA. (b, Bottom panel) the loss of binding energy (mfe: -15.5 kcal/mol, relative to wild type) is caused by introducing two point mutations (cytidine (C) to adenosine (A), as shown by the blue color) therefore disrupting the binding properties of hsa-miR-181a*. (c) Reporter assay of Nanog 3'UTR and mutant Nanog 3'UTR in cells transfected with the mimic or inhibitor of miR-181a* oligonucleotides. Bar represents mean \pm SD ($n = 3$). Statistical analysis, * $P < 0.001$.

isolation kit (Miltenyi Biotec, Cologne, Germany) according to the manufacturer's protocol. Adherent CD34⁺ progenitor cells were isolated as previously described with modification.¹⁶ Briefly, isolated CD34⁺ cells were added to 24-well or 35-mm tissue-culture treated dishes (Nunc, Roskilde, Denmark) at a density of $2.5\text{--}5 \times 10^5$ cells in α -minimum essential medium to isolate the adherent CD34⁺ cell population. After 30-minute incubation, nonadherent cells were removed and adherent cells were rinsed three times with phosphate-buffered saline or α -minimum essential medium. The adherent CD34⁺ cell population was grown in a serum-free medium (CellGro; CellGenix, Freiburg, Germany) containing three cytokines: 250 ng/ml of stem cell factor, 250 ng/ml of interleukin-6, and 250 ng/ml of interleukin-3 (Invitrogen, Paisley, UK) in 0.5% penicillin/streptomycin antibiotics. Cells were incubated at 37 °C in 5% CO₂.

Total viable cells were counted using the trypan exclusion assay. For the alkaline phosphatase assay, $\sim 2.5 \times 10^5$ progenitor cells in 24-wells were transfected twice with the oligonucleotides at 100 nmol/l and the cells were harvested 72 hours later as described in the alkaline phosphatase assay kit (Cell Biolabs, San Diego, CA and Millipore, Billerica, MA). Equal protein load at 100 μ g of protein was used for the assays and the reaction was measured after 1-hour incubation at 37 °C at 405 nmol/l in an ELISA reader.

MicroRNA profiling. The detailed miRNA protocol is described previously.³⁷ Adherent and nonadherent cells were added into a PCR tube and heat-treated at 95 °C for 5 minutes. Then the miRNAs were reverse transcribed into cDNAs by 460 miRNA-specific stem-looped primers. Then these miRNA cDNAs were

amplified by 18 cycles of PCR by 460-specific forward primers and a universal reverse primer. Finally, the cDNAs were split and individual miRNA was measured by TaqMan probe-directed real-time PCR. All reactions were duplicated. The PCR was done as following by AB7900 with 384-well plates: first, 95 °C for 10 minutes to activate the Taq polymerase. Then 40 cycles of 95 °C for 15 seconds (for denaturation) and 60 °C for 1 minute (for annealing and extension) were performed. Two replicates were performed for each sample. Two independent allogenic donor samples were used in the analysis. In order to obtain sufficient total RNAs, the adherent CD34⁺ progenitor cells were combined from the two donors.

RT-PCR and quantitative real-time RT-PCR. The miRNA-181a* mimic and inhibitor were purchased from Dharmacon, Lafayette, CO. The oligonucleotides were transfected (at day 0 and again at 24 hours) at 100 nmol/l into 1×10^5 adherent CD34⁺ stem cells using Nanofectamine reagent following the manufacturer's recommendation (PAA, Pasching, Austria). Total RNA was harvested using the RNAqueous-Micro kit (Ambion, Paisley, UK) 72 hours post-transfection. The RNA was quantified using the Nanodrop 2000 micro-sample quantifier. Five hundred nanogram of total RNA from each sample was reverse transcribed using the One-Step RT-PCR kit from Qiagen (Hilden, Germany) following the manufacturer's recommendation. Expression of human Nanog was measured semiquantitatively by PCR using primer pair from R&D Systems (Minneapolis, MN) after 32 cycles at 94 °C for 45 seconds, 55 °C for 45 seconds and 72 °C for 45 seconds. GAPDH primers: Forward (5'GTGAAGGTCGGAGTCAACG3') and Reverse (5'GGTGAAGACGCCAGTGGACTC3') was used as a loading control after 30 cycles at 94 °C for 45 seconds, 60 °C for 45 seconds and 72 °C for 1 minute. The samples were separated on agarose gel and analyzed using UVP Geldoc system (UVP, Upland, CA). Four sets of band intensity across three independent experiments were used for semiquantitative analysis. For the quantitative real-time RT-PCR, 500 ng of total extracted RNA (as described above) was processed for elimination of genomic DNA followed by reverse transcription using the QuantiTect Reverse Transcription kit from Qiagen. The first strand cDNA synthesis was then amplified for quantitative analysis of human Nanog (NM_024865) and the reference gene, human glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (NM_002046) using QuantiFast SYBR Green PCR Kit from Qiagen. Amplification was performed using Applied Biosystems 7900HT FAST-Real-Time System with 40 cycle conditions at 95 °C–15 seconds and 60 °C–45 seconds with a total volume of 25 µl per sample. Amplified products were then analysed using Applied Biosystems RQ Manager 1.2.1. Four independent experiments were amplified in triplicates for quantitative analysis. Student *t*-test scoring was performed at 99% confidence intervals.

Bioinformatic analysis. Sequence annotations and data were downloaded from version hg18 of the UCSC table browser's RefSeq Genes track.³⁸ The genomic loci Nanog, STAT3, Hic1, Sox2, HoxB4, and POU5F1 (Oct4) were scanned for putative target sites for miR-181a* (5'-ACCAUCGACCGUUGAUUGUACC-3'). More specifically, the genes' annotated 3' UTR sequences were scanned for (i) seed sites with perfect reverse-complementarity to the oligonucleotide's seed sequence (nucleotides 2–7

from the 5' end) and (ii) sites with strong overall complementarity to the oligonucleotide. Such sites could be target sites for miRNA-like translational suppression and mRNA degradation, and small interfering RNA-like mRNA cleavage. The former scan used a custom python script, whereas the second scan used RNAhybrid³⁹ with default parameter settings to evaluate potential hybridization between the oligo and target sequence. None of the five genes' 3' UTRs contained miRNA-like seed sites for the oligonucleotide. Of the sites that formed the most stable interactions with the oligonucleotide, the Nanog site was the most stable site that contained paired bases at the putative AGO2 cleavage site. The AGO2 cleavage site would presumably be between nucleotides 10 and 11 from the 5' end.⁴⁰

Nanog 3' UTR construct, cloning, and dual-luciferase reporter assay. Nanog 3' UTR was amplified from HeLa genomic DNA by nested PCR using two different forward primers and a single reverse primer (primers are listed below). For both PCRs, 50 µl sample volumes were prepared using 0.3 µmol/l each of *Forward* and *Reverse* primers, 1× Accuprime Pfx Reaction Mix (Invitrogen), and 1.0 unit of Accuprime Pfx DNA Polymerase (Invitrogen). One hundred nanogram of genomic DNA and 0.5 µl of PCR product were used as templates in the first and the second PCR, respectively. The *Forward 2* and the *Reverse* primers each contained two restriction enzyme cut sites; XhoI and SgfI for *Forward 2* and *NotI* and *EcoRI* for *Reverse*. The Nanog 3' UTR construct was cloned into the psiCHECK 2.2 vector (Promega, Madison, WI), using the unique restriction enzyme cut sites for *NotI* and *XhoI*. Two point mutations were inserted in the Nanog 3' UTR target site of hsa-miR-181a* using two mutagenic primers and psiCHECK 2.2 with Nanog 3' UTR insert as template. A 25.5 µl PCR sample volume was prepared using 10 ng of template, 0.5 µmol/l of each primer, 1× Reaction buffer (Agilent Technologies, Santa Clara, CA), dNTP mix (0.2 µmol/l each, Finnzymes), and 1.25 units of *PfuTurbo* DNA polymerase (Stratagene). Sequence of mutated hsa-miR-181a* target site (mutations in bold and underlined): GTGAAGTGGCGAGGCTTGGC. List of primer sequences: Forward 1, AGCAACCAGACCAGAACATCCAG; Forward 2, GCGATCGCTCGAGAGATGAGTGAAACTGATATTACTCAATTTTCAGTCTGG; Reverse, GAATTCGCGGCCGATGTTAAGCTGTATATTTACTCATTGAAACTCGG; psiCHECK+, AGGACGCTCCAGATGAAATG; Nanog seq, TCACTGCAAGCTCCGTCTCC; Rev psiCHECK, CAAACCCTAACCCAGGCTTA; Nanog Mut Fr, GGCTGGAGTGAAGTGGCGAGGCTTGGCTC; Nanog Mut Rev, GAGCCAAGACCTCGCCACTTCACTCCAGCC. For the reporter luciferase reporter assay, 100 nmol/l of miR181a* mimic or its inhibitor (Dharmacon) were transfected into CD34 progenitor cells (2.5×10^5 cells/well) in a 24-well plate for 24 hours. The Nanog 3'UTR wild-type or mutant with two point mutations was cloned downstream of the *Renilla* translational stop codon in psiCHECK-2 vector (Promega). 100 ng of the 3-UTR-Nanog (3-UTR-Nanog-psiCHECK-2) or its mutated variant was cotransfected into the cells pre-conditioned with miR181a* mimic or inhibitor for further 24 hours before harvesting. Empty (psiCHECK-2) vector was used for normalization and 3-UTR-Nanog alone was used as a control. *Renilla* and Firefly luciferase assay

was carried out using the Dual-Luciferase Reporter Assay System (Promega). Readings were measured the VICTOR plate reader (Waltham, MA) (Perkin-Elmer VICTOR).

Statistical analysis. Statistical analysis was performed using Prism 3.0 software. Results are shown as the means \pm SD. Differences between groups were assessed using the two-tailed Student *t*-test.

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