Expression of miR-210 during erythroid differentiation and induction of γ-globin gene expression

Nicoletta Bianchi1, Cristina Zuccato1, Ilaria Lampronti1, Monica Borgatti1 & Roberto Gambari1,2,*
1BioPharmaNet, Department of Biochemistry and Molecular Biology, University of Ferrara, Via Fossato di Mortara, 74-44100 Ferrara, 2Biotechnology Center, University of Ferrara, Via Fossato di Mortara, 64-44100 Ferrara, Italy

MicroRNAs (miRs) are a family of small noncoding RNAs that regulate gene expression by targeting mRNAs in a sequence specific manner, inducing translational repression or mRNA degradation. In this paper we have first analyzed by microarray the miR-profile in erythroid precursor cells from one normal and two thalassemic patients expressing different levels of fetal hemoglobin (one of them displaying HPFH phenotype). The microarray data were confirmed by RT-PCR analysis, and allowed us to identify miR-210 as an highly expressed miR in the erythroid precursor cells from the HPFH patient. When RT-PCR was performed on mithramycin-induced K562 cells and erythroid precursor cells, miR-210 was found to be induced in time-dependent and dose-dependent fashion, together with increased expression of the fetal γ-globin genes. Altogether, the data suggest that miR-210 might be involved in increased expression of γ-globin genes in differentiating erythroid cells.

INTRODUCTION

MicroRNAs (miRs) ([http://microrna.sanger.ac.uk/sequences/](http://microrna.sanger.ac.uk/sequences/)) are a family of small (19 to 25 nucleotide in length) noncoding RNAs that regulate gene expression by sequence-selective targeting of mRNAs (1, 2), inducing translational repression or mRNA degradation, depending on the degree of complementarities between miRs and the target sequences (3, 4). Considering that a single miR can target several mRNAs and a single mRNA might contains in its 3’UT sequences several signals for molecular recognition by miRs, it is calculated that 10-30% of human mRNAs are target of microRNAs (4), leading to the control of highly regulated processes, such as differentiation, cell cycle and apoptosis (3, 4).

In this respect, few reports are available on microRNAs expression and functions in erythroid cells (5-13). For instance, microRNAs in erythropoiesis were studied by Felli et al., who identified miR-221 and miR-222 as being highly expressed in human cord blood derived haematopoietic CD34⁺ progenitor cells (5). Erythroid-specific miRNAs were also identified by Georgantas et al., who demonstrated miR-155 as a microRNA involved in the control of both myeloid and erythroid differentiation (7).

The aim of this study was to compare the expression of microRNAs in erythroid precursor cell populations isolated from a normal donor, a patient affected by β-thalassemia expressing low levels of fetal hemoglobin (HbF), and a β-thalassemic patient exhibiting hereditary persistence of fetal hemoglobin (HPFH). A second objective was to verify the content of miRs preferentially expressed in HPFH cells in the K562 cell system and in erythroid precursor cells from peripheral blood induced to γ-globin gene expression by mithramycin (14-18).

RESULTS

Characterization of globin genes expression in erythroid cell cultures exhibiting different levels of fetal hemoglobin

The biochemical features of the three erythroid two-phase primary cell cultures employed in our miR-profiling analysis are the following. As expected, erythroid cells from the normal donor (US) accumulate α-globin and β-globin mRNA and produce high level of HbA and very low levels of HbF. Erythroid cells from Th-1 (a β-major-thalassemic patient) accumulate very low levels of β-globin mRNA, low levels of γ-globin mRNA and levels of α-globin mRNA comparable to the normal donor; accordingly, erythroid cells from this patient express low levels of β-globin mRNA, low levels of γ-globin mRNA and levels of α-globin mRNA comparable to the normal donor; accordingly, erythroid cells from this patient express low levels of HbF (15% ± 3.5, n = 3). As recently published by our group, erythroid cells from Th-17 accumulate, in addition to the α-globin, very high levels of γ-globin mRNA, HbF being the major hemoglobin produced by erythroid cells of this HPFH patient (>85% ± 5.5, n = 5) (19). All the cellular cultures displayed more than 80% erythroid differentiated cells, as judged by benzidine-staining (data not shown).

MicroRNA profiling analysis

The microarray-based screening was performed using the microRNA chip Agilent and LNA-modified oligonucleotides, which contains 470 miR probes and RNA isolated from erythroid cells from one normal and two thalassemic patients expressing different levels of fetal hemoglobin (one of them displaying HPFH phenotype).
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Fig. 1. (A) Profiles of miRNA expression in erythroid cell cultures. The analysis was performed with total RNA from US, Th1 and Th17 samples, as indicated. Heatmap of 194 miRNA expression profiling found expressed in at least one sample is depicted using a color-bar approach. Lower part of the panel: restricted analysis to the 30 miRNAs expressed at high levels in Th-1, Th-17 and US samples. (B, C) Comparison between microarray (white histograms) and quantitative RT-PCR (black histograms) data, obtained using RNA from Th1 (B) and Th17 (C) EPO-induced erythroid precursor cells. miR-let-7c was used as internal control to normalize RNA input.

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As far as analysis of the possible role and/or association with the phenotype of the employed biological samples, we pointed out our attention on miRs which are similarly expressed in cells from Th-1 and US (range Th-1/US from 0.9 to 1.1, see Table 1). Among these 30 miRs, miR-210 displays the highest level of expression in cells from Th-17 (Th-17/US being 16.73, see Table 1). Therefore, miR-210 should be tentatively considered as associated with high expression of HbF, which is one of the most striking parameters which differentiate Th-17 on one side from Th-1 and US samples on the other. The miRNA let-7c could represent one of reference sequence to quantify miR expression, because its content does not change significantly between the assayed samples (data not shown).

Validation of the miR microarray analysis with quantitative real-time RT-PCR
Quantitative RT-PCR (Q-RT-PCR) was employed as a method for validation of the microarray data. Due to the already demonstrated involvement of miR-155, miR-221 and miR-222 in erythropoiesis (6, 11), these microRNAs were chosen for this analysis. The comparison between microarray and RT-PCR data is shown in Fig. 1 (B and C). When the expression in erythroid cells from the unaffected subject is taken as 1, the level of miR-155, miR-221 and miR-222 were found to be 0.59, 0.37, and 0.44 in Th-1, and 0.92, 4.01, and 3.42 in Th-17 samples, in agreement with the trend displayed by the microarray data. The results obtained for miR-210 clearly demonstrate that the differences found in the microarray data are reproducibly observed using the quantitative real-time RT-PCR approach.

In order to verify possible changes in the expression of miR-210 following erythroid differentiation and/or increased expression of γ-globin genes, two cellular model systems were employed, (a) K562 cells induced to erythroid differentiation
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Table 1. Microarray analysis of miR-profiling. Data represent fold content in respect to standard hybridization reactions

<table>
<thead>
<tr>
<th>miRNA</th>
<th>US Normalized</th>
<th>TH1 Normalized</th>
<th>TH17 Normalized</th>
<th>TH1/US</th>
<th>TH17/US</th>
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<td>miR-130b</td>
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by mithramycin (MTH) and (b) EPO-treated erythroid precursor cells from normal donors cultured in the presence of mithramycin. Our group has previously demonstrated MTH as a powerful inducer of erythroid differentiation and \( \gamma \)-globin gene expression in erythroid cells (14, 15). As far as human erythroid precursors, MTH is one of the most powerful inducers of HbF (15).

Functional studies on human erythroid cells treated with mithramycin: increased expression of miR-210 is associated with K562 differentiation

Fig. 2A shows the fold expression of the analyzed miRs when RNA from K562 cells treated for 5 days with 20, 30, 40 nM MTH is compared to that isolated from control untreated cells. As it is clearly appreciable, during erythroid induction of K562 cells miR-210 is the only miR whose expression increases in correlation with the increase of the concentration of mithramycin. The accumulation of the other miRs (miR-155, miR-221, miR-222) tends to decrease. The proportion of benzidine-positive cells was 18% (20 nM MTH), 52% (30 nM MTH) and 78% (40 nM MTH).

In a second experiment, K562 cells were induced to erythroid differentiation with 30 nM MTH, RNA isolated after different days and analyzed in RT-PCR experiments using primers/probes amplifying \( \alpha \)-globin mRNA, \( \gamma \)-globin mRNA, miR-210, miR-155, miR-221 and miR-222 sequences. Fig. 2B shows the results of the benzidine-test, demonstrating that the increase of the proportion of differentiated cells occurs after 4 days of cell culture, reaching plateau levels after 5-7 days of treatment. These results confirm previously published data on the effects of mithramycin and related compounds on erythroid differentiation of K562 cells (14). In agreement with Fig. 2B, RT-PCR analysis demonstrated a sharp increase of accumulation of \( \alpha \)-globin and \( \gamma \)-globin mRNAs (Fig. 2C). With respect to accumulation of miRs, Fig. 2D indicates that at 30 nM MTH, the content of miR-155, miR-221 and miR-222 do not
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Fig. 2. (A) Induction of miR-210 in response to MTH-treatment. Fold expression of the analyzed miRs are indicated when Q-RT-PCR was performed on RNA from K562 cells treated for 5 days with 20, 30, 40 nM MTH; data represent fold increase in respect to control untreated cells (average ± S.D. from three independent experiments). (B-D) Kinetics of increase of the proportion of benzidine positive cells (average ± S.D. from four independent experiments). (B) α-globin (○) and γ-globin (●) mRNAs (C) and miR-210 (●), miR-155 (△), miR-221 (▲) and miR-222 (□) sequences following treatment with 30 nM MTH (D). Data depicted in panels C and D represent fold increase in respect to control untreated cells (average ± S.D. from three determinations).

In this paper we have first analyzed by microarray the miR-profile in erythroid precursor cells from one normal and two thalassemic patients expressing different levels of fetal hemoglobin (one of them displayed HPFH phenotype). The microarray data were confirmed by RT-PCR analysis, and allowed us to identify miR-210 as an highly expressed miR in the erythroid precursor cells from the HPFH patient. When RT-PCR was performed on MTH-induced K562 cells and erythroid precursor cells, we demonstrate that miR-210 is induced in time-dependent and dose-dependent fashion during MTH-mediated erythroid induction of K562 cells. Interestingly, miR-210 is induced following MTH-treatment also in erythroid precursor cells from human donors.

As far as the other miRs here analyzed, the major difference between K562 cells and normal erythroid progenitors is the expression of miR-155, which is induced in erythroid progenitors but not in K562 cells treated with MTH. On the contrary, the other miRs, including the erythroid-associated miR-221
and miR-222 are not induced in K562 cells and display low-levels of induction also in MTH-treated erythroid progenitors. We can speculate that miR-155 is associated with later stages of differentiation, according with results reported by Masaki et al. (10).

Altogether, these data identify a novel miR-210, whose expression is enhanced in association with erythroid differentiation and induction to HbF production. As far as the role of miR-210, our data do not allow conclusive considerations. However, it is very interesting to note that miR-210 has been recently associated with hypoxia (22, 23-28). This has been first demonstrated by Kulshreshtha et al., who described for the first time the microRNA signature of hypoxia, which includes high expression of miR-210 (22). Accordingly, miR-210 is induced by hypoxia and links hypoxia with cell cycle regulation (28). Interestingly, hypoxia has been recently demonstrated to alter progression of the erythroid program (29). Low pO2 might indeed modulate the relative amounts and types of hemoglobins produced by erythroid cells, leading to increased HbF during stress erythropoiesis (29-33).

Understanding the developmental progression of globin gene expression and reactivation of γ-globin gene expression and HbF production in the adult is pursued as an important therapeutic strategy for sickle cell anemia and β-thalassemia (29). Altogether, the data reported in this paper suggest that the hypoxia-associated miR-210 might be involved in increased expression of γ-globin genes in differentiating erythroid cells.

MATERIALS AND METHODS

Human cell lines and culture conditions
Human leukemia K562 cells (17) were cultured in humified atmosphere of 5% CO2/air in RPMI 1640 medium (Sigma, St Louis, MO, USA), 10% foetal bovine serum (FBS; Analytical de Mori, Milan, Italy), 50 units/ml penicillin and 50 μg/ml streptomycin (20). Erythroid differentiated cells containing hemoglobin were detected by specific reaction with 0.2% benzidine in 5 M glacial acetic acid, 10% H2O2 (18).

Patients and erythroid precursor cultures
The blood samples were from Ospedale S. Chiara, UO di Oncologia Pediatrica, Pisa, Italy. The homozygous β°39 thalassemia Th-1 patient expresses low levels of HbF (about 2.5%). The Th-17 patient is a 13-year-old girl with a novel β° thalassemia mutation associated with a deletion of the delt-alpha-globin gene region, and exhibiting HPFH (HbF being over 88%) (19). The two-phase liquid culture procedure was employed (20, 21). Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation and seeded in α-minimal essential medium, 10% FBS (Celbio, Milano, Italy), 1 μg/ml cyclosporine A (Sandoz, Basel, Switzerland), 1 U/ml human recombinant erythropoietin (EPO) (Tebu-bio, Magenta, Milano, Italy), 10 ng/ml stem cell factor (SCF) (Inalco, Milano, Italy), and 10% conditioned medium from the 5637 bladder carcinoma cell line (20). After 7 days incubation in this phase I culture, non adherent cells were harvested, washed, and then cultured in fresh medium composed of α-medium, 30% FBS, 1% deionized bovine serum albumin (BSA), 10⁻⁵ M β-mercaptoethanol, 1.5 mM L-glutamine, 10⁻⁶ M dexamethasone, 1 U/ml human recombinant erythropoietin (EPO) (Tebu-bio, Magenta, Milano, Italy) and 10 ng/ml stem cell factor (SCF) (Inalco, Milano, Italy). This part of the culture is referred to as phase II (21).
RNA isolation
Cells were isolated by centrifugation at 1,000 rpm for 10 min at 4°C, washed in PBS, lysed in Tri-reagent™ (Sigma Aldrich, St. Louis, MO, USA), according to the manufacturer instructions. The 2100 bioanalyzer was used to determine the integrity and measure the concentration of total RNA samples (Agilent Technologies, Instrument DE54700480, Eukaryote Total RNA Nano Series II xly).

MicroRNA analysis
For microRNA expression study two approaches were followed, (a) miR profiling and (b) quantitative RT-PCR. RNA analysis using microRNA microarray chips has been carried on by Agilent Technologies (22) using a platform containing 470 human miRNA probes for mature and precursor of microRNAs, each spotted 5-20 times. RNA was isolated from erythroid precursor cells from a unaffected subject (US), Th-1 and Th-17, labeled and hybridized on microRNA microarray chips as previously described (22), using 2 μg of RNA from each sample, biotin labeled during reverse transcription using random hexamers. Raw data were normalized and analyzed by GeneSpring GX software version 7.3 (Agilent Technologies). The GeneSpring software generated a unique value for each microRNA, determining the average of the results from four spots. The data are presented as means ± standard error of the mean (SEM). Student’s t-test was performed for statistical evaluation with P < 0.005 considered significant. For microRNA quantization reverse transcriptase (RT) reactions were performed using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and real-time PCR were performed using the specific human TaqMan Micro RNA Assay Kits from Applied Biosystems for hsa-miR-210, hsa-miR-155, hsa-miR-221, hsa-miR-222, hsa-let-7c and the 7700 Sequence Detection System version 1.7 (Applied Biosystems, Foster City, CA, USA). Relative expression was calculated using the comparative cycle threshold method and as reference genes the endogenous control human 18S kit human 18S rRNA and hsa-let-7c microRNA.

RT-PCR analysis of globin gene expression
For gene expression analysis 20 ng of total RNA were reverse transcribed by using random hexamers. Quantitative real-time PCR assay was carried out using gene-specific double fluorescently labeled probes. The nucleotide sequences used for real-time PCR analysis of α, β- and γ-globin mRNAs and β-globin gene are reported elsewhere (22).

Statistics
All the data were normally distributed and presented as mean ± S.D. Statistical differences between groups were compared using one-way ANOVA (Analyses Of VAriance between groups) software. Statistical significance was assumed at P < 0.05.

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