Blood Cells, Molecules and Diseases 54 (2015) 364–368

Contents lists available at ScienceDirect

Blood Cells, Molecules and Diseases

journal homepage: www.elsevier.com/locate/bcmd

Daily variations in the expression of miR-16 and miR-181a in human leukocytes

Diego de Siqueira Figueredo [a], Daniel Leite Góes Gitaí [b], Tiago Gomes de Andrade [a,*]

[a] Laboratory of Molecular Chronobiology, Universidade Federal de Alagoas, Campus Arapiraca, Alagoas, Brazil
[b] Laboratory of Cellular and Molecular Biology, Universidade Federal de Alagoas, Maceió, Alagoas, Brazil

ARTICLE INFO

Article history:
Submitted 11 December 2014
Accepted 11 January 2015
Available online 17 January 2015

(Communicated by M. Narla, DSc, 11 January 2015)

Keywords:
Circadian
miR
Hematopoiesis
Human leukocytes
Housekeeping genes

ABSTRACT

Circadian rhythms are controlled by a molecular mechanism that is organized in transcriptional and translational feedback loops of gene expression. Recent studies have been demonstrating the involvement of microRNAs (miRs) in post-transcriptional/translational control of circadian rhythms. In the present study we aimed to analyze the daily variations of miR-16 and miR-181a expression in human leukocytes. These miRs were independently associated with hematopoiesis and circadian rhythms in previous studies using experimental models. Peripheral blood from 6 subjects was sampled in a 24 hour period for expression analysis using quantitative real-time PCR (RT-qPCR). Initially, we evaluated the expression stability of RNU6-2, RNU1A-1, RNU5A-1, SNORD-25, SCARNA-17 and SNORA-73A as candidate genes for normalization of RT-qPCR data. The combination of the four most stable genes (SNORA-73A/SCARNA-17/SNORD-25/RNU6-2) was indicated to provide a better normalization of miRs expressions. The results show a daily variation of miR-181a and miR-16 expression in human leukocytes, suggesting a potential participation of these genes in the modulation of the circadian rhythms present in blood cells.

1. Introduction

Several evidences indicate that hematopoiesis and immune response are under a circadian regulation [1–4]. In fact, leucocytes and lymphocyte subsets vary in number and proportion following a circadian rhythm. Granulocytes, macrophages, natural killer cells, extrathymic T cells, γδ T cells and $CD8^+$ T cells exhibited diurnal variation with higher levels during the day, in contrast to T cells, αβ T cells, B cells and $CD4^+$ cells that have increased levels during the night [5]. Neutrophils, eosinophils, monocytes and lymphocytes oscillated significantly during the day [6].

Circadian changes were also observed in the profile of leukocyte trafficking and inflammatory responses in arrhythmic hamsters [7]. In molecular level, above 8% of the macrophage transcriptome oscillate in a circadian pattern, including many important regulators for pathogen recognition and cytokine secretion, suggesting that the circadian clock control inflammatory immune responses [8]. The clock genes *Per1*, *Per2*, *Per3*, *Bmal1*, *Cry1*, *Cry2* and *CKIe* showed circadian expression in total human leukocytes [9–11].

Self-sustained circadian rhythms of clock genes expression were also observed in cultures of human mononuclear cells isolated from peripheral blood [12]. Freshly isolated CD4 + T cells showed robust rhythms of clock genes expression, IL-2, IL-4 and IFN-c production and PER2::LUCIFERASE reporter activity, suggesting that endogenous T cell clock rhythms are self-sustained under constant culture conditions [13]. Furthermore, clock genes have altered expression in leukocytes of individuals with leukemia [11,14] and acute systemic inflammation [15].

Recent studies have demonstrated that the number of transcripts with circadian expression is lower than the number of proteins, which may be a result of posttranscriptional, translational and posttranslational regulation mechanisms [16–20]. microRNAs (miRs) are small non-coding transcripts responsible for the negative regulation of gene expression by mRNA degradation or inhibition of translation [21] and have been implicated in the circadian control of gene expression in different organisms, such as *Arabidopsis* [22–24], *Drosophila* [25,26], chicken [27] and mice [28,29]. Recently, we have described a diurnal expression of miR-27b in human leukocytes and several candidate miRs potentially involved in the control of circadian rhythms [30].

miR-16 and miR-181a are important genes for hematopoesis [31] and presented a daily variation or a circadian expression evaluated in other tissues [32,33]. miR-16 presented daily variations in rat jejunum and the over-expression of this gene suppressed *cyclins D1–3*, *cyclin E1* and *cyclin-dependent kinase 6*, specific G1/S regulators and produced G1 arrest [32]. Validated targets or pathway genes included some associated with circadian rhythms, such as BCL-2, BRCA1 and *Myc* [34–45].

miR-181a showed a circadian variation in the mice liver and was negative correlated with *Clock* expression [33]. *Bcl-2*, *Prox1* and GABA receptor, involved in circadian rhythms, are validated targets of

* Corresponding author at: Universidade Federal de Alagoas/Campus Arapiraca. Rodovia AL 115, Km 6.5. CEP: 57309-005 Arapiraca, Alagoas, Brazil.
E-mail address: deandrade.tiago@pq.cnpq.br (T.G. de Andrade).

http://dx.doi.org/10.1016/j.bcmd.2015.01.004
1079-9796/

miR-181a [31,46–55]. However, despite these evidences, there are no published studies in hematology investigating the expression pattern of these genes in a circadian period. The aim of this study was to evaluate if miR-16 and miR-181a have daily variations in their expression in human leukocytes, which could be associated with the modulation of the rhythms observed in hematopoesis and immune response.

2. Material and methods

2.1. Subjects

For the analysis of daily variation of miR-16 and miR-181a in human leukocytes, we selected 6 young men, named herein as S1–S6, of the same ethnicity and intermediate chronotype according to the Horne–Ostberg Morningness–Eveningness Questionnaire [56]. These individuals did not experience shift-work or jet lag, were not smokers, and did not regularly use medications. They also presented normal hematological findings. The subjects were told that during the week that preceded the study they should perform their daily activities under a regular routine of sleeping and eating, should avoid excessive light at night, should not ingest foods with caffeine or energetics, and should not perform extensive physical activities. On the day of blood sampling, food was available from 07:00 to 08:00 h (breakfast), 09:30 to 10:30 h (snack), 12:00 to 13:00 h (lunch), 15:30 to 16:30 h (snack), and 19:00 to 20:30 h (dinner), with water available ad libitum. The study was approved by the Committee on Ethics of the Federal University of Alagoas (#007138/2011-41).

2.2. Blood sampling and RNA isolation

Blood was collected in EDTA tubes every 4 h along a circadian cycle. Red blood cells were lysed with Buffer EL (Qiagen, Germany), and RNA purification of the remaining cells was performed with miRNeasy Mini Kit (Qiagen, Germany). The samples were then stored at −80 °C for further analysis.

2.3. cDNA synthesis

The cDNA was synthesized from 1 μg of total RNA using the miScript Reverse Transcription Kit (Qiagen, Germany) according to manufacturer's protocol. The reactions were adjusted to a final volume of 20 μL with RNase-free water. All cDNA samples were diluted (1:10) in RNase free water for analysis.

2.4. Quantitative real-time PCR

The qPCR reactions were performed using StepOne Plus (Applied Biosystems, Foster City, CA, USA) with 2 μL of miScript Primer Assays (Qiagen, Germany) for miR-16 or miR-181a, 10 μL of 2× QuantiTect SYBR Green PCR Master Mix, 2 μL of 10× miScript Universal primer,

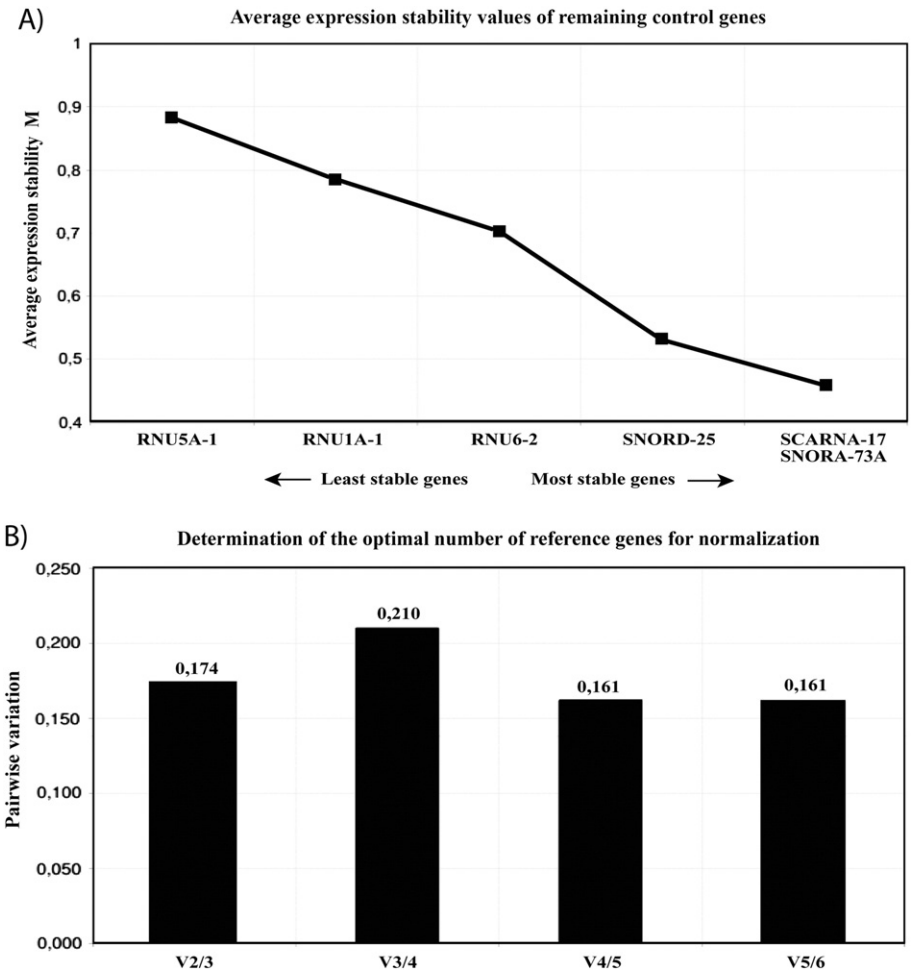

Fig. 1. Selection of the most suitable reference genes for normalization in human leukocytes samples using geNorm analysis. A) Expression stability measures (M) of the six reference genes analyzed. The x-axis from left to right indicates the ranking of the genes according to their expression stability; lower M values indicate higher expression stability. B) Determination of the optimal number of reference genes for normalization was conducted. The software calculates the normalization factor from at least two genes at which the variable V defines the pairwise variation between two sequential normalization factors.