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Review Article

Integrated expression profiling of multiple RNA species by real-time PCR

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ABSTRACT

MicroRNAs (miRNAs) are endogenous, non-coding RNAs comprising approximately 21–23 nucleotides that regulate gene expression by binding to and targeting messenger RNA (mRNA) for translational repression or degradation. miRNAs have been shown to regulate cellular processes including proliferation, differentiation, and development and to play an important role in immune system function. The expression of miRNAs is misregulated in numerous diseases, including cancers of immunological origin. To better understand the role of miRNA in T-cell activation, we used a real-time PCR-based system to analyze changes in miRNA expression following activation of Jurkat T-cells with the inducing agents Phorbol Myristyl Acetate (PMA) and Ionomycin (CI) and detected several miRNAs that showed differential regulation following treatment. Using this system, miRNAs and their mRNA targets, along with other non-coding RNAs, can be simultaneously detected and quantified using SYBR® Green real time-PCR, enabling comprehensive, genome-wide expression profiles of multiple RNA species.

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1. Introduction

MicroRNAs (miRNAs) are endogenous, noncoding RNAs of about 21–23 nucleotides that bind to the 3′ untranslated region of specific mRNAs, leading to the repression of protein expression and promoting mRNA degradation. Initially, miRNAs are transcribed as long, primary miRNAs (pri-miRNAs) that vary greatly in length. These pri-miRNAs are then cleaved by the protein Drosha, resulting in a shorter hairpin loop structure known as precursor miRNA (pre-miRNA). Once the pre-miRNA is exported from the nucleus, the hairpin loop is removed by Dicer, leaving a double-stranded miRNA structure, one strand of which is loaded onto the RNA-induced silencing complex (RISC). Mature miRNAs anneal and bind to their mRNA target of interest via RISC, leading to mRNA degradation or translation inhibition. Since miRNAs do not require an exact nucleotide match to bind to their target, each miRNA can potentially downregulate hundreds of targets.

miRNAs regulate numerous functions, including gene expression during development, differentiation, cell proliferation, and apoptosis. Since their discovery, more than 21,600 mature miRNAs have been identified in 168 different species [1]. Importantly, the discovery of stable miRNA species circulating in blood has led to increased research focus on disease-related variations in serum and plasma miRNA expression and the possibility that such variations could serve as noninvasive biomarkers for disease. For advanced information on high throughput miRNA profiling for biomarker discovery and validation, please refer to Lader and Shaffer [2]. Misregulation of miRNA expression is associated with several cancers and other diseases, and miRNA is thought to regulate several aspects of immune system function, including the development and differentiation of B- and T-cells, the proliferation of monocytes and neutrophils, antibody switching, and the release of inflammatory mediators [3]. In T-cells, miRNAs are believed to be key regulators of lineage induction pathways and to play a role in the induction, function, and maintenance of the regulatory T-cell lineage.

T-cell activation involves a complex cascade of signal transduction pathways linking T-cell receptor engagement at the cell membrane to the transcription of multiple genes within the nucleus. It is a highly regulated process that requires the coordination of multiple signaling pathways that activate proliferation, cytokine production, and differentiation. Additionally, once the antigen is cleared, mechanisms are required for reduction of the T-cell response. Understanding the role of miRNAs in regulating the immune response may lead to more effective treatments for immunological diseases, such as autoimmunity and cancer.

2. Methods

2.1. RNA purification

Total RNA was prepared from HeLa S3 cells, various mouse tissues, or Jurkat T-cells using the miRNeasy® Mini Kit (QIAGEN).

Abbreviations: miRNA, microRNA; snoRNA, small nucleolar RNA; piRNA, Piwi-interacting RNA; PMA, Phorbol Myristyl Acetate; Cl, Ionomycin.

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2.2. Generation of cDNA

Reverse transcription reactions were carried out using the miScript® II RT Kit, using the miScript HiFlex Buffer included in the kit (QIAGEN). With this system, all RNA species (mature miRNA, precursor miRNA, other noncoding RNAs, and mRNA) are polyadenylated and reverse transcribed into cDNA using oligo-dT primers. The oligo-dT primers have a 3′ degenerate anchor and universal tag sequence on the 5′ end allowing amplification of different RNA species. Real-time PCR detection of the RNAs was then performed using the assays described below.

2.3. RNA quantification

For detection of miRNA from HeLa cells: the miScript PCR System (QIAGEN) and primers designed in-house were used with 1 ng cDNA for real-time PCR analysis of 4 miRNAs (Let-7a, miR-16, miR-21, and miR-25). For detection of mRNA, QuantiTect® Primer Assays (QIAGEN, www.qiagen.com/GeneGlobe) were used with 1 ng cDNA and the QuantiTect SYBR Green PCR Kit (QIAGEN) for real-time PCR analysis of GAPDH, PPIA, CDC2, and Erk2. For detection of snoRNAs: the miScript PCR System was used with primers designed in-house and 0.5 ng cDNA per reaction for real-time PCR analysis of 21 snoRNAs and 5S ribosomal RNA. For detection of piRNAs: cDNA was prepared using 1 μg RNA from each mouse tissue. The miScript PCR System was used with primers designed in-house and 1 ng cDNA/reaction for real-time PCR analysis of 3 piRNAs and the miRNA Let-7a. For miRNA expression profiling in Jurkat T-cells, the miScript PCR System was used with a range of miScript Primer Assays (QIAGEN) for real-time PCR analysis of 328 miRNAs. Data for the 111 miRNAs expressed in Jurkat T-cells were normalized to that of U6B.

2.4. Jurkat T-cell activation

Jurkat T-cells were seeded at 4000 cells/well and grown untreated, or stimulated with PMA, or PMA and Cl for 4 h. Cells were then washed with culture medium, and grown for an additional 24 h before harvest. After 24 h, total RNA, including small RNAs, was prepared using the miRNeasy Mini Kit.

3. Results and discussion

With their potential to regulate numerous targets, miRNAs are emerging as key regulators of gene expression in many biological processes, including the induction, function, and maintenance of immune cells. To analyze these processes more closely, we used a system that enables integrated profiling of multiple RNA species from a single cDNA reaction, and studied the effect of activating agents on miRNA expression in Jurkat T-cells, a model T-cell line. Several miRNAs known to be involved in T-cell activation, as well as novel miRNAs, were identified.

3.1. Integrated analysis of multiple RNA species

The analysis of multiple RNA species from a single cDNA reaction is advantageous, as it allows simultaneous detection of miRNA and their potential mRNA targets, as well as small, noncoding RNAs that can be used to normalize miRNA quantification. We therefore used the miScript PCR System to carry out reverse transcription of total RNA. Using this system, multiple miRNAs and mRNAs (Fig. 1a), as well as a range of small nucleolar RNAs (snoRNAs) and 5S ribosomal RNAs, were detected in HeLa S3 cells (Fig. 1b). Several of these snoRNAs, such as U6B, and ribosomal RNAs, such as 5S, are commonly used as reference RNAs to normalize expression levels of miRNAs.

We were also able to detect Piwi-interacting RNAs (piRNAs) [4], a newly reported class of germline-specific small RNAs (29–30 nt) that were first described in mouse testis, indicating that the miScript PCR System is capable of detecting a wide variety of small RNAs (Fig. 1c). Comprehensive, genome-wide expression profiles of multiple RNA species from a single cDNA sample is important to fully understand changes in miRNA/mRNA levels following treatment of T-cells with activating agents.

3.2. Generation of an miRNA expression profile in Jurkat T-cells

Jurkat T-cells, an acute T-cell leukemia cell line, were used as a model system to study the expression of various miRNAs in both untreated cells, and in cells treated with activating agents. The expression of 328 different miRNAs (chosen from miRBase V 8.0; http://microrna.sanger.ac.uk/sequences) was determined to generate a comprehensive expression profile of miRNA expression in untreated Jurkat T-cells. Of the 328 miRNAs analyzed, 111 miRNAs were detected (Fig. 2).

3.3. Changes in miRNA levels following treatment with activating agents

T-cell activation involves tightly regulated, complex signaling pathways that activate genes involved in cell activation, proliferation, and survival. To better understand the role of miRNAs in T-cell activation, we quantified changes in miRNA expression following treatment of Jurkat T-cells with the inducing agents Phorbol Myristyl Acetate (PMA), which activates Protein Kinase C (similar to diacylclycerol) and leads to activation of NFB and AP1, and Ionomycin (Cl), which activates Ca^{2+}/calmodulin-dependent signaling pathways and leads to activation of NFAT [5]. Of the 111 miRNAs detected in the expression profile obtained from resting Jurkat T-cells (Fig. 2), 30 showed greater than a 3.5-fold change in regulation 24 h after treatment with PMA or PMA + Cl (Fig. 3).

miRNAs have the potential to regulate T-lymphocyte activation by targeting highly differentially expressed genes that are involved in aspects critical for cell activation, proliferation, and survival. In this study, several miRNAs known to be involved in T-cell regulation were upregulated in response to treatment with PMA and Ionomycin, including miR-146a (overexpressed almost 30-fold) and miR-155 (overexpressed by approximately 18-fold). Several algorithms have been created and are freely available for predicting the mRNA target sites of miRNA. mRNA targets were identified using miRanda [6] and TargetScan [7], which detected potential binding sites for miR-155 and miR-146a on mRNA encoding the cyclin-dependent kinase inhibitors (CDKI) 2A, 2D, 1B, and 3. This implies that upregulation of miR-146a and miR-155 could down-regulate inhibition of cyclin-dependent kinases due to repression of CDKIs, resulting in increased cell growth.

Several studies have identified a role for miR-146a and miR-155 in modulating T-cell function. miR-155 has been widely studied in the context of T-cell activation and has been shown to be upregulated in activated T-cells. Significantly increased proliferation results from inhibition of miR-155 upregulation following T-cell activation [8].

miR-146a levels are low in human naïve T-cells and are induced upon T-cell receptor stimulation [9]. A recent study has shown that miR-146a modulates activation-induced cell death in T-cells in order to eliminate the T-cell once the pathogen has been removed. The Fas-associated death domain (FADD) is a target of miR-146a, and a negative correlation has been observed between miR-146a induction and FADD protein levels in primary CD4 + T lymphocytes

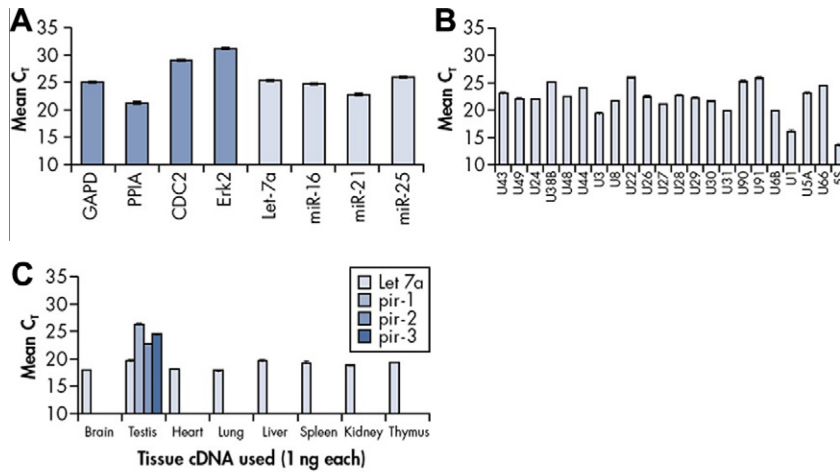

Fig. 1. Simultaneous analysis of multiple RNA species. (A) A single cDNA synthesis reaction enabled detection of multiple miRNAs and mRNAs. Total RNA was prepared from HeLa S3 cells using the miRNeasy Mini Kit. The miScript PCR System and 1 ng cDNA were used with miScript Primer Assays for real-time PCR analysis of 4 miRNAs (Let-7a, miR-16, miR-21, and miR-25). QuantiTect Primer Assays were used with 1 ng cDNA and the QuantiTect SYBR Green PCR Kit for real-time PCR analysis of 4 mRNAs (GAPDH, PPIA, CDC2, and Erk2). (B) Total RNA was prepared from HeLa S3 cells using the miRNeasy Mini Kit. The miScript PCR System was used with in-house designed assays and 0.5 ng cDNA per reaction for real-time PCR analysis of 21 snoRNAs and 5S ribosomal RNA. (C) Total RNA was prepared from various mouse tissues using the miRNeasy Mini Kit. cDNA was prepared using 1 μg RNA from each tissue. The miScript PCR System was used with in-house designed assays and 1 ng cDNA per reaction for real-time PCR analysis of 3 piRNAs and the miRNA Let-7a. The piRNAs were only detected in testis tissue while Let-7a was detected in all tissues.

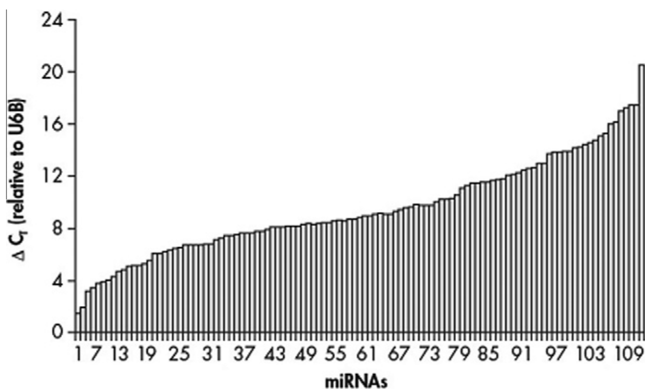

Fig. 2. Generation of a miRNA expression profile in resting Jurkat T-cells. Total RNA was prepared from untreated Jurkat cells using the miRNeasy Mini Kit. The miScript PCR System was used with multiple miScript Primer Assays and 0.5 ng cDNA per reaction for real-time PCR analysis of 328 miRNAs. This graph shows the difference in C_T values between the target miRNA and the reference small nuclear RNA, U6B.

that were stimulated with PMA and Ionomycin, suggesting that miR-146a may modulate adaptive immunity [9].

Several miRNAs downregulated in our study potentially target mRNAs involved in apoptosis. Caspase 3 contains a putative binding site for miR-23a/b, while Caspase 7 is a potential target of miR-29a and miR-30e. Decreased expression of these miRNAs could upregulate apoptotic signaling pathways, which may have an effect on the immune response and removal of T-cells once a pathogen has been cleared.

Numerous studies have identified that misregulated expression of several of the miRNAs that were differentially regulated in our study is associated with the development of cancer. For example, miR-132, which was upregulated in our study, is differentially regulated in 6 solid cancer types. It is predicted to target Rb and its overexpression has been linked to decreased pRb levels, which could result in the overexpression of numerous E2F transcription factors and increased cell proliferation [10]. miR-29, downregulated in our study, has been linked with aggressive CLL phenotypes. Studies on acute myeloid leukemia (AML) indicate a tumor suppressive function for this miRNA [11]. It is evident that miRNAs provide important controls for cell proliferation and function.

4. Concluding remarks

Understanding the role of miRNA on mRNA regulation is complicated by the fact that multiple miRNAs often coordinate to differentially regulate their targets. Therefore, miRNA may be

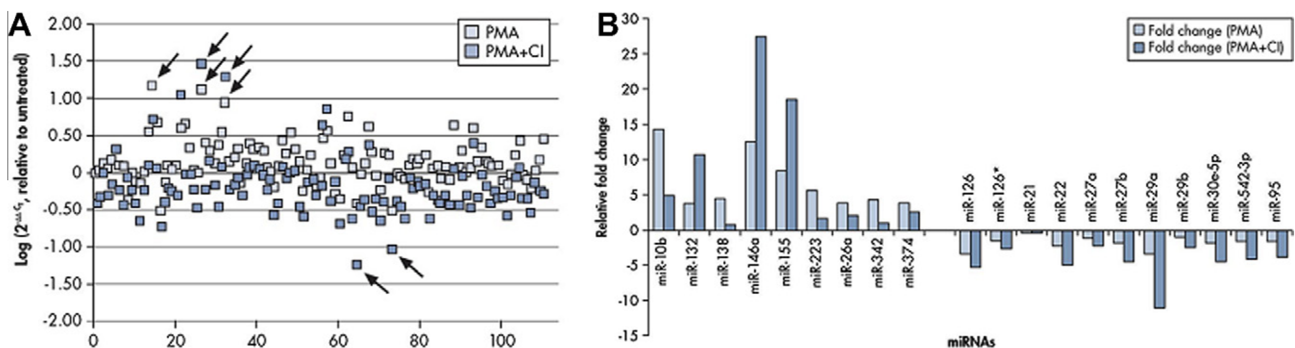

Fig. 3. Differential miRNA expression following treatment with activating agents. (A) Jurkat cells were untreated, treated with PMA, or treated with PMA and CI. After 24 h, total RNA was prepared using the miRNeasy Mini Kit. The miScript PCR System was used with multiple miScript Primer Assays for real-time PCR analysis of 328 miRNAs. Data for the 111 miRNAs expressed in Jurkat cells were normalized to that of U6B and are presented here as log of $2^{-\Delta\Delta CT}$ relative to the expression in untreated cells. (B) Differential regulation of specific miRNAs is shown.

upregulated in some cells, and downregulated in others to effect different cellular functions. In order to ensure that accurate analysis of miRNA expression takes place, it is essential to include the proper controls, such as snoRNAs to normalize quantification, and to analyze all RNA species from the same cDNA sample, to ensure the cell stimulus is consistent for both miRNA and mRNA.

In this study, we detected several miRNAs that showed differential regulation following treatment of Jurkat T-cells with activating agents. Further investigation is ongoing in order to fully understand their role in T-cell function.

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