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Editors

Macro Roles for MicroRNAs in the Life and Death of Neurons

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Foreword

The discovery of microRNAs has revealed an unexpected and spectacular additional level of fine tuning of the genome and how genes are used again and again in different combinations to generate the complexity that underlies for instance the brain. Since the initial studies performed in C.elegans, we gave gone a far way to begin to understand how microRNA pathways can have an impact on health and disease in human. Although microRNAs are abundantly expressed in the brain, relatively little is known about the multiple functions of these RNA molecules in the nervous system. Nevertheless, we know already that microRNA pathways play major roles in the proliferation, differentiation, function and maintenance of neuronal cells. Several intriguing studies have linked microRNAs as major regulators of the neuronal phenotype, and have implicated specific microRNAs in the regulation of synapse formation and plasticity. Dysfunction of microRNA pathways is also slowly emerging as a potential important contributor to the pathogenesis of major neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease. These novel insights appear to be particular promising for the understanding of the very frequent and badly understood sporadic forms of these diseases as compared to the genetic forms. Thus, the better understanding of the implications of this novel field of molecular biology is crucial for the broad area of neurosciences, from the fundamental aspects to the clinic, and from novel diagnostic to potentially therapeutic applications for severe neurological and maybe psychiatric diseases. The present volume gathers contributions to the Colloque Médecine et Recherche on the implications of microRNAs in neuroscience organized by the Fondation Ipsen, in Paris, on April 20, 2009. It had as objective to bring together neuroscientists from different areas of research to discuss their current insights into the wonderful world of microRNAs, and to hear and discuss their research and views about microRNA biology in neuronal processes and in brain disorders.

Bart de Strooper
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Profiling the microRNAs

Kenneth S. Kosik, Thales Papagiannakopoulos, Na Xu, Kawther Abu-Elneel, Tsunglin Liu, and Min Jeong Kye

Abstract Profiling has contributed to the rapid growth of knowledge concerning microRNAs (miRNAs). The ability to measure all the known miRNAs by hybridization or by quantitative PCR has yielded insights into oncogenesis, stem differentiation, development, and disease. The steps toward discovery have often begun with profiling, followed by target prediction for miRNAs that are differentially expressed and then experimental validation of the targets. Here we present some methodological detail on the profiling procedures used in the lab and their application.

1 Introduction

microRNAs have emerged as a critical layer of post-transcriptional regulation over the expression of many genes (Bartel 2004; Stefani and Slack 2008). Among the super-kingdoms of life - Archaea, Bacteria, and Eukarya - microRNAs are found in Eukarya. Within Eukarya, they are present in the animal and plant kingdoms and absent in Fungi. Information about microRNAs remains scant in Protista.

microRNAs (miRNAs) regulate both the stability and translatability of their mRNA targets by binding to an imperfectly matched sequence within the target mRNA. Descriptions of miRNA function often focus on their role as post-transcriptional regulators of target mRNAs or on their organismal roles. However, a poorly understood territory lies between these biological levels of action. The effects of miRNA targeting are reflected in the proteome and, in some cases, ramify back through the transcriptional profile. Thus, mRNAs that may not be direct targets of miRNAs may exhibit more dramatic changes than their direct targets

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and may lie closer to the phenotypic consequences of miRNA inhibitory effects than direct targets.

miRNAs are particularly prevalent within the nervous system, where they contribute to the complexity and function of the mammalian brain (Mercer et al. 2008). One role of miRNAs in the nervous system appears to be related to plasticity and, more speculatively, to the epigenetic and transcriptional programs that underlie long-term memory storage.

To understand and develop full descriptions of these many facets of miRNA biology, detailed and sensitive profiling of miRNA levels is necessary, as well as mRNA profiles and the analytical tools to link these data sets.

2 The first miRNA profiling

We performed some of the earliest miRNA profiling by spotting membranes with tri-mer oligonucleotides (antisense to microRNAs) of 54–72 nt at a final concentration of 7 μM (Krichevskyet al. 2003). The oligonucleotides were spotted on the GeneScreen Plus (NEN) membranes with a 1536 pin plate replicator (V&P Scientific). To confirm the specificity of hybridization, a series of oligonucleotides with three mismatches (G → C or C → A) were included on the array. These mismatches resulted in a significant drop in signal from these spots compared to their cognates. Five to ten micrograms of total RNA from brain tissue was filtered through Microcon YM-100 concentrators to obtain a low molecular weight fraction of RNA enriched in molecules under 60 nucleotides. Despite this filtering, several highly expressed pre-miRNAs in the 70 nucleotide range were detected. A synthetic 21-nt RNA with a sequence that does not correspond to any miRNA, but is an exact complement to a random spotted sequence, was added to the RNA sample at a known concentration as a reference for normalization. The Low Molecular Weight (LMW) RNA was then end-labeled with 30 μCi of γ^{33}P dATP (3,000 Ci/mmole) by T4 polynucleotide kinase and purified. A typical experiment included three independent RNA samples for each experimental condition.

Among the findings we reported using this method was the highly consistent increase in levels of miR-21 in glioblastomas (Chan et al. 2005). Subsequently, miR-21 has been shown to be the microRNA most likely to be elevated in a wide range of solid tumors. In a follow-up study, we showed that miR-21 targets multiple important components of the p53, transforming growth factor-beta (TGF-beta), and mitochondrial apoptosis tumor-suppressive pathways (Papagiannakopoulos et al. 2008). Down-regulation of miR-21 in glioblastoma cells led to de-repression of these pathways, causing repression of growth, increased apoptosis, and cell cycle arrest. These findings clearly established miR-21 as an oncogene that targets a network of p53, TGF-beta, and mitochondrial apoptosis tumor suppressor genes in glioblastoma cells.

With this technique and additional supportive data, we also reported that brain-specific miR-124a and miR-9 molecules affect neural lineage differentiation in

embryonic stem cell-derived cultures (Krichevsky et al. 2006) and published the first report of systematic miRNA gene profiling in cells of the hematopoietic system (Monticelli et al. 2005).

3 miRNA profiling by multiplex real time PCR

Commercialization of hybridization arrays with fluorescent dyes and glass slides came quickly. However, our profiling departed from the hybridization approaches and, in collaboration with Applied Biosystems, we profiled miRNAs using multiplex real-time RT-PCR. In one of our earliest experiments with this method, we used a 187-plex RT-PCR adapted for the rat sequences from Lao et al. (2006) to profile miRNAs in laser-captured cell bodies and neurites of dissociated hippocampal neurons (Kye et al. 2007). Briefly, multiplex reverse transcription primers were generated with a common 20 nucleotide stem loop followed by eight nucleotides complimentary to the 3′-end of a given miRNA (e.g., 5′-AACTATAC-3′for rno-let-7a). Reverse transcriptase reactions were performed in 5 μl containing 1x cDNA Archiving Kit buffer (Applied Biosystems), 10 units MMLV reverse transcriptase, 1.25 mM of each dNTP, 1.3 units RNase inhibitors (Applied Biosystems), and 2.5 nM of each reverse primer. Our sample RNA in this case was rat hippocampal dissociated culture taken by laser capture microdissection. Reactions were 30 cycles at 20°C for 30 sec, 42°C for 30 sec, and 50°C for 1 sec and one cycle at 85°C for 5 min to inactivate the enzyme.

cDNA was pre-amplified with a common reverse primer (UR) to the 20 nucleotide stem loop and multiplexed ∼18 nucleotide forward primers with a common 5′-end and a 3′-end matching 12-17 nucleotides of the 5′-end of a given miRNA (e.g., 5′-TCCAGCTCCTATATGAT-3′ for rno-let-7a). The amplification was performed in 25 μl containing 1x Universal Master Mix (Applied Biosystems), 50 nM of each forward primer, 5 μM of UR, 6.25 units AmpliTaq Gold polymerase (Applied Biosystems), 0.5 mM of each dNTP, 2 mM MgCl2, and 5 μl of the initial reverse transcriptase amplification. Reactions were one cycle at 95°C for 10 min, 55°C for 2 min, and 14 cycles at 95°C for 1 sec and 65°C for 1 min.

The product of this amplification was diluted four times and 0.1 μl was used for real-time PCR performed in 10 μl containing 1x Universal Master Mix (Applied Biosystems), 0.25 μM FP, 0.1 μM TaqMan® probe, and 5 μM UR. The miRNA specificity for each reaction was provided by the miRNA-specific sequences of the forward primers and TaqMan® probes. Real-time PCR was performed in a Biosystems 7500HT 96-well plate Sequence Detection System using 40 cycles of 95°C for 15 sec and 60°C for 1 min. All reactions were run in duplicate. The threshold cycle (Ct) was determined as the fractional cycle number at which the fluorescence passes the fixed threshold**.** All Ct values were averaged over two to four repeated biological experiments, each with two averaged real-time PCR experimental replicates. Most recently, Applied Biosystems decided to commercialize this product as

a series of single-plex real-time PCR reactions and current methodologies differ slightly from what is described above.

A convenient facet of the multiplex approach is the flexibility to include mRNA detection in the same sample for real-time PCR. In our case, we used the same laser capture microdissected samples. Reverse transcriptase reactions were performed in 5 µl containing 1x cDNA Archiving Kit buffer (Applied Biosystems), 10 units MMLV reverse transcriptase, 1.25 mM of each dNTP, 1 unit RNase inhibitor (Applied Biosystems), 2 mM random primer, and 1 µl of the laser capture microdissection samples. Reactions were at 20°C for 10 min, 37°C for 120 min, and 85°C for 5 min to inactivate the enzyme. cDNA was pre-amplified using gene-specific forward primers and reverse primers. The amplification was performed in 25 µl containing 1x Universal Master Mix (Applied Biosystems), 50 nM of each forward primer, 50 nM of each reverse primer, 6.25 units AmpliTaq Gold polymerase (Applied Biosystems), 0.5 mM of each dNTP, 0.75 mM MgCl2, and 5 µl of the initial reverse transcriptase amplification. Reactions were one cycle at 95°C for 10 min, 55°C for 2 min, and 14 cycles at 95°C for 1 sec and 65°C for 1 min. The product of this amplification was diluted four times and 0.1 µl was used for real-time PCR reactions detecting mRNAs. It was performed in 10 µl containing 1x Universal Master Mix (Applied Biosystems), 500 nM forward primer, 200 nM TaqMan® probe, and 500 nM UR.

As noted above, the results from the miRNA profiling by quantitative PCR are given in Ct values. The cycle number at the threshold level of log-based fluorescence is defined as Ct number. Because larger Ct values tend to have greater fluctuation, we set 28 as the cutoff value above which the fluctuation makes precise determination of the Ct less reliable and below which the Ct value is highly reliable. This feature of the data can be clearly shown on a scatter plot in which two identical samples are profiled and the points where the line splays are consistently at a Ct value of 28. In a study of rat hippocampal neurons (Kye et al. 2007), among the 187 miRNAs in the raw data, 30 had an NTC Ct <28 (high NTC noise): 36 Cts were above the cut-off in all samples, four had a large fluctuation in duplicated experiments, and 13 had a large fluctuation between samples (s.d.>1). A further five miRNAs were excluded with a mean Ct in the cell body >28 even though their among-sample fluctuation was small. After filtering, we were left with 99 miRNAs.

Real-time reverse transcription PCR was useful for detecting a microRNA signal in an in vivo expression set of mRNAs (Liu et al. 2007). In this study, RNA was extracted from 12 human primary brain tumor biopsies. We determined genome-wide mRNA expression levels by microarray analysis and a miRNA profile by real-time reverse transcription PCR. Correlation coefficients were determined for all possible mRNA-miRNA pairs, and the distribution of these correlations was compared to the random distribution. Within the correlations we sought endogenous fluctuations in the data set that were statistically distinguishable from the many other fluctuations in the data set. In fact, an excess of high positive and negative correlation pairs was observed at the tails of these distributions. Among the 70 most