Journal of Cytology & Histology
ISSN: 2157-7099

Research Article Open Access

Dicer Silencing by siRNA in Cerebellar Granule Neurons

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Abstract

RNA-mediated gene silencing is a fundamental mechanism of gene expression regulation. A main component of RNA silencing machinery, the RNase III Dicer enzyme, catalyzes the processing of double-stranded RNAs (dsRNAs) into ≈21-25 nucleotide-long small interfering (si)RNAs and micro (mi)RNAs, an essential step in the biogenesis of small non-coding RNA molecules. In previous report was reported the Dicer localizes with ER and Golgi network in post-mitotic neurons, increasing its expression during the development and maturation in cultured neurons. With the aim to study the components of RNA-induced silencing complex (RISC), it was investigated the effect of Dicer silencing on survival in cultured cerebellar granule neurons (CGNs). In this immnofluorescence study was observed that Dicer silencing by siRNA facilitates the apoptotic neuronal cell death in CGNs.

Keywords: RNA induced silencing complex; Dicer; RNAi; Post-transcriptional gene regulation; Post-mitotic neurons; Apoptosis

Introduction

MiRNAs tune gene expression at the post-transcriptional level by mRNA degradation and inhibition of the translation of target mRNAs [1]. As first, miRNAs are transcribed by RNA polII, generating an initial transcript named primary miRNA (pri-miRNA), which spans in length from 100 to 1000 nucleotides and contains a 60-80 nucleotides stem-loop structure [2]. The ribonuclease (RNAse) III-like Drosha enzyme and the RNA-binding protein DGCR8/Pasha, process pri-miRNAs in a hairpin intermediate called pre-miRNA [2]. In the cytoplasm the pre-miRNA is processed by RNase III enzyme Dicer, which cuts out the loop region of the hairpin, releasing the mature miRNA, which is incorporated into RISC. RISC contains a key component an Argonaute family protein, Ago2, which directly binds the miRNA and correspond to effector complex of the miRNA pathway [3].

In the last decade, the molecular and cellular studies of RNA-mediated gene silencing in the nervous system were performed [4]. In our previous work was described the sub-cellular localization of the RNAi machinery in post-mitotic neurons and in particular was observed that Dicer was associated with ER/Golgi network and its expression increases during the development and maturation in cultured CGNs [5]. Recently, several works analyzed the importance and involvement of Dicer in neuronal survival *in vivo* and *in vitro* [6-13]. In order to evaluate the essential Dicer expression in neuronal survival, we observed the effects of Dicer silencing on death of CGNs by an immunofluorescence study. This work is a preliminary description on the functions of the RISC components Dicer in primary cultured neuronal cells.

Methods

CGNs, RNA interference and immunofluorescence

CGNs were obtained from dissociated cerebella of 8-day-old Wistar rats as previously described [5]. SiGLO Green was used as transfection indicator and siGENOME ON-TARGET plus SMART pool Rat Dicer1, number XM-216776, both by Dharmacon as siRNAs. In single transfection, the siRNAs molecules were used at 50 nM, whereas when co-delivered, they were used a 1:1 ratio not exceeding 100 nM (50 nM each). After 4 DIV neurons were transfected with siGLO Green and/or Dicer siRNAs by using Lipofectamine 2000 (Invitrogen). 24-96 h post-transfection, the neurons were fixed for immunofluorescence analysis as described [5]. The coverslips were incubated with rabbit polyclonal antibody anti-dicer 1:100 (Imgenex) and monoclonal antibody anti-caspase-3 (Promega) and nuclei were stained with Hoechst 33258 (Sigma). Slides were mounted and examined by conventional epifluorescence microscope (Olympus BX51). Images were captured by a SPOT RT3 camera and elaborated by IAS software.

Results

Dicer was mapped in the Golgi-reticulum network in neurons [5]. With the aim to elucidate a potential involvement of Dicer in CGNs survival process [14], Dicer was knocked-down by siRNA transfection as shown in Figures 1 and 2. After silencing, the Dicer staining is markedly reduced, whereas in non-transfected neurons or transfected with siGLO Green oligonucleotide, it decorated the Golgi-reticulum area as shown in Figures 1A,1D, 2A and 2D. CGNs transfected with siGLO Green indicator show the 'green neurons' with intact nuclei and normal Dicer signal (data not shown). The reduced Dicer expression was associated to condensed nuclei (cell death). The number of not viable neurons increased from 48 h (20%) as shown in Figure 1E and1F to 96 h (50%) after siRNA Dicer transfection as shown in Figures 2E and 2F. In order to characterize the neuronal cell death, was performed a double immunofluorescence with antibodies recognizing Dicer and caspase-3 activated in silenced neurons, without siGLO green marker, showing that the caspase-3 signal is associated to dying neuronal cells whereas the Dicer positive neurons were negative to caspase-3 as shown in Figures 3A-3D. This result indicates that Dicer silencing facilitates the caspase-3 activation and the nuclei condensation suggesting an apoptotic cell death instead necrotic/toxic effects as shown in Figures 3.

Discussion

Dicer is a member of the ribonuclease RNase III family, that

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Received April 17, 2019; **Accepted** May 08, 2019; **Published** May 14, 2019

Citation: Barbato C (2019) Dicer Silencing by siRNA in Cerebellar Granule Neurons. J Cytol Histol 10: 539.

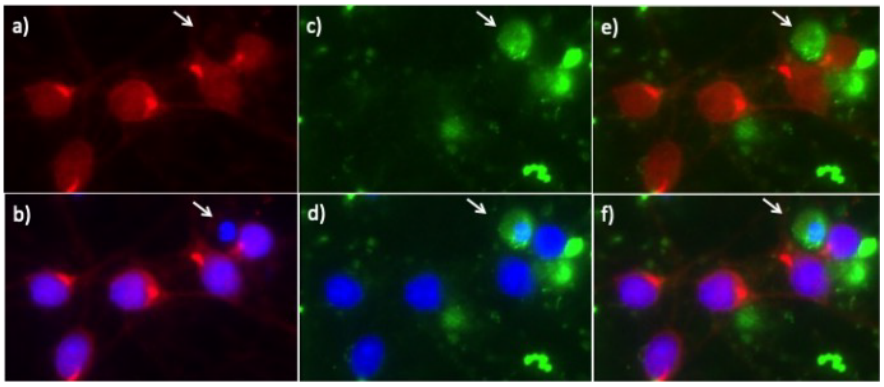

Figure 1: Dicer expression and silencing 48 h post-transfection in CGNs. Immunofluorescence of CGNs co-transfected with siGLO Green and Dicer siRNAs. **(a)** anti-dicer staining (red); **(b)** combined images showed dicer red signal was absent in cell with condensed nuclei (white arrow); **(c)** siGLO Green (green) is a transfection marker; **(d)** merge indicate that dicer silencing was effective in transfected neurons. The green dots represent the normal aggregates formation between nucleic acids and lipids used for the transfection; **(e)** combined images with anti-dicer staining and Dicer siRNAs/siGLO Green indicates neurons without Dicer; **(f)** the triple images shows Dicer associated to Golgi-reticulum in viable neurons; red signal was reduced in dying silenced neurons (green).

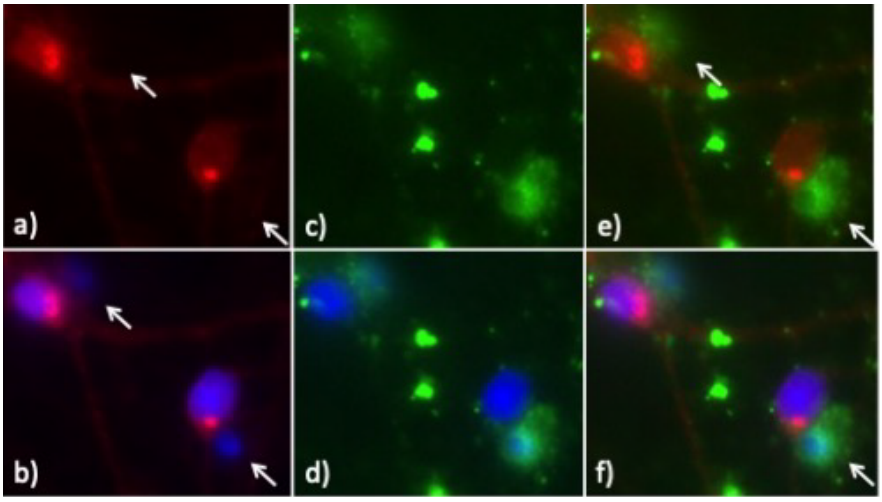

Figure 2: Dicer siRNAs 96 h post-transfection in CGNs. Indirect immunofluorescence of CGNs co-transfected with siGLO Green and Dicer siRNAs. **(a)** Cytoplasmic anti-dicer staining (red); **(b)** White arrows indicate neuronal cells with condensed nuclei without dicer expression; **(c)** Cerebellar neurons transfected with Dicer siRNAs; **(d)** Shows a condensed nuclei; **(e)** At 96 h was observed a doubling increase of Dicer siRNAs transfected neurons, showing clearly **(f)** that the silenced neurons are dying.

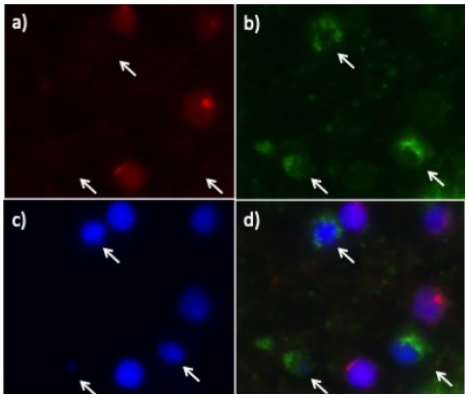

Figure 3: Dicer silencing and caspase-3 activation in CGNs. **(a)** Specific anti-dicer staining (red); **(b)** Caspase-3 activated (green) is used as a marker for cell death; **(c)** Nuclei were stained with Hoechst 33258; **(d)** The combined triple labeled images clearly indicate that the dicer expression was absent where the caspase-3 was activated, green signal (white arrows indicate condensed nuclei).

processes double-stranded RNAs and pre-miRNA into ≈21-25 nucleotide-long siRNAs and miRNAs [15,16]. Recently, Dicer was explored in CGNs cultured *in vitro*, demonstrating that it is localized in the Golgi-reticulum structures and its expression increase during the development and maturation [5]. Here was showed by indirect immunofluorescence, which the Dicer silencing in CGNs is accompained by increase of neuronal cell death. After siRNA Dicer transfection in CGNs, the neuronal cell death increase of 30% from 48 h to 96 h as shown in Figures 1 and 2. Non transfected neurons showed a canonical Dicer localization, whereas reduced Dicer expression was observed only in dying silenced neurons, indicating that Dicer protects cerebellar neurons from cell death. To establish if the neuronal death is apoptotic, CGNs were stained with anti-dicer and caspase-3 activated, a marker of CGNs apoptotic cell death [17]. In positive neurons to caspase-3 activated, showing an altered morphology and nuclei condensation, Dicer was almost absent. The presence of caspase-3 activated only in Dicer silenced neurons, showed an antiparallel profile, suggesting that Dicer reduce the activation of cell death pathway,

probably by miRNAs post-transcriptional regulation. Recently was observed the dysregulation of miRNA biogenesis in adult neurons during aging and the Dicer activity promotes survival of cultured dopaminergic neurons, demonstrating that its downregulation or inactivation induces a reduced survival and mild protection from thapsigargin-induced endoplasmic reticulum stress [12]. The negative association between Dicer and thapsigargin was observed also in CGNs [5]. In previous study was showed that the deletion of Dicer in Purkinje neurons resulted in progressive neurodegeneration [18], whereas in dopaminergic neurons, both apoptosis and neurodegeneration [19]. In post-mitotic neurons the conditional loss of Dicer resulted in increased apoptosis at early stage of development but not later in the development [20]. In this work was suggested that specific subpopulation of neurons depends on Dicer function for survival, or on the other hand, might be fundamental the timing of Dicer inactivation. In silenced CGNs, the apoptotic death was more prominent at late time post-transfection, corresponding to *in vitro* development phase of these neurons [21,22]. In this regard, it will be important evaluate the effects of RNAi of Dicer in CGN at 8DIV a late stage of cell culture, but it is not reliable transfect the cerebellar granule at this time. Moreover, Dicer expression correlates with the period of rapid proliferation in the developing cerebellum, and its deletion results in accumulation of DNA damage [10]. The time dependent effects of Dicer down-regulation in post-mitotic neurons are consistent with other reports [6,23].

Conclusion

In conclusion, it is possible to suggest that the Dicer interacting proteins, timely and spatially regulated, might to be essential not only in the biogenesis of most small regulatory RNAs, but also to Dicer non-canonical functions in post-mitotic neuronal cells. These preliminary observations prompt us to explore the molecular and cellular aspects of RISC complex in post-mitotic neuronal cells.

Acknowledgments

This work was supported by Compagnia di Sanpaolo, Programma Neuroscienze Grant, MicroRNAs in Neurodegenerative Diseases (2009-2011 to CB). I wish to acknowledge the help and support provided by MT Ciotti and C Cogoni.

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