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Assessment of tissue distribution and subcellular localization of miR-302 and miR-21 by means of in situ hybridization technique

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Abstract

MicroRNAs (miRNAs) are a group of short non-coding RNAs implicated in numerous fundamental cellular processes, and their disregulations have been linked to several pathologic conditions, mainly cancers. Determining tissue distribution of miRNAs is a prerequisite for understanding their exact functions during development, tissue homeostasis and abnormality. In situ hybridization is a powerful technique to delineate the sub-cellular localization and tissue distribution patterns of mRNAs as well as miRNAs. Due to the important role of miRNAs in tumorigenesis, we optimized an ISH technique for detection of two well-known miRNAs (miR-302 and miR-21) in formalin-fixed paraffin-embedded (FFPE) tumor samples along with a pluripotent embryonal carcinoma cell line, NTERA-2 (NT2). After fixation of cells on slides/sectioning of FFPE blocks, proteinase K digestion, probe concentration, antibody development and light sensitive color reaction were optimized for both the FFPE samples and cell line. Signals for U6 snRNA, as an internal control, were detected in the nuclei of the cells. MiR-21 and miR-302 expression was detected in the cytoplasm of FFPE samples of seminoma carcinoma and in NT2 cell line, respectively. In this study, we optimized ISH for miRNA detection in FFPE samples and NT2 cell line.

Keywords**:** *in situ* hybridization, microRNA, miR-302, miR-21

Introduction

MicroRNAs (miRNAs) are short (18-22 nt) non-coding RNAs that post-transcriptionally regulate gene expression by direct degradation of their mRNA targets or inhibition of their translation. MiRNAs have prominent roles in regulation of cell proliferation, differentiation and apoptosis. They have been also implicated in different pathological conditions, especially in tumorigenesis. Contribution of miRNAs to carcinogenesis was first introduced by Calin et al. (Calin et al., 2002). Since then an enormous body of investigations has focused on their mechanism of action in cancer initiation and progression, as well as recognizing their role as potential cancer biomarkers and therapeutic targets (Schetter et al., 2008; Spizzo et al., 2009; Iorio et al., 2005). In this aspect, several techniques have been utilized or innovated to predict and experimentally validate new miRNAs, quantify their expression and localize their distributions within tumor tissues.

In situ hybridization (ISH) is carried out by a labeled oligonucleotide (called a probe) which is designed to complementary hybridize to a specific sequence (DNA, RNA, miRNA) inside the cells, and therefore precisely localizes its presence within the cell or tissue section (Carter et al., 2005). DNA probes are usually used for diagnostic purposes and chromosomal abnormalities (Zitzelsberger et al., 1994), while RNA probes are used for gene expression analysis (Jorgensen et al., 2010). Due to their critical role in tissue homeostasis, development and diseases, miRNA localization and expression timing is of vital importance in understanding their exact role in development and initiation and progression of diseases. ISH technique allows miRNA expression detection at the cellular level, and therefore it demonstrates the cellular source of a given miRNA. However, it does not provide a precise quantitative measurement of miRNA expression level.

The main challenges in ISH technology for miRNA detection are: 1) the unstable nature of RNA which demands RNase-free procedures during the whole experiment, 2) the small sizes of the miRNAs, and 3) the very similar sequences of some miRNAs (Jorgensen et al., 2010; Nuovo, 2010a). To improve the specificity and affinity of

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probe binding, different technologies have been employed. Among them locked-nucleic acid (LNA) substituted oligonucleotides sound promising. LNA is a nucleotide with a 2'-O, 4'-C-methylene-β-D-ribofuranosyl nucleotide bond that locked in an RNA mimicking sugar. With an increase in the melting temperature (Tm), oligonucleotides containing LNAs show higher hybridization affinity to the target RNA and an increase in the signal to background ratio. Moreover, they have higher binding specificity which makes them suitable for specific miRNA hybridization, since the miRNAs have very short length and some of them might differ in only 1-2 nucleotides (Vester and Wengel, 2004; Silahtaroglu et al., 2004; Silahtaroglu et al., 2007; Nuovo, 2010b).

Here, we are reporting an optimized ISH procedure for miRNA detection in FFPE samples of seminoma carcinoma and a human embryonal carcinoma cell line, NT2.

Materials and Methods

Bacterial strains, plasmids and media

Before starting the procedure, all the glasswares were incubated at 180ºC for 8 h to remove any potential RNase contamination. All the solutions were prepared with DEPC-treated water, and if it was possible, autoclaved after preparation. It is very important to maintain RNase-free conditions during the whole procedure. Once the procedure starts, it cannot be halted or the slides allowed to be air dried. The only steps that can be prolonged are the washing steps with phosphate buffered saline (PBS). In general, the whole protocol of ISH could be divided in 2 days.

Deparaffinization

FFPE blocks of seminoma carcinoma were collected from department of pathology, Shariati hospital (Tehran-Iran). Blocks were sectioned into 4-6 µm thickness, and were fished out from a water bath filled with DEPC (Cinnagen, Iran) treated water. For being firmly attached on slides, sections were incubated at 37°C and for overnight, instead of heating them on 60°C hot plate. On first day, the sections were deparaffinized using xylene for 10 times immersion and then 2 times of incubation each for 5 minutes, then slides were rehydrated with serial dilution of ethanol (100, 96 and 70%) each for 10 times immersion and then incubated for 5 minutes within each dilution. Finally slides were incubated with PBS (Invitrogen, USA) for 5 minutes.

Prehybridization treatment

Proteinase K (Fermentas, Lithuania) was diluted to 15 µg/mL concentration in proteinase K buffer (Tris-Cl 100 mM, EDTA 50 mM, NaCl 500 mM; PH=8) and slides were incubated with this solution for 20 minutes at 37°C in ThermoBrite hybridizer (Fisher Scientific, USA). After protein digestion, slides were washed with fresh PBS (prepared with DEPC treated water) for 5 minutes and then the tissues were fixed in increasing serial dilutions of ethanol (70, 96 and 100 %) each for 10 immerse and 1 minute of incubation. Slides were then air-dried under laminar hood.

Hybridization

Hybridization buffer (50% Formamide, 5X SSC (20x SSC preparation will be discussed further on), 0.1% Tween 20, 9.2 mM citric acid, 50 µg/mL heparin (Sigma, Germany; Cat # H3393), 500 µg/mL yeast RNA (Sigma, Germany; Cat # R6750)) was prepared freshly. 5' digoxigenin (DIG)-labeled miRCURY LNA microRNA detection probes (Exiqon, Denmark) were diluted to 50 nM in hybridization buffer and slides were incubated in 15 µl of the hybridization solution, covered with coverslips and sealed with fixogum (Germany). Slides were then incubated in ThermoBrite hybridizer for 3 minutes at 90°C to denature the dsRNAs, followed by 1 hour at hybridization temperature (Tm of the probe -21°C). Melting temperature for each LNA probe depends on the GC content of the oligonucleotides sequence and it is provided by the company in the probes' data sheet. Table 1 shows the probe sequences and hybridization temperatures for each miRNA and U6 snRNA.

Table 1. Probe sequences and hybridization temperatures for each gene.

Probe name	Sequence	Hybridization temperature
hsa-miR-302	5'- AAGCATGGAAGCACTTA-3'	47 ºC
hsa-miR-21	5'-TCAACATCAGTCTGATAAGCTA-3'	51 ºC
hsa/mmu/rno-U6 snRNA	5'-CACGAATTTGCGTGTCATCCTT-3'	55 ºC

Probes are labeled at 5' end and they have an extra nucleotide as 5'-overhang which facilitates the hapten (DIG) recognition by the antibody.

Stringency wash

Descending serial dilutions of standard sodium citrate buffer (SSC) (20x solution: NaCl 300 mM, Sodium Citrate 30 mM; PH=7) were prepared (5x, 1x, 0.2x) and stringency wash was performed for 5 minutes in each solution at hybridization temperature (47ºC for miR-302, 51ºC for miR-21 and 55ºC for U6) for each probe, followed by 5 minutes incubation in 0.2x SSC and 5 minutes in PBS, both at room temperature. Tissue sections are not allowed to dry out during this and subsequent stages.

Blocking and antibody treatment

Using PAP pen (Sigma, USA), a hydrophobic barrier was delineated around the tissue sections, then the slides were incubated with 2% sheep serum (Sigma, USA) in BSA/PBS-T (2 mg/mL) for 15 minutes at room temperature in humidifying chamber to block nonspecific binding of the first antibody. Then alkaline phosphatase (AP)-conjugated anti-DIG antibody (Roche, Germany) was diluted 1:800 in blocking buffer and covered the slides for overnight at 4 □C, in humidifying chamber.

Second day; Alkaline Phosphatase reaction

Slides were washed with PBS (3 times each for 5 minutes at room temperature) and incubated with Alkaline Phosphatase buffer (Tris-Cl 100 mM, $MgCl_2$ 50 mM, NaCl 100 mM, 0.1% Tween 20; PH= 9.5) for 3 times, each for 5 minutes at room temperature. Light-sensitive color reaction was performed with 4-nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolylphosphate (NBT/BCIP) ready-to-use tablets (Roche, Germany) for 3 hours at 30ºC in a humidified chamber. BCIP is a substrate for alkaline Phosphatase, and dephosphorylated BCIP itself can be oxidized with NBT. Both reduced NBT and oxidized BCIP generate a blue insoluble precipitant. Then slides were dehydrated in ascending serial dilutions of ethanol (70, 96 and 100%), before being counterstained with nuclear fast red (for miRNAs) or eosin (for U6 snRNA) for visualization of signals with light microscopy (Figure 1).

In situ Hybridization for NT2 cell culture

NT2 cells were cultured in DMEM (Dulbecco's Modified Eagle's Media) (Gibco, USA), supplemented with 10% FBS (Sigma, USA). At the confluency of 60-70%, cells were trypsinized and the pellet was resuspended in 1 mL of DMEM. After checking cells' morphology with microscope, various numbers of cells were attached on coated slides with Cytospin centrifuge (Hettich, Germany). Different speeds and spin times were tested, before reaching the optimized conditions. Slides were then fixed with freshly made 4% paraformaldehyde (1 g paraformaldehyde, 25 mL PBS; PH=7.4) for 10 minutes followed by 3 times washing with PBS, each for 2 minutes. Slides were again washed with PBS-Triton X100 (0.5%) for 10 minutes and then hybridization and other steps of the procedure were performed as described for FFPE tissue sections.

Results

Optimizing ISH conditions with U6 snRNA

Different Proteinase K treatments (10, 20 and 30 minutes) and different probe concentrations (30 nM and 60 nM) were employed to optimize ISH conditions for FFPE samples. Incubation with 15 µg/ml Proteinase K for 30 minutes and 60 nM probe concentration in hybridization step were found to be the optimized conditions for signal detection of U6. Hybridization temperature for U6 probe was 55ºC. Blue signals of U6 snRNA were detected in the nuclei of the cells. Similar conditions were found to be applicable for NT2 cell line (Figure 2). In each experiment, a negative control with no probe treatment was used to confirm the specificity of hybridization. These slides were treated only with hybridization buffer, and as expected generated no visible signal.

MiR-21 cytoplasmic signals were detected in FFPE samples

We used the same obtained optimum conditions of ISH to detect miR-21 in the cytoplasm of seminoma carcinoma. The hybridization temperature was 51ºC and stringency washes were also performed at the same temperature. The signal intensity of miR-21 was compared with the intensity of U6 snRNA signals (Figure 3). No signal was observed in negative controls which further proves the specificity of miR-21 signals.

MiR-302 is localized within the cytoplasm of NT2 cells

We selected NT2 cell line due to the reports on high expression of miR-302 cluster in these cells (Hohjoh and Fukushima, 2007; Lee et al., 2008). We initially used hematoxilin and eosine (H&E) staining to optimize NT2 cell attachment and fixation situations. We then used 9 x 10^3, 18 x 10^3 and 36 x 10^3 cells and 800, 1000 and 1400 rpm and

6 minutes of spin for cell attachment to the slides. Moreover, we checked methanol and paraformaldehyde fixation for the cells. Finally, 8 x 10^3 cells and 800 rpm for 6 minutes of spin were selected as the suitable situations for attachment of the cells. The hybridization temperature for miR-302 probe was 47ºC. Our ISH results showed a cytoplasmic localization of miR-302 in this cell line (Figure 4). In our negative controls which had no probe treatment, there was no blue signal that was observed for miR-302 (data not shown).

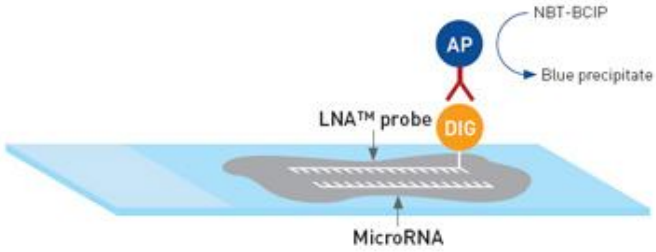

Figure 1. A schematic diagram of in situ hybridization procedure with LNA probe.

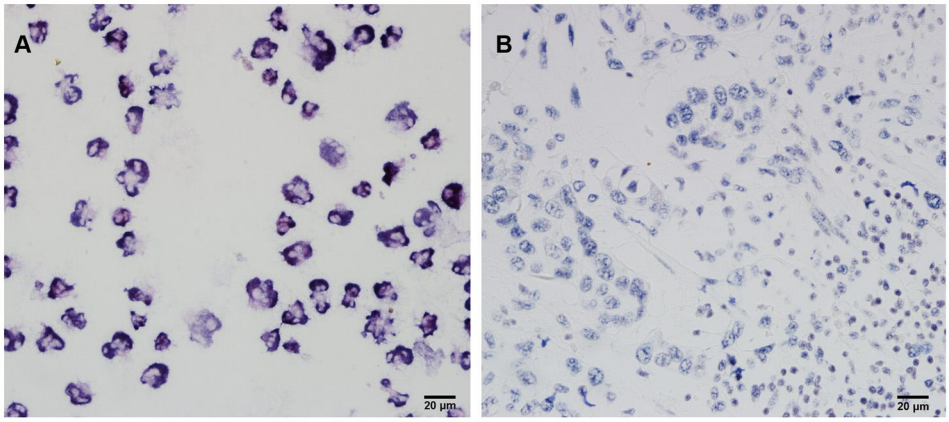

Figure 2. Nuclear signals of U6 snRNA; A) in NT2 cell line, B) in seminoma carcinoma FFPE tissue.

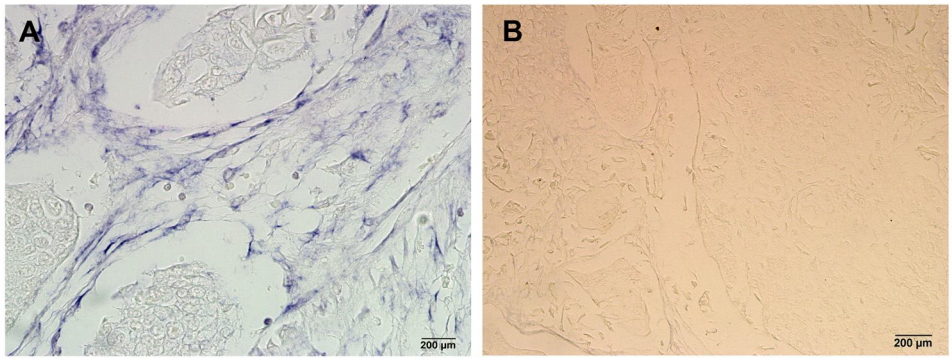

Figure 3. A) MiR-21 cytoplasmic signals in FFPE tissue of seminoma carcinoma, B) Negative control without probe treatment.

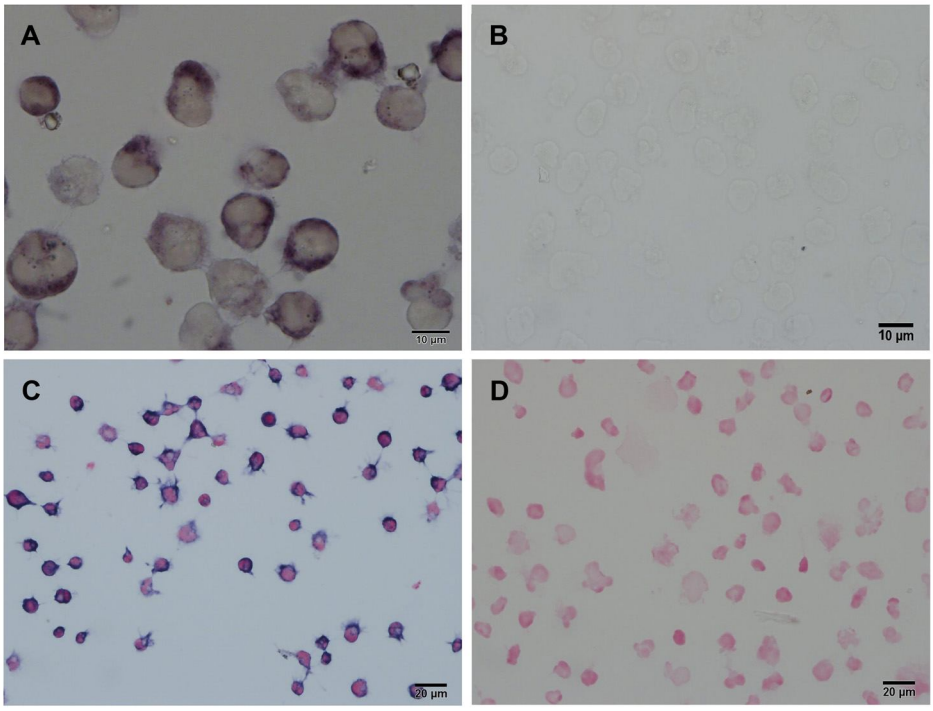

Figure 4. Cytoplasmic signals of miR-302, A) without counterstain, B) Negative control, C) slides were counterstained with nuclear fast red, blue signals show miR-302 localization, D) Negative control which is counterstained with nuclear fast red. There is no blue cytoplasmic signal in negative controls without probe treatment.

Discussion

ISH is a robust technique for visualization of nucleic acids at cellular and tissue levels. We were able to detect miR-21 sub-cellular localization in seminoma carcinoma tissues. MiR-21 is a well-known oncomir and its overexpression has been reported along with cancer cell proliferation, apoptosis, tumor invasion, and metastasis (Reis et al., 2012; Folini et al., 2010; Yan et al., 2011). The optimized procedure can be used for other FFPE samples with minor changes due to tissue specific characters.

Our study on NT2 cells showed cytoplasmic distribution of miR-302. Different studies have reported this miRNA to be localized both at nucleus and cytoplasm depending on its role and its targets, which points out to its complex level of function Liao et al., 2010; Jeffries et al., 2011). In this study, we detected both miRNAs in the cytoplasm of the studied cells or tissues and for each specimen different procedure is needed. During fixation and paraffinization of tumor tissues, some cross-links form between proteins and RNAs inside the cells. Due to their small size, miRNAs are totally covered with cellular proteins. So, we need Proteinase K digestion in order to strip the miRNA, but this step is not needed for miRNA detection in cell lines. Following the protein digestion, we used different procedures for miRNA fixation in different samples. The present protocol is a robust and practical procedure for miRNA detection which will provide more accurate data at the tissue level.

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References

1- Calin G.A., Dumitru C.D., Shimizu M., Bichi R., Zupo S., Noch E., Aldler H., Rattan S., Keating M., Rai K., Rassenti L., Kipps T., Negrini M., Bullrich F. and Croce C. M., (2002) Frequent deletions and down-regulation of microRNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. proceedings of the National Academy of Sciences of the USA 99: 15524-15529.

2- Schetter A. J., Leung S. Y., Sohn J. J., Zanetti K. A., Bowman E. D., Yanaihara N., Yuen S. T., Chan T. L., Kwong D. L., Au G. K., Liu C. G., Calin G. A., Croce C. M. and Harris C. C. (2008) MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. The Journal of American Medical Association 299: 425-436.

3. Spizzo R., Rushworth D., Guerrero M. and Calin G. A. (2009) RNA inhibition, microRNAs, and new therapeutic agents for cancer treatment. Clinical

Lymphoma and Myeloma 3: S313-318.
4. Iorio M. V., Ferracin M., Liu C. G., Veronese A., Spizzo R., Sabbioni S., Magri E., Pedriali M., Fabbri M., Campiglio M., Menard S., Palazzo J. P., Rosenberg A., Musiani P., Volinia S., Nenci I., Calin G. A., Querzoli P., Negrini M. and Croce C. M. (2005) MicroRNA gene expression deregulation in human breast cancer. Cancer Research 65: 7065-7070.
5. Carter B., Fletcher J. and Thompson R. (2010) Analysis of messenger RNA expression by in situ hybridization using RNA probes synthesized via in vitro transcription. Methods 52: 322-331.
6. Zitzelsberger H., Szucs S., Weier H., Lehmann L., Braselmann H., Enders S., Schilling A., Breul J., Hofler H. and Bauchinger M. (1994) Numerical abnormalities of chromosome 7 in human prostate cancer detected by fluorescence in situ hybridization (FISH) on paraffin-embedded tissue sections with centromere-specific DNA probes. Journal of Pathology 172: 325-335.
7. Jorgensen S., Baker A., Moller S. and Nielsen B. (2010) Robust one-day in situ hybridization protocol for detection of microRNAs in paraffin samples using LNA probes. Methods 52: 375-381.
8. Nuovo G. (2010 a) Introduction to the Methods issue on in situ hybridization. Methods 52: 269-270.
9. Vester B. and Wengel J. (2004) LNA (Locked Nucleic Acid): High-Affinity Targeting of Complementary RNA and DNA. Biochemistry 43: 13234-13241.
10. Silahtaroglu A., Pfundheller H., Koshkin A., Tommerup N. and Kauppinen S. (2004) LNA-modified oligonucleotides are highly efficient as FISH probes. Cytogenetic Genome Research 107: 32-37.
11. Silahtaroglu A., Nolting D., Dyrskjot L., Berezikov E., Moller M., Tommerup N. and Kauppinen S. (2007) Detection of microRNAs in frozen tissue sections by fluorescence in situ hybridization using locked nucleic acid probes and tyramide signal amplification. Nature Protocols 2: 2520 - 2528.
12. Nuovo G. (2010 b) In situ detection of microRNAs in paraffin embedded, formalin fixed tissues and the co-localization of their putative targets. Methods 52: 307-315.
13. Hohjoh H. and Fukushima T. (2007) Marked change in microRNA expression during neuronal differentiation of human teratocarcinoma NTera2D1 and mouse embryonal carcinoma P19 cells. Biochemical and Biophysical Research Communications 362: 360-367.
14. Lee N. K., JS, Cho W., Lee M., Steiner R., Gompers A., Ling D., Zhang J., Strom P., Behlke M., Moon S. H., Salvaterra P. M., Jove R. and Kim K. S. (2008) miR-302b maintains “stemness” of human embryonal carcinoma cells by post-transcriptional regulation of Cyclin D2 expression. Biochemical and Biophysical Research Communications 377: 434-440.
15. Reis S., Pontes-Junior J., Antunes A., Dall’Oglio M., Dip N., Passerotti C. C., Rossini G. A., Morais D. R., Nesrallah A. J., Piantino C., Srougi M. and Leite K. R. (2012) miR-21 may acts as an oncomir by targeting RECK, a matrix metalloproteinase regulator, in prostate cancer. BioMed Central Urology 12:14.
16. Folini M., Gandellini P., Longoni N., Profumo V., Callari M., Pennati M., Colecchia M., Supino R., Veneroni S., Salvioni R., Valdagni R., Daidone M. G. and Zaffaroni N. (2010) miR-21: an oncomir on strike in prostate cancer. Molecular Cancer 9: 12.
17. Yan L., Wu Q., Zhang Y., Li Y., Liao D., Hou J. H., Fu J., Zeng M. S., Yun J. P., Wu Q. L., Zeng Y. X. and Shao J. Y. (2011) Knockdown of miR-21 in human breast cancer cell lines inhibits proliferation, in vitro migration and in vivo tumor growth. Breast Cancer Research 13: R2.
18. Liao J., Ma L., Guo Y., Zhang Y., Zhou H., Shao P., Chen Y. Q. and Qu L. H. (2010) Deep sequencing of human nuclear and cytoplasmic small RNAs reveals an unexpectedly complex subcellular distribution of miRNAs and tRNA 3′ trailers. Public Library of Science One 5: e10563.
19. Jeffries C., Fried H. and Perkins D. (2011) Nuclear and cytoplasmic localization of neural stem cell microRNAs. RNA 17: 675-686.