NANODIAMONDS AS AN INNOVATIVE SYSTEM FOR INTRACELLULAR DELIVERY OF miRNA-34a IN PROSTATIC CANCER THERAPY

BITTI Giuseppe[1,2], ABATE Marianna[2,4], NEUHÖFEROVÁ Eva[3], KINDERMANN Marek[3], PETRÁKOVÁ Vladimíra[3], BOCCELLINO Mariarosaria[2], QUAGLIUOLO Lucio[2], FILOVA Eva[4], BENSON Veronika[3], CARAGLIA Michele[2] , AMLER Evzen[4]

[1]*Charles University, Second Faculty of Medicine, Prague, Czech Republic, EU*

[2]*University of Campania "Luigi Vanvitelli", Department of Biochemistry, Biophysics and General Patology, Napoli, Italy, EU*

[3]*Institute of Microbiology, Czech Academy of Sciences, Prague, Czech Republic, EU*

[4]*Institute of Experimental Medicine, AS CR,Laboratory of Tissue Engineering, Prague, Czech Republic, EU*

Abstract

The microRNA(miRNA)-34a is an important regulator of tumor suppression. It controls the expression of several target proteins involved in cell cycle, differentiation and apoptosis, and antagonizes processes that are necessary for basic cancer cell viability as well as cancer stemness, metastasis, and chemoresistance. It is downregulated in numerous cancer types, including prostatic cancer, and inhibits malignant growth by repressing genes involved in various oncogenic signaling pathways. Given the anti-oncogenic activity of miR-34a, here we proved the substantial benefits of a new therapeutic concept based on nanotechnology delivery of miRNA mimics. In order to monitor the miRNA-34a replacement, we used a fluorescent nanodiamond particles (FND) system with linked miRNA-34a mimic, which was delivered to PC3 and DU145 prostatic cancer cell lines. We used functionalized nanodiamonds coated with polyethylenimine to transfer miRNA-34a into PC3 and DU145 prostatic cancer cell lines and we measured the zeta-potential of these complexes before using them for *in vitro* experiments. A replacement of miRNA-34 was observed by monitoring levels of miRNA-34 via real-time PCR. Moreover, our *in vitro* experiments demonstrated that miRNA-34a replacement, using this FND delivery system, decreased viability and induced apoptosis in prostatic cancer cell lines. Our findings suggest the replacement of oncosuppressor miRNA-34a provides an effective strategy for cancer therapy and the FND-based delivery systems seems to be an excellent strategy for a safe and effective targeting of the tumor.

Keywords: miR-34a, nanodiamonds, delivery, prostate cancer, apoptosis

1. INTRODUCTION

1.1. The microRNA-34a

MicroRNAs are a class of short, single non-coding RNAs (19-22 nt), that have been identified in many eukaryotic organisms. They can prevent protein expression through cleavage of specific target mRNAs or through inhibition of their translation and may conceivably play a key role in tumorigenesis [1]. Some of miRNA molecules are associated with tumor proliferation and their level is increased inside cancer cells in comparison to healthy cells. Those miRNAs which contribute to carcinogenesis by inhibiting tumor suppressor genes, are considered oncogenic miRNAs (oncomiRs), while downregulated miRNAs, that normally prevent cancer development by inhibiting the expression of proto-oncogenes, are known as tumor suppressor miRNAs [2]. We focused on miRNA-34a, for which it was proved a decreased level in samples from patients with prostate cancer [3]. MiR-34a may exert its tumor suppressor role via targeting several signaling molecules involved in

various stages of prostate cancer progression. DNA damage and oncogenic stress strongly induced miR-34 expression in a p53-dependent pathway. The distinctive features of p53 activity are promoted by miR-34 activation: induction of cell cycle block and apoptosis through down-regulation of different proteins [4]. Given the miR-34 family role as tumor suppressor in cancer, we tried to increase the levels of miR-34a in prostate cancer cell lines through replacement with miRNA mimics transfected by *in vitro* experiments using nanodiamonds as a delivery system.

1.2. Nanodiamonds

The possible use of nanoparticles for diagnostic and therapeutic applications has always been very appealing. The increased interest about their usage with the increased presence of nanomaterials in commercial product has raised concerns about its potential environmental pollution and toxicity effects [5]. Nanodiamonds (NDs) are carbon based nanostructures with a nanoscale size (tens to hundreds of nanometers). Given the small size of the individual nanodiamond particles, the ratio of their surface to their volume is much greater than within the large diamonds. That means that they can be abundantly coated with numerous functional molecules. Their size is also suitable for transportation of molecules into the cell cytoplasma. Moreover, biocompatibilty of nanodiamonds has been studied under various conditions and so far nanodiamonds are considered to be non-toxic and highly biocompatible, even though no rigorous clinical trials have been done yet and the questions of expulsion of nanodiamonds or their possible accumulation in organs remain to be answered. The use of so-called high-pressure high-temperature (HPHT) nanodiamonds has opened a possibility to track the transfection directly using engineered fluorescent nitrogen-vacancy (NV) centers. NV centers in nanodiamonds are widely used for fluorescence-based detection techniques because they provide photostable luminescnece with emission near-infrared spectra and lack any photoblinking [6]. These properties have been utilized in a vast range of applications, from physics to biological applications such as single particle tracking inside cells or long-term *in vivo* particle tracking [7]. Finally, the HPHT fluorescent nanodiamonds surface is negatively charged upon oxidation, and strongly binds the prototypical transfection reagent polyethylenimine (PEI) for delivery of biomolecules as miRNAs.

2. METHODS

PC3 and DU145 prostate cancer cell lines were maintained in RPMI1640 and DMEM media respectively supplemented with 10 % of fetal bovine serum and 1 % of antibiotics. MicroRNA-34a mimic and a scrambled microRNA (scr) as a negative control were delivered into the cells using NDs-PEI and RNAiMAx lipofectamine as a standard transfection reagent (Tr.re.) in order to compare two different types of transfection methods. Prior to use, the nanodiamonds were dissolved in water and sonicated with a probe for 30 min. The resulting transparent colloid was filtered using a 0.2 µm PVDF microfilter. Nanodiamonds diluted in water were coated with 0.09 mg/mL of polyethylenimine (PEI 800) adding the same volume of both solutions. The mix was vortexed overnight, after which the solution was centrifuged in 9000 g for 30 min to create a ND-PEI pellet. This pellet was diluted in RNAse/DNAse free water to reach a final concentration of 1 mg/µL. To link microRNA we mixed 12.5 µL of 1 mg/µL ND-PEI with 5 µL of miRNA-34a and scrambled miRNA. In order to evaluate the electrokinetic surface potentialpure NDs, ND-PEi and ND-PEI-miRNA34a, zeta potential measurements were recorded with a Zetasizer NANO ZS system (Malvern Instruments). Cells were maintained *in vitro* for all the experiments. Cells were seeded in 96-well plates in order to evaluate cytotoxicity using LDH assay after a 48 hrs incubation after treatment and cell proliferation was evaluated using WST-1 assay after 48 and 72 hrs incubations. Cells were seeded in 12-well plates and after 48 and 72 hrs incubations total RNA was isolated. Those RNA samples were used to perform real-time PCR in order to evaluate the expressions levels or miRNA-34 and RNU44 as a control housekeeping gene. Finally, cells were seeded in 12-well plates and after 48 and 72 hrs incubations they were tested to perform flow cytometry analyses in order to evaluate the apoptotic and necrotic populations using an Annexin V-Propidium iodide double staining assay. Double negative cells were

viable, propidium iodide positive cells were necrotic, Annexine V positive cells were in early apoptosis and double positive cells were in late apoptosis.

3. RESULTS AND DISCUSSION

3.1. Zeta-potential

In order to demonstrate the charge changes on NDs particles, we measured the zeta-potentials of the individual complexes. The negative zeta potential of uncoated NDs (−27.9 mV) became positive after coating with PEI (+49.3 mV). Binding of microRNA-34a led to restoration of negative zeta potential (−24.3 mV). These results prove our assumption that the PEI positive charge is compensated by microRNA binding.

3.2. Evaluation of NDs cytotoxicity

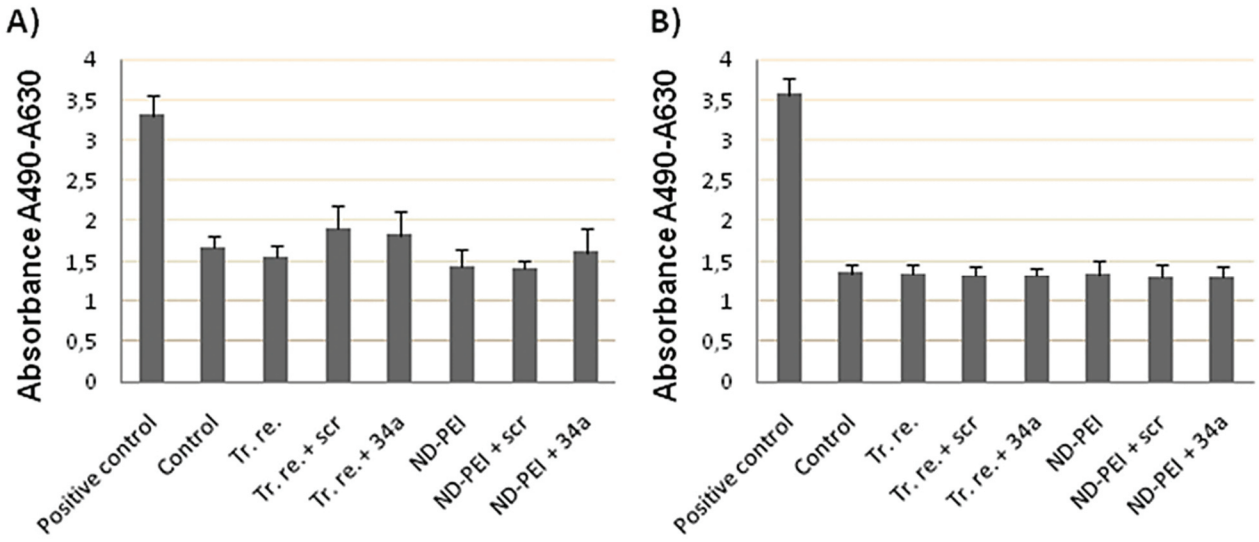

Figure 1 Cytotoxicity analyses in PC3 cell line (A) and DU145 cell line (B)

In order to evaluate the possible cytotoxic effect of NDs, we treated PC3 and Du145 cell lines with miRNA-34a and scramble miRNA using ND-PEI complexes and RNAiMax (Tr.re.) as transfection methods and, after 48 hrs incubation, LDH assays were performed. Control samples were obtained from non-treated cells, and positive control samples were obtained treating cells with lysis solution at the end of incubation period. We observed no significant cytotoxic effects in samples treated with NDs in both prostate cancer cell lines. Moreover, ND-PEI samples results were similar to non-treated samples and to samples treated with the standard transfection reagent (**Figure1**).

3.3. Evaluation of cell proliferation

WST-1 assay was performed in order to evaluate cell proliferation in PC3 and DU145 treated with miRNA-34a and scramble miRNA transfected using ND-PEI complexes and standard transfection reagent. Absorbance measurements were recorded 48 and 72 hrs incubations of treatment and control samples were obtained from non-treated cells. We evaluated percentage of cell proliferation normalizing the absorbance results obtained from each samples with control samples (100 %). We observed a decrease of cell proliferation in both cell lines in samples treated with ND-PEI-miRNA34a with results similar to samples treated with the standard transfection reagent. The most significant difference was in PC3 cell line after 72 h incubation (Tr.re. + miRNA-34a = 71 %, ND-PEI-miRNA34a = 66 %) (**Figure 2**).

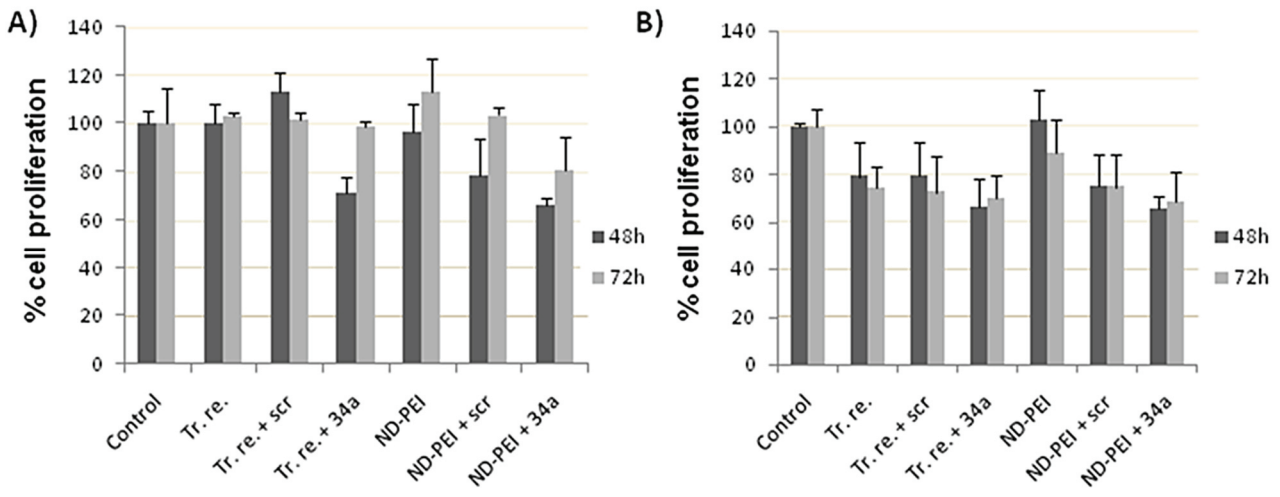

Figure 2 Cell proliferation analysis in PC3 cell line (A) and DU145 (B) cell line

3.4. Detection of miRNA-34a expression

After 48 and 72 hrs incubations after treatment with miRNA-34a and scramble miRNA transfected with both transfection methods, we isolated total RNA and subsequently we performed Real-time PCR in order to evaluate gene expressions of miRNA-34a. We observed a significant increase of miRNA-34a expression levels in the samples treated with ND-PEI complexes with values higher than the values from samples treated with the standard transfection reagent. In DU245 cell line, the replacement of miRNA-43 levels was more successful after 48 hrs of incubation. Results were normalized using RNU44 as a control housekeeping gene (**Figure 3**).

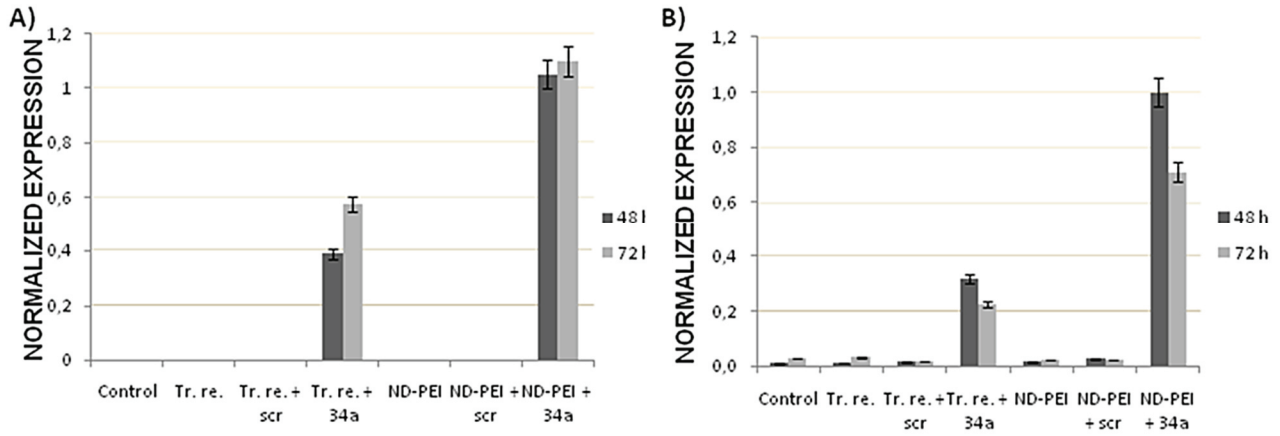

Figure 3 Real-Time PCR analysis od miRNA-34 expression in PC3 cell line (A) and DU145 cell line (B)

3.5. Apoptosis analysis

Flow cytometry analysis was performed to evaluate apoptosis in PC3 and DU145 cell lines after 48 and 72 hrs incubations from the treatment with miRNA-34a and scramble miRNA transfected with ND-PEI and the standard transfection reagent. We used Annexin V-Propidium Iodide double staining method in order to separate viable cells, necrotic cells and apoptotic cells. In PC3 cell line we observed a higher increase (49 %) of early and late apoptotic populations in the sample treated with ND-PEI-miRNA34a after 48 and 72 hrs incubations, while the samples treated with the standard transfection reagent showed a slight increment

only in the late apoptotic population (48 hrs =36 %, 72 hrs = 26 %). However, in the DU145 cell line we observed only a slight increase (30 %) of early apoptotic population in ND-PEI samples compared to Tr.re. samples (13 %) after 48 hrs incubation (**Table 1**, **Figure 4**).

Table 1 Percentages of viable (V) and apoptotic (A, early and late) population cell populations

%	Control		Tr. re.		Tr. re.+ scramble		Tr. re. + miRNA34a		ND-PEI		ND-PEI + scramble		ND-PEI + miRNA34a	
Population	V	A	V	A	V	A	V	A	V	A	V	A	V	A
PC3 48 h	83	10	51	23	54	19	51	36	51	20	55	23	30	49
PC3 72 h	84	8	53	31	62	30	66	26	58	19	64	12	37	49
DU145 48 h	84	5	68	16	71	10	63	13	68	5	64	5	42	30
DU145 72 h	74	11	60	27	57	12	63	25	62	10	65	10	55	25

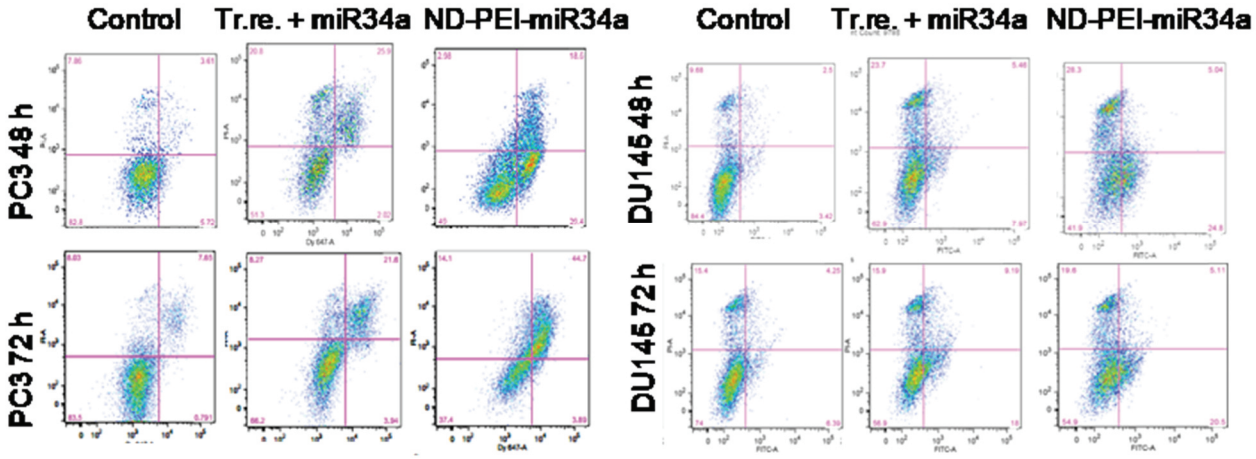

Figure 4 Apoptosis analysis in PC3 and DU145 cell lines.
Abscissa = Annexin V and ordinate = Propidium Iodide.

4. CONCLUSION

Our scientific goal was to compare the transfection efficiency between a nanodiamonds-based system and standard transfection reagent in order to delivery miRNA-34a, a well known tumor suppressor miRNA, into prostate cancer cell lines. We prepared nanodiamonds coated with PEI in order to make positive charged complexes able to bind miRNA-34a mimic. The success of the PEI coating and the miRNA binding was proved measuring zeta-potential of each complexes. We performed *in vitro* experiments treating PC3 and DU145 prostate cancer cell lines with ND-PEI complexes and a lipofectamine transfection reagent in order to transfect miRNA-34 mimic. LDH assay was performed and no significant cytotoxicity was showed in the samples treated with ND-PEI-miRNA34a. Moreover, using WST-1 assay, we observed a significant decrease in cell proliferation in samples treated with ND-PEI-miRNA34a, especially in PC3 cell line and after 72 h incubation. However using Real-time PCR, we observed in both cell lines a very significant increase of miRNA-34a levels in samples treated with ND-PEI-miRNA34, demonstrating a better replacement of miRNA-34a levels when transfected with nanodiamonds. Because it is known that a replacement of miRNA-34a can induce apoptosis, we performed a flow cytometry analysis. In samples treated with ND-PEI-miRNA34a we found a significant increase of the apoptotic cell population in PC3 cell line after 48 and 72 h incubatio and a slight increase of apoptotic cell population in DU145 cell line after 48 h incubation, Our preliminary results suggest that the ND-based delivery systems seems to be a promising strategy to deliver miRNA-34a into prostate cancer in order to achieve a safe and effective targeting of the tumor cells regulation.

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