Manuscript Title: Differentiation induction and proliferation inhibition by a cell-free approach for delivery of exogenous miRNAs to neuroblastoma cells by mesenchymal stem cells

Samaneh Sharif, Ph.D\textsuperscript{1*}; Mohammad Hossein Ghahremani, Ph.D\textsuperscript{1,2†} and Masoud Soleimani, Ph.D\textsuperscript{3†}.

\textsuperscript{1}Department of Molecular Medicine, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran. Email: sharif_s@razi.tums.ac.ir

\textsuperscript{2}Department of Toxicology & Pharmacology, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran. Email: mhghahremani@tums.ac.ir

\textsuperscript{3}Department of Haematology, Tarbiat Modares University, Tehran, Iran. Email: soleim_m@modares.ac.ir

Running Title: Cell-free delivery of miRNAs to neuroblastoma cells

\textsuperscript{*}Corresponding author email: sharif_s@razi.tums.ac.ir, Tel: 982143052000

\textsuperscript{†}These two authors contributed equally in this study.
Abstract

**Objective:** Neuroblastoma (NB) is one of the frequently observed malignant solid tumors of childhood and infancy, accounting for 15% of pediatric cancer deaths. Recently, the approach of differentiation therapy has shown considerable promise in effective treatment of NB patients.

MiR-124 is a kind of nervous system-specific miRNA that increases during neuronal differentiation and may be one of the potential therapeutic targets in the treatment of NB. However, despite its well-established therapeutic potential, its efficient delivery to the targeted tumor cells is a challenging task. Mesenchymal stem cells (MSCs) are multipotent adult progenitor cells that are also widely indicated to have antitumor properties and the capability of migrating toward cancer cells and tumors. The objective of this study was to show that human adipocyte-MSCs (hAD-MSCs) have the potential of delivering exogenous miRNAs to NB cells to induce differentiation and decrease proliferation of these cells.

**Material and methods:** In this experimental study, hAD-MSCs are isolated, cultured and differentiated. M17 human NB cells are also cultured. A specific kind of miRNA, i.e. miR-124, is delivered successfully with hAD-MSCs to M17 NB cells by means of direct or indirect (exosome-based) contacts.

**Result:** It is shown that the indirectly delivered miR-124 considerably decreases the proliferation of NB cells and induces their differentiation.

**Conclusion:** The results offer the opportunity to use the delivered exogenous miRNAs by the derived exosomes from hAD-MSCs as a novel cell-free stem cell-based therapy for NB cancer.

**Keywords:** Neuroblastoma; MiR-124; Exosome; Mesenchymal stem cells; Differentiation; Proliferation.
Introduction

Neuroblastoma (NB) is one of the frequently observed malignant solid tumors in children that accounts for nearly 7% of childhood cancers and more than 15% of pediatric cancer deaths (1). The most commonly used treatments for metastatic NB are powerful chemotherapy or intensive chemotherapy with autologous hematopoietic rescue after excision of the initial tumor (2). Despite the great advances of multimodal treatments of NB, its prognosis in metastatic cases is still relatively poor (3). As it is widely accepted, NB is caused by imperfect differentiation of neural crest cell precursors of the sympathetic nervous system (4). Therefore, the approach of differentiation therapy can be the most appropriate and effective treatment method for NB. The ability of undifferentiated fatal cells in differentiating to mature cells results in arrest of cell growth and apoptosis (5). 13-cis-retinoic acid (RA), as differentiation agent, is one of the standard mainstays of therapy in high risk NB patients (6). This type of treatment results in a significant increase in patient survival. However, at the same time, more than half of the treated patients develop recurrence. It must also be noted that RA therapy may result in the adverse effects of cell toxicity and inflammation. Therefore, development of novel therapy for NB is an urgent issue.

MicroRNAs (miRNAs) are noncoding RNAs that play important roles in the coordinated regulation of gene sets (7). Indeed, studies of the regulatory mechanism of miRNAs has attracted much attention in recent years. They are able to regulate different cellular processes like proliferation, differentiation, apoptosis, invasion and angiogenesis (8). When miRNAs are expressed in specific tissue types, they can effectively contribute to differentiation. MiR-124 is a kind of nervous system-specific miRNA that regulates neurite outgrowth during neuronal differentiation (9). Levels of miR-124 increase during neuronal differentiation and it plays an outstanding role in development of neurons (10). Its overexpression in human glioblastoma multiforme cells induces neuronal phenotype (11). It is also shown that overexpressing miR-124 in stem cells results in terminal neuronal differentiation with reduced malignancy (12). One must note that despite the well-established therapeutic potential of miR-124, its correct delivery to the targeted tumor cells is a challenging task.

In recent years, studies suggested that mesenchymal stem cells (MSCs) have tropism to tumor sites and produce antitumor effects (13). They can also act as delivery vehicles for therapeutic miRNAs to transfer them to the region of cancer cells (14). In vivo experiments indicated that
MSCs promote NB differentiation and suppress tumor proliferation (15). As we reported elsewhere (8), Wharton's jelly-MSCs have the capability to deliver miR-124 to glioblastoma multiforme cells and decrease cell migration and proliferation and confer chemosensitivity. Based on these studies, it can be expected that use of MSCs for transferring miR-124 to cancerous cells may also be a potential and promising candidate for NB treatment.

In this study, we report on transferring miR-124 to the NB cells by the MSCs. It is shown that human adipocyte MSCs (hAD-MSCs) deliver miR-124 to NB cells and this delivery regulates gene targeting and changes their function. It is demonstrated that the indirectly delivered miR-124 by the secreted exosomes from hAD-MSCs significantly decreases the proliferation of NB cells and induces their differentiation.

Materials and methods
In this section, preparation of the cell samples and the methods used for characterizations are fully described.

Isolation, culture and differentiations of hAD-MSCs
HAD-MSCs were obtained from healthy donors undergoing esthetic surgery. To isolate the hAD-MSCs from the tissue, an isolation method that is fully described in a previous report (16) was used. Digestion of tissue was performed at 37 °C with 1 mg/ml collagenase type I (Gibco). After centrifugation of the suspension, hAD-MSCs were cultured in supplemented DMEM by 10% FBS, 100 g/ml streptomycin (Invitrogen), 100 U/ml penicillin (Invitrogen), and 2 mM L-glutamine (Invitrogen). Replacement of the medium of the cultured cells and remove of the non-adherent cells were done after 48 h. After 3 weeks, detachment of hAD-MSCs was performed when the cells were reached at 70–80% confluences. hAD-MSCs were characterized by examining the positive expression of CD73, CD105 and CD90 (Abcamb) markers and the negative expression of hematopoietic stem cell markers HLA-DR (Abcam), CD34 and CD45 (PE, eBiosciences). In order to study the multipotential differentiation of cells, special media were used to induce hAD-MSCs to differentiate into osteocytes and adipocytes. By addition of dexamethasone 10 M and insulin (6 ng/ml), adipogenic differentiation was induced in the plated cells. Similarly, for osteogenic differentiation, ascorbic acid (50 µg/ml), dexamethasone 10M (Sigma Aldrich) and sodium β-glycerophosphate (10 mM) were used. After 3 weeks, the plates
were washed and the cells were stained with Alizarin Red (17) and Oil Red O (18) to confirm their osteogenic and adipogenic differentiation, respectively.

Culture of M17 cell

In this study, the cloned M17 human NB cell lines from SK-N-Be (2) NB cell line were used (ATCC manassas, VA). HEK T293 and human M17 NB cell lines were purchased from Pasteur Institute of Iran.

The M17 Cells were cultured in plastic flasks using as culture medium a 50:50 mixture of DMEM (GibcoBRL) and F12 mediums supplemented (to final concentration) with 10% Fetal Bovine Serum (FBS, Gibco), 100 U/mL penicillin, 0.1 mg/mL streptomycin, 2 mM L-glutamine and 1% nonessential amino acids (Invitrogen) (19).

miR-124 Transfection

Cy3 (Life Technologies, Invitrogen) was used for labelling RNA duplexes corresponding to has-miR-124. Transfections of 3–4 passaged hAD-MSCs were performed using the Lipofectamine 3000 kit (LifeTechnologies, Inc.) according to the manufacturer's instructions (20).

Preparation of Exosomes

Culture of the transfected hAD-MSCs with miR-124-Cy3 and control miR was done in MSC medium using Gibco™ Exosome-Depleted FBS (Thermo Fisher Scientific). After incubating for four days, isolation of the exosomes from the supernatants of the hAD-MSC cultures was performed using the exosome precipitation solution, ExoQuick (System Biosciences). The protein content was evaluated by the Micro BCA assay kit (Sigma-Aldrich) (21).

Co-culturing hAD-MSCs with M17 NB Cells

Following the manufacturer’s protocol, the M17 NB cells were labelled with green fluorescence CMFDA Cell Tracker (Molecular Probes). After labelling the cells, they were mixed at a ratio of 50:100 by the transfected hAD-MSCs by miR-124 and plated in 8-well plates. After 72 h, flow cytometric analysis was performed for confirming the delivery of miR-124 to M17 cells.

To check the delivery of miR-124 mimic with indirect contact (i.e. via the secreted exosomes from hAD-MSCs), membranes with 0.4 μm-pore diameter were used in a transwell chamber to inhibit cell infiltration (hAD-MSCs-miR-124-Cy3 and M17 cells). Plating of the transfected
hAD-MSCs with miR-124-Cy3 was carried out in the transwell inserts. At the same time, seeding of the M17 NB cells was performed in the lower well. 72 h later, collection of the M17 NB cells was done and flow cytometric analysis was used to ensure that miR-124-Cy3 was delivered to M17 cells.

**Quantitative real-time PCR**

QIAzol reagent (Qiagen) was used for extraction of the total RNA. Synthesis of complementary DNA (cDNA) was also done by reverse transcriptase (Fermentas) and random hexamers for gene primers. Triplicate real-time PCR analysis with SYBR Premix Dimer EraserTM (TaKaRa) was used and the results were analyzed using the REST and the Rotor-Gene 6.1 (Corbett) softwares. The PCR primers and their respective reverse complements were as follows: h-Tubullin beta III forward GGA GTA TTT GGA TGA CAG AAA C; reverse GAT TAC CAC TGG AGT CTT C (product length:238 bps), MAP2 forward AGT TCC AGC AGC GTG ATG (product length:164 bps); reverse TAGTCTAAGCTTAGC TGAGAATCTACCGA, GAPDH forward GAC AAG CTT CCC GTT CTC AG; reverse GAG TCA ACG GAT CTG GTG (product length:132 bps). GAPDH mRNA was used as an internal control. The real-time PCR protocol was as follows: 2 min at 95°C, 5 sec at 95°C for denaturation, 30 sec at 60°C for annealing, 10 sec at 72°C for amplification, and 40 cycles of extension (22).

**Assessing viability of cells and Apoptosis Assay**

In order to investigate the cells' viability, M17 cells were seeded at a density of 5 × 103 in 96-well plates and incubated for one night while keeping the temperature constant at 37°C. Addition of the derived exosomes from control-miR and hAD-MSC-miR-124-Cy3 to the M17 cells was done after 24 h. Flow cytometric analysis was used to detect the delivery of the exosomes that contained miR-124. When it is ensured that the delivery of miR-124 to the M17 cells is made, viability of the treated M17 cells with miR-124 and control miR was examined by MTT assay 24, 48 and 72 h after incubating with miR-124. Apoptosis induction in the treated M17 cells with transferred miR-124 and control miR was measured using the purchased TUNEL kits from the Roche Pharmaceutical Company. The cultivated M17 NB cells with the secreted exosomes from hAD-MSCs-Con-miR (M17-hAD-MSCs-Con-miR) were treated with derived exosomes from hAD-MSCs-miR-124 (M17-hAD-MSCs-miR-124). The cultivated M17 NB cells were also directly transfected with miR-124 (M17-miR-124) and its control (M17-con-mir).
In this study, all the collected data were analyzed by statistical package for social sciences (SPSS software).

Results

As can be verified in Figs. 1a-1c, flow cytometric analyses indicated consistent expression of CD73 and CD90 and CD105 in the hAD-MSCs. Furthermore, the cells were negative for HLA-DR, CD34 and CD45 (see Figs. 1d-1f). The multipotency of the hAD-MSCs was also confirmed by adipogenic and osteogenic differentiation (see Figs. 1g and 1h). Transfection efficiency was evaluated about 80% by Fluorescence microscopy (see Figs. 2a and 2b). In the following sections, the results that confirm the delivery of hAD-MSCs to M17 NB cells and their subsequent effects in inducing apoptosis and neuronal differentiation in NB cells are presented.

Confirming the delivery of miR-124 mimic to M17 NB cells

According to previous reports (23-25), MSCs have the ability of cell-to-cell communicating via gap junctional intercellular channels (direct contact) or by secreting different factors like cytokines, vesicles and extracellular matrix molecules (indirect contact) that promote neurogenesis. Furthermore, MSCs can also be genetically modified to be able to release specific growth factors, cytokines and miRNAs in the form of exosomes (26, 27). In this paper, the potential of hAD-MSCs for delivering exogenous miRNA mimics to M17 NB cells was examined. Specifically, the focus of this study was on miR-124 delivery because this miRNA has already been reported to have a major role in differentiation of NB cells (12). To this end, after 2 days of co-culturing the hAD-MSCs-miR-124 with the M17 NB cells (direct contact), the combination was studied with the aid of two channel flow cytometry technique. Confirmation of direct miR-124 transfer from hAD-MSCs-miR-124 to M17 NB cells was obtained by detection of Cy3 in M17 NB cells (see Fig. 2c). Moreover, in transwell-cultured hAD-MSCs, miR-124-Cy3 detection indicated that M17 cells were Cy3- positive and hence, miR-124-Cy3 was indirectly transferred from hAD-MSCs to M17 NB cells (see Fig. 2d). Figure 2c confirms that miR-124 was indirectly transferred with the derived exosomes from hAD-MSCs to M17 NB cells. The shown two-colour flow cytometry dot plots in Fig. 2 represent the percentage of the co- and the transwell-cultured cells.
Concomitant proliferation decrease and apoptosis induction in M17 NB cells through delivery of miR-124 by hAD-MSCs

To determine whether transferring miR-124 to M17 NB cells by hAD-MSCs may have an effect on proliferation in addition to induction of apoptosis, the derived exosomes from hAD-MSC-miR-124 cells were used for treating the M17 cells. The result of the MTT assay showed that the delivery of miR-124 reduced the proliferation of the M17 cells (see Fig. 3a). In order to further check whether the delivery of miR-124 can induce apoptosis in M17 NB cells, the TUNEL test was carried out. As shown in Fig. 3b, the results of the TUNEL assay approved that miR-124 induced apoptosis in M17 NB cells.

MiR-124 mimic delivery by hAD-MSCs stimulates the neuronal differentiation of M17 NB cells

As it is already mentioned in the previous sections, the sole delivery of miR-124 to NB cells without any intermediate element has been reported to induce differentiation in the cells (12, 28). The goal of this study was to examine the role of MSCs as intermediate carriers to facilitate the delivery of miR-124 to M17 NB cells and inducing further differentiation. In order to investigate this, the M17 cells were treated with the exosomes derived from hAD-MSCs-miR-124 and showed that the expression of β-tubulin III and MAP2 significantly enhanced in comparison with the control (see Fig. 4). This results confirm that miR-124 delivery induced differentiation of M17 NB cells. In addition, as can be verified in Fig. 3, the treated M17 cells with the exosomes derived from hAD-MSCs-Con-miR also induced cell differentiation. In the same manner, in the treated M17 cells with the exosomes secreted from hAD-MSCs-miR-124, the induction of differentiation was rather spectacular. The increased differentiation in this case can be ascribed to the joint action of neurotropic factors secreted from both hAD-MSCs and miR-124. Furthermore, one must also note that compared with the undifferentiated cells, the differentiated M17 cells exhibited a more neuron-like shape. This is consistent with previous findings that miR-124 overexpression noticeably induces differentiation of NB cancer cells (12, 28).

Discussion

Recently, miRNAs have been appeared as the main potential therapeutic targets in cancers (29) and expression alternation of miRNAs in different neurological disorders have been the subject of several studies (30-32). Indeed, it is already shown that administration of miR-based therapy
will provide therapeutic approaches in pathological conditions of the central nervous system cancers (33). However, despite their therapeutic potential, the existing problems in the way of controlled delivery of therapeutic agents to targeted neural cells are mostly considered as the major reasons for the poor outcomes of treatment.

In regenerative medicine, MSCs are known to be hopeful sources for cell therapy since they have immunomodulation, trophic factor secretion and transdifferentiation properties (34). Furthermore, the secreted exosomes from MSCs that assist in restraining tissue injuries, lead to re-entry of cell cycle in resident cells and induce tissue self-repair, are being examined for various applications in neural, musculoskeletal or cardiac repair (35). In recent years, various studies have indicated that MSCs can be effectively used for treating different disorders in the central nervous system, including Parkinson’s disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS) and stroke (36, 37). MSCs show tropism to malignant cells, migrate to tumor microenvironments, exert antitumor effects and also have this ability to act as delivery vectors for anticancer agents (38, 39). MSCs and neural stem cells are also considered as promising candidates for overcoming the blood-brain barrier by delivering drugs and RNAs to tumors or neurodegenerative disorders (40).

In this study, the ability of hAD-MSCs to act as delivery vectors for transferring miR-124 to cocultured NB cancer cells is demonstrated and it is proposed as a promising approach for the targeted delivery of miRNA-based therapy to NB cancer cells. Previously, Bianchi et al. reported that functional cross-talks among hMSCs and NB cell lines can be effective only within short range interaction and showed that intravenously inoculated hMSCs in different NB models did not reach the tumor sites (15). Nevertheless, they also showed that intratumorally injected hMSCs in a subcutaneous NB model decreased tumor growth and enhanced the survival time. On the contrary, Kimura et al. have reported that intraperitoneally administered hMSCs can migrate and affect the tumor of TH-MYCIN mouse model (41). In a recent clinical study (42), efficiency of treatments of bone marrow metastasis in NB by infected autologous MSCs with ICOVIR-5 is also demonstrated. Excellent treatment tolerance and full clinical response is reported for this new type of treatment (42). It is also shown in our previous work (8) that Wharton’s jelly-MSCs deliver exogenous miRs to glioblastoma multiform cells and its functional effects is fully studied. It is shown that the labeled miR-124 can be delivered effectively with Wharton’s jelly-MSCs to U87 glioblastoma multiform cells through exosome-
dependent or independent manners. Consistent with these reports, in this study, it is also found that hAD-MSCs can successfully deliver miR-124 to the co-cultured M17 NB cells by localizing the Cy3-labeled miR-124. Furthermore, it is found that miR-124 delivery by the secreted exosomes from hAD-MSCs to M17 NB cells defines a new and efficient cell-free method for delivery of miRNAs to target cells.

As it is already well-known, miR-124 is a neuronal specific miRNA that has a great impact on neurogenesis and differentiation of neural cells (43). According to previous reports, miR-124 has the ability to act as proliferation inhibitor and with suppression of CDk6, induce cell differentiation (44, 45). Also it is shown in our previous work that MiR-124 overexpression increases the level of expression of MAP2, β-Tubulin III, NF-M, SYN and Nestin markers and induces functional differentiation in M17 NB cells (28). In this study, it is further found that secretion of exosomes from hAD-MSCs-miR-124 promotes miR-124 delivery to the M17 NB cells and reduces their proliferation. Control of proliferation is a critical event in terminal differentiation program of tumor cells (46). Therefore, in the current work, after successful delivery of miR-124 with the secreted exosomes from hAD-MSCs to the M17 NB cells and approving their proliferation decrease, induction of differentiation in these cells was also investigated. The obtained results confirm that exosome delivery of miR-124 to M17 NB cancer cells can be an efficient cell-free approach for differentiation therapy of NB cancer. In a previously published report, it is indicated that secreted neurotrophic factor from MSCs solely can induce differentiation in neuronal progenitor cells (47). In the present study, it is shown that the induction of differentiation in the M17 NB cells that were treated with exosomes of hAD-MSCs-miR-124, compared with the control (M17 NB cells that were treated with exosomes of hAD-MSCs-Con-miR), is the outcome of two distinct factors, i.e. miR-124 and hAD-MSCs. Considering these results, we are of the opinion that using hAD-MSCs as vectors for miR-124 delivery to NB cells seems to indicate a new way for treatment of NB cancer.

**Conclusion**

In summary, MSC exosome as a novel cell-free stem cell-based therapy and the genetically modified exosomes have yielded positive therapeutic results (48). In this study, it is noted that hAD-MSCs can efficiently deliver exogenous miR-124 to NB cells and result in decrease of their proliferation and induction of differentiation. The represented results offer the opportunity to use delivered exogenous miRs by hAD-MSCs as a novel cell-free stem cell-based therapy for NB
cancer. Future studies can be directed towards more investigations on allogeneic MSCs by mouse tumor models, which is necessary to more evidently confirm the antitumor effects of MSCs.

**Ethical considerations**

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Research Ethics Committee of Tehran University of Medical Sciences decision No. 92-01-87-21665-85626.

**Authors’ Contributions**

S.S. Participated in study design, data collection, evaluation, and drafting. M.H.G., M.S.; Were responsible for overall supervision and provided critical revision of the manuscript. All authors read and approved the final manuscript.

**Acknowledgements**

This work is a part of PhD thesis of Samaneh Sharif that is financially supported by the Department of Molecular Medicine, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran. There is no conflict of interest in this study.

**References**

5. Cruz F D and Matushansky I 2012 Solid tumor differentiation therapy—is it possible? *Oncotarget* **3** 559
8. Sharif S, Ghahremani M and Soleimani M 2018 Delivery of exogenous miR-124 to glioblastoma multiform cells by Wharton’s jelly mesenchymal stem cells decreases cell proliferation and migration, and confers chemosensitivity *Stem Cell Reviews and Reports* **14** 236-46
29. Peng Y and Croce C M 2016 The role of MicroRNAs in human cancer Signal transduction and targeted therapy 1 15004
32. Mouradian M M 2012 MicroRNAs in Parkinson's disease Neurobiology of disease 46 279-84
33. De Smaele E, Ferretti E and Gulino A 2010 MicroRNAs as biomarkers for CNS cancer and other disorders Brain research 1338 100-11
35. Biancone L, Bruno S, Deregibus M C, Tetta C and Camussi G 2012 Therapeutic potential of mesenchymal stem cell-derived microvesicles Nephrology Dialysis Transplantation 27 3037-42
37. Junn E and Mouradian M M 2012 MicroRNAs in neurodegenerative diseases and their therapeutic potential Pharmacology & therapeutics 133 142-50
43. Li K K W, Pang J C-s, Ching A K-k, Wong C K, Kong X, Wang Y, Zhou L, Chen Z and Ng H-k 2009 miR-124 is frequently down-regulated in medulloblastoma and is a negative regulator of SLC16A1 Human pathology 40 1234-43
44. Pierson J, Hostager B, Fan R and Vibhakar R 2008 Regulation of cyclin dependent kinase 6 by microRNA 124 in medulloblastoma Journal of neuro-oncology 90 1-7
45. Matushansky I, Radparvar F and Skoulitchi A 2003 CDK6 blocks differentiation: coupling cell proliferation to the block to differentiation in leukemic cells Oncogene 22 4143
46. Przyborski S A, Hardy S A and Maltman D J 2008 Mesenchymal stem cells as mediators of neural differentiation Current stem cell research & therapy 3 43-52
47. Vishnubhatla I, Corteling R, Stevanato L, Hicks C and Sinden J 2014 The development of stem cell-derived exosomes as a cell-free regenerative medicine Journal of Circulating Biomarkers 3 3-2
Fig. 1 Flow cytometric analysis of hAD-MSCs for a CD73, b CD90, and c CD105 (positive markers); d CD34, e CD45, f HLA-DR (negative markers). Light microscopy pictures show g adipogenic and h osteogenic differentiation of hAD-MSCs (Scale bar = 200 μM).
Fig. 2 Flow cytometry results confirms miR-124 delivery to M17 NB cells by hAD-MSCs. HAD-MSCs were transfected with Cy3-labeled miR-124. After 24 h, the labeled M17 NB cells with Green Cell Tracker CMFDA were added to the hAD-MSCs cultures. Expression of the fluorescent miR-124 in M17 NB cells was analyzed 24 h later by flow cytometry. The results indicated the transfer of miR-124-Cy3 from hAD-MSCs to M17-CMFDA cells. 

a) HAD-MSCs were transfected with Cy3-labeled miR-124. Transfection efficiency was evaluated about 80% by fluorescence microscopy (P value < 0.05). 

b) M17 NB cells co-cultured with hAD-MSCs-miR-124-Cy3, left panel: the NB cells alone; middle panel: analysis of the NB cells and hAD-MSCs for CMFDA and Cy3; right panel: Cy3 in co-cultured cells. 

c) The derived exosomes from hAD-MSCs were added to NB cells with transwell, left panel: the NB cells alone; middle panel: analysis of the M17 NB cells for CMFDA and Cy3; right panel: Cy3 alone in the M17 NB cells.
The confirming results of inducing differentiation in M17 NB cells after delivery of miR-124 by hAD-MSCs.

a) The MTT assay results that represent the cell proliferation in miR-124 treated cells in every 24 h interval and b) TUNEL staining results indicating that the delivered miR-124 induced considerable apoptosis 24 h after exposure, nuclei of TUNEL-positive cells indicate that most of the cells were suffered from apoptosis. The cultivated M17 NB cells with the secreted exosomes from hAD-MSCs-Con-miR (M17-hAD-MSCs-Con-miR) were treated with derived exosomes from hAD-MSCs-miR-124 (M17-hAD-MSCs- miR-124). The directly transfected M17 NB cells with miR-124 (M17-miR-124) and its control (Scale bar = 50 μM).

M17-Con-miR

M17-miR-124

M17-MSCs-Con-miR

M17-MSCs-miR-124

TUNEL

DAPI

Merge
Fig. 4 The qRT-PCR analysis results that show the increase of mRNA level of β-Tubulin III and MAP2 (P values <0.05) and indicate that hAD-MSCs delivered miR-124 and induced differentiation in M17 NB cells in comparison with the control.