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RESEARCH ARTICLE

Experimental verification of a conserved intronic microRNA located in the human *TrkC* gene with a cell type-dependent apoptotic function

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Abstract Tropomyosin receptor kinase C (*TrkC*) is involved in cell survival, apoptosis induction and tumorigenesis. We hypothesized that, similar to $p75^{NTR}$ receptor, some of the diverse functions of *TrkC* could be mediated by a microRNA (miRNA) embedded within the gene. Here, we experimentally verified the expression and processing of two bioinformatically predicted miRNAs named *TrkC*-miR1-5p and *TrkC*-miR1-3p. Transfecting a DNA fragment corresponding to the *TrkC*-premir1 sequence in HEK293t cells caused ~300-fold elevation in the level of mature *TrkC*-miR1 and also a significant downregulation of its predicted target genes. Furthermore, endogenous *TrkC*-miR1 was detected in several cell lines and brain tumors confirming its endogenous generation. Furthermore, its orthologous miRNA was detected in developing rat brain. Accordingly, *TrkC*-miR1 expression was increased during the course of neural differentiation of NT2 cell, whereas its suppression attenuated NT2 differentiation. Consistent with opposite functions of *TrkC*, *TrkC*-miR1 overexpression promoted survival and apoptosis in U87 and HEK293t cell lines, respectively. In conclusion, our data report the discovery of a new miRNA with overlapping function to *TrkC*.

Keywords *TrkC-miR1* · Survival · U87 · Neural differentiation · Neurotrophin receptor

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Introduction

Neurotrophin tyrosine kinase receptor type 3 (*NTRK3*) or tropomyosin receptor kinase C (*TrkC*) (NC_000015.9) is a member of the Trk family of neurotrophin receptors that mediates multiple effects including neuronal differentiation and survival [1]. This gene has also the potential to act either as an oncogene or as a tumor suppressor gene [2]. Expression of Trk family receptors in some cell types could promote cell proliferation or differentiation [3]. *TrkC* is also implicated in regulating growth and survival of many human cancer tissues [2, 4, 5]. This gene activates three downstream pathways where their components are under intense investigation [1].

MicroRNAs (miRNAs) are endogenously made, highly conserved, 18–27 nucleotide-long non-coding RNAs produced in many organisms [6]. miRNA precursors (pri-miRNA) contain complicated secondary structure that is quickly processed into one or more ~70 nucleotide-long hairpin-structured pre-miRNA molecule. Pre-miRNA is further processed into its mature form, located either at 5′ or 3′ side of the stem loop [7, 8]. In mammalian cells, mature miRNA often function through pairing of its seed sequence with the 3′-UTR of its target genes, which results in either mRNA degradation or protein translation inhibition [9]. Non-canonical pairing has also been reported for seedless miRNA recognition elements (MRE) [10, 11].

To date, about 2000 human miRNAs have been registered in mirbase databank (http://www.mirbase.org/). However, ~55,000 miRNA genes are estimated to exist in the human genome [12]. Accordingly, numerous

bioinformatics tools have been developed for prediction of novel miRNAs. These software's are designed based on secondary structure information, phylogenetics conservation, similarity to the known miRNAs, stability of hairpins and genomic location of the candidate sequences relative to the known miRNAs [13, 14].

Here, we employed bioinformatics tools to search for hairpin structures within the human *TrkC* gene. One of the predicted stem loops had all of the bioinformatics features for production of a real miRNA. This conserved miRNA was experimentally validated and further detected in the human and rat tissues. Overexpression of this miRNA created expected phenotypes such as suppression of predicted target genes and other functions related to the *TrkC* signaling pathway.

Materials and methods

Bioinformatics prediction of miRNA and its target genes

To search for the possible hairpin structures within the gene, SSC profiler (http://mirna.imbb.forth.gr/SSCprofiler.html) and miPRED (http://www.bioinf.seu.edu.cn/miRNA) programs were employed. CID-miRNA (http://mirna.jnu.ac.in/cidmirna/) was used for prediction of Drosha processing sites along with Microprocessor SVM program (https://demo1.interagon.com/miRNA/). Conservation of the *TrkC*-miR1 and its precursor sequence was examined using Mireval (http://tagc.univ-mrs.fr/mireval/) along with blat search for human genome and other organisms in UCSC database (http://genome.ucsc.edu/). Furthermore, *TrkC*-miR1 was predicted using MatureBayes (http://mirna.imbb.forth.gr/MatureBayes.html), Pmirp (http://ccst.jlu.edu.cn/ci/bioinformatics/MiRNA) MiRNA Spotter (http://mcube.nju.edu.cn/jwang/lab/soft/miRNA/), MiRmat (http://mcube.nju.edu.cn/jwang/lab/soft/MiRmat/index.html), and Mirz (http://www.mirz.unibas.ch) online tools. Using mirbase, sequence similarities between *TrkC*-premir1 and mature *TrkC*-miR1 from different species were searched. RNAFOLD algorithm (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) was also used for RNA structure prediction.

DIANA-microT (http://diana.cslab.ece.ntua.gr/pathways/) and RNAHybrid tools (http://bibiserv.techfak.uni-bielefeld.de) were used to find potential target genes for the novel miRNA. To search for the potential promoter upstream of the putative stem loop, Alibaba 2.1 (http://www.generegulation.com/pub/programs/alibaba2/index.html), P-Match (http://www.gene-regulation.com/cgi-bin/pub/programs/pmatch/bin/p-match.cgi), Tfsitescan (http://www.ifti.org/cgi-bin/ifti/Tfsitescan.pl), and Promoter 2.0 Prediction Server (http://www.cbs.dtu.dk/services/Promoter/) bioinformatics tools were used. To find the pathways in which *TrkC*-miR1 is involved, Diana-mirpath (http://diana.cslab.ece.ntua.gr/pathways/), DAVID (http://david.abcc.ncifcrf.gov/), and geneset2 miRNA (http://mips.helmholtz-muenchen.de/proj/gene2mir/) online tools were used.

Cell lines and tissue samples

U87MG, A172, 1321N1, Daoy, SK-N-MC and HCT116 cell lines were cultured in RPMI-1640 media (Invitrogen), supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma), and with 10 % fetal bovine serum (FBS) (Invitrogen), and incubated in 37 °C with 5 % CO_2. HEK293t and NT2 cells [15] were cultured in DMEM-HG (Invitrogen) containing 10 % FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Tissue samples (seven meningioma transition type 1, eight glioma and three other brain tumor types) were obtained from Imam Hospital, Tehran, Iran and stored at −80 °C. All cell lines were obtained from Pasteur Institute, Iran.

DNA constructs

For cloning the region corresponding to the *TrkC*-miR1 and its antisense, about 700 bp fragment of the human *TrkC*-intron 14 was PCR amplified using Int14-F and Int14-R primers (Table 1) and cloned into pEGFP-C1 expression vector (Clontech) downstream of the GFP gene both in sense and antisense directions. Human genomic DNA template was extracted from white blood cells using standard protocol [16]. As scrambled control, a previously described hairpin structure sequence [17] was cloned into the pEGFP-C1 vector. 3′UTR sequences of the *MDNI* and *HBEGF* were cloned in psiCHECK vector downstream of the luciferase gene for dual luciferase assay analysis using Promega kit. All the vectors were sequenced for verification of correct insert.

RNA preparation

Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions and treated with RNAase-free DNAaseI (Fermentas).

Detection of *TrkC*-miR1 and its precursor

According to the protocol [18], cDNA was made from polyadenylated RNAs and each cDNA sample was amplified using specific primers (Table 1) in a 7500 Real-Time PCR system (Applied Biosystems), using the following conditions for 45 cycles: stage 1, 95 °C for 5 s; stage 2, 60 °C for 20 s; stage 3, 72 °C for 34 s. RT-qPCR was performed according to MIQE guidelines using

Table 1 Primer and oligo sequences used in the research

Primer name	Organism	Primer sequence, 5′–3′	Amplicon size (base pairs)
TRKC	Human	Forward: CCTGTGTCCTGTTGGTGGTTCTC	195
		Reverse: GAGTCATGCCAATGACCACAGTGTC	
TRKC	Rat	Forward: ACCATGGCATCACTACACCTTC	229
		Reverse: CTTAGATTGTAGCACTCAGCCAG	
TrkC-premir1	Human	CTCCTCCCGCAAACTGTTTTGG	
TrkC-miR1-5p	Human/Rat	GTCTGACCAACCTCCTCCC	
TrkC-miR1-3p	Human	AGGAGGAGGAGGTCAGG	
U48	Human	Forward: TGACCCCAGGTAACTCTGAGTGTGT	
U6	Rat	Forward: GGTGGGAAGTGAGGGAGAGGG	
Anchored OligodT	–	GCGTCGACTAGTACAACTCAAGGTTCTTCCAGTCACGACG(T)18N	
Universal-outer	–	GCGTCGACTAGTACAACTCAAG	
Universal-inner	–	AACTCAAGGTTCTTCCAGTCACG	
TGFBR1	Human	Forward: CATTTTTCCCAAGTGCCAGT	235
		Reverse: ACACCCCTAAGCATGTGGAG	
HBEGF	Human	Forward: ACCTATGACCACACAACCATCCTG	200
		Reverse: TAGCAGTCCCCAGCCGATTCCT	
MDN1	Human	Forward: CCTCATGGACACGATCTTCCAGCC	131
		Reverse: TCTCAGCTGCAACCTTCTCCTCC	
GAPDH	Human	Forward: GCCACATCGCTCAGACAC	115
		Reverse: GGCAACAATATCCACTTTACCAG	
B2m	Rat	Forward: CCGTGATCTTTCTGGTGCTT	318
		Reverse: TTTTGGGCTCCTTCAGAGTG	
TrkC-Intron 14	Human	Int14-F : CAGGGGACTGCTTCTGAGCCTC	709
		Int14-R: TTGAATCCTATCCTCTCCACACCTC	
MDN1-3′UTR	Human	Forward: CTCGAGGAGACTTAACTGTGGTCAGAAGGT	1122
		Reverse: GCGGCCGCGGTACATGCTTTGGGACACTTGG	
HBEGF-3′UTR	Human	Forward: CTCGAGCTGCTACCTCTGAGAAGACAC	1328
		Reverse: GCGGCCGCCTTCCAGAAAGGACCATGACAG	

SYBRPremix Ex TaqTM II PCR Mastermix (Takara, Japan) in experimental duplicates. Expression data were analyzed using endogenous U48 small nucleolar RNA (SNORD48), *B2m* and *GAPDH* as the reference genes and were normalized using the $2^{-\Delta C_t}$ and $2^{-\Delta\Delta C_t}$ method [19].

NT2 cell differentiation

The NT2 cells were treated with retinoic acid (RA) to induce neural differentiation following manufacturer's protocol [15]. Also, the vector expressing anti-*TrkC*-mir1 was transfected in NT2 cells, 10 days after starting differentiation. Then, the expression of differentiation markers was monitored.

Overexpression and knockdown of *TrkC*-premir1 in cell lines

One microgram of pEGFP-C1 expression vector containing *TrkC*-miR1 precursor was engulfed in lipofectamin 2000 (Invitrogen) and used for transfection of HEK293t and U87 cell lines. 24 h later, GFP microscopy (by Nikon eclipse Te2000-s) ensured successful transfection.

Azacytidine treatment

Azacytidine (Sigma-Aldrich) was dissolved in sterile water and used to treat HCT116 cells with a final concentration of 10 μM for 48 h. Total RNA was then extracted from the cells and used for qRT-PCR analysis.

Cell cycle analysis

Cells were transfected with either overexpression or knockdown cassettes of *TrkC*-premir1 and were harvested 36 h after transfection and stained with propidium iodide (PI) and annexin V (Roche) according to the manufacturer's protocol. All samples were analyzed with a FACS

Calibur flowcytometer with Cell Quest software (BD Biosciences).

Statistical analysis

RT-qPCR data were analyzed using DataAssist software V3.0 [20]. Other statistical analysis was performed with GraphPad Prism 5.04 (GraphPad, San Diego, CA, USA). For apoptosis studies, data showing percent of early apoptotic cell population within the negative group and test group were compared with each other by repeated measures ANOVA test followed by Bonferroni test using GraphPad.

Results

Computational prediction of novel miRNAs

SSC profiler predicted hairpin structures within the *TrkC* gene capable of producing novel miRNAs. Among 150 predicted stem loops, a highly conserved 103-nucleotide sequence located in 14th intron of the gene (hg17, chr15: 86290421–86290524) contained Drosha and Dicer recognition sites and a minimum free energy score. This predicted pre-miRNA producing stem loop, was named *TrkC*-premir1 and predicted mature miRNA was named *TrkC*-miR1 (Fig. 1). Moreover, Maturebayes software predicted *TrkC*-miR1-5p and *TrkC*-miR1-3p as bona fide miRNAs. Although *TrkC*-miR1-5p showed similarity to the hsa-miR-6133, hsa-miR-4750-3p and hsa-miR-4728-3p, and *TrkC*-miR1-3p showed similarity to the hsa-miR-6894-3p, hsa-miR-4298 and hsa-miR-6831-5p, no identical miRNA for the *TrkC*-miR1 had been reported in mirbase yet. Based on UCSC genome browser, the sequence and structure of both *TrkC*-premir1 (Fig. 1d) and *TrkC*-miR1-5p (Fig. 1e) are conserved in mammals. Furthermore, miRNA Spotter, MiRmat, Pmirp, and MatureBayes all recognized *TrkC*-premir1 with significant scores. Several promoter prediction tools predicted an independent promoter for *TrkC*-miR1 at approximately 1400 bp upstream of the corresponding stem loop. Prominent transcription factors such as GATA-6, Sp1, CREB, Oct-1 and NF-κappaB were predicted to interact with this putative promoter region.

TrkC-premir1 overexpression follows by downregulation of predicted target genes

The highest levels of mature *TrkC*-miR1-5p and *TrkC*-miR1-3p expression (~300 times) were detected 48 h post-transfection of HEK293t cells using *TrkC*-premir1-expressing vector (Fig. 2a). The size and authenticity of the amplified products were further confirmed by sequencing; After RT-qPCR products were cloned in the TA vector, three independent clones had the same nucleotide sequence as it was predicted for *TrkC*-miR1-5p (Fig. 2b). *TrkC*-miR1-3p-amplified product was also cloned in TA vector and sequenced (Fig. 2b). The minimum size of these sequences was submitted to EMBL-EBI database accessible by EBI accession#: HF679088 and HG529982 for *TrkC*-miR1-5p and *TrkC*-miR1-3p, respectively.

Both *TrkC*-miR1-5p and *TrkC*-miR1-3p were introduced to Dianna lab software, which generated about 50 and 500 predicted target genes, respectively (Sup. 1). The highest scored target for *TrkC*-miR1-5p was *MDN1* (Midasin homologue MIDAS-containing protein) gene which had three highly conserved MRE in its 3′-UTR. *HBEGF* (Heparin-binding EGF-like growth factor precursor) was another highly scored predicted target gene for *TrkC*-miR1-5p, showing 3 MREs in its 3′-UTR. On the other hand, DAVID and RNAhybrid softwares predicted TGβR1 (transforming growth factor, beta receptor I) gene as a putative target for the *TrkC*-miR1-3p. *TrkC* host gene was also predicted as a low-scored target for *TrkC*-miR1-3p (Sup. 1). Subsequently, RT-PCR result indicated these predicted target genes were expressed in HEK293t and U87 cell lines (data not shown). When *TrkC*-premiR1 was overexpressed in HEK293t cells a significant downregulation of *MDN1*, *HBEGF*, and *TGβR1* expression level was detected by RT-qPCR (Fig. 2c). Consistently, qRT-PCR analysis failed to detect any expression alteration for *TrkC* and Sox2 (as non-predicted targets), following *TrkC*-miR1 overexpression (Fig. 2c).

Further, dual luciferase assay supported a direct interaction of *TrkC*-miR1-5p with high-scored targets *MDNI* and *HBEGF*, with 25 and 19 % reduction in luciferase count, respectively (Fig. 2d).

Detection of *TrkC*-miR1 in the human brain tumors and cell lines

To examine the existence of an endogenous *TrkC*-miR1, RNA samples were extracted from human glioblastoma (U87MG and A172), astrocytoma (1321N1), medulloblastoma (Daoy), and neuroblastoma (SK-N-MC) cell lines. Then, *TrkC*-miR1-5p, *TrkC*-miR1-3p, and *TrkC*-premir1 were specifically amplified from the related cDNA samples using RT-qPCR. The highest expression level of *TrkC*-miR1-5p was detected in the non-glioblastoma cell; however, such a pattern of expression was not detected for *TrkC*-miR1-3p or even *TrkC* host gene (Fig. 3a).

Endogenous *TrkC*-miR1 was also detected in brain tumor biopsies. Although, both *TrkC*-miR1-5p and *TrkC*-miR1-3p were expressed at relatively low levels in the most brain tumor samples, they had the highest level of expression in glioma samples (Fig. 3b).

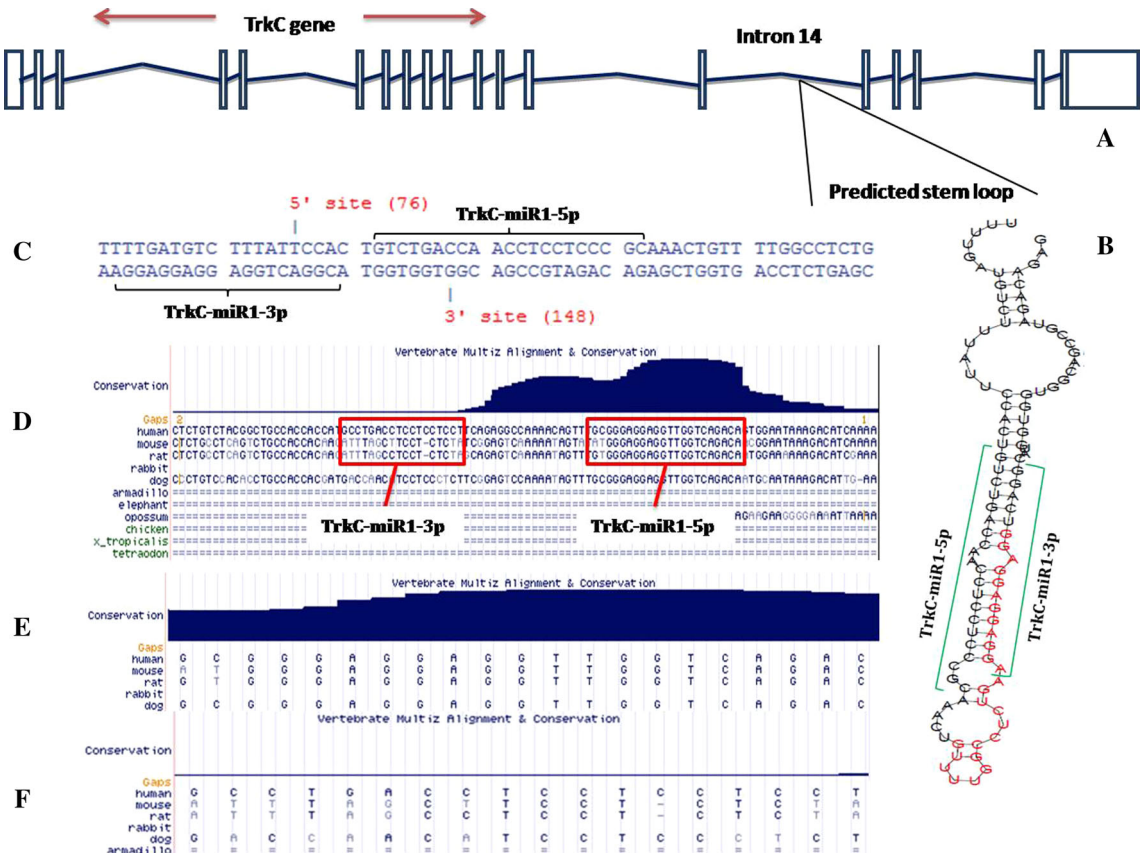

Fig. 1 Predicted *TrkC*-miR1 and its precursor within the human *TrkC* gene. **a** *TrkC* gene exons and introns adapted from Ensemble are shown with *rectangular* and *broken line shapes*, respectively. **b** Relative location of a stem loop in the 14th intron of human *TrkC* gene predicted to produce *TrkC*-premir1. *Red* colored sequence is predicted mature form of *TrkC*-miRNA. **c** Predicted Drosha cutting sites is shown on the sequence of hairpin structure. **d–f** Blat search terminates conservation of *TrkC*-premir1 between human, mouse, rat and dog. *TrkC*-premir1 has strong conservation (section D) only in an area of approximately 20 nucleotides that is equivalent of *TrkC*-miR1-5p (section E). *TrkC*-miR1-3p is mostly conserved at seed sequence. *TrkC*-mir1 sequences are marked in all *panels*

Effect of *TrkC*-premir1 expression alteration on the survival of cell lines

HEK293t and U87 cells were transfected with either overexpression or knockdown cassettes of *TrkC*-miR1 and RT-qPCR results confirmed *TrkC*-miR1 expression level alteration in these cells (Fig. 4a, b). Then, flow cytometry data indicated >20 % sub-G1 cell cycle elevation in HEK293t cells following *TrkC*-miR1 overexpression (Fig. 4c). Annexin V test also indicated that ~20 % of transfected HEK293t cells were in early apoptosis (Fig. 4d). Consistently, knockdown of *TrkC*-miR1 in HEK293t cells did not change the rate of apoptosis (Fig. 4e). In contrast, survival rate was increased in U87 cells following *TrkC*-premir1 overexpression which this effect was attenuated when *TrkC*-miR1 was suppressed (Fig. 4f–h). Results of MTT assays were also in accordance with the flow cytometry conclusions, emphasizing on *TrkC*-miR1 cellular functions (Fig. 4i).

TrkC-mir1 expression alteration during NT2 cell differentiation

TrkC and *TrkC*-miR1 expression was compared during the NT2 embryonal carcinoma cells differentiation process (Fig. 5a). *TrkC* and *TrkC*-miR1-5P expression pattern were similar until 2 weeks. However, *TrkC*-miR1-5p expression level was increased while *TrkC* expression level was decreased at the end of the process. To investigate the effect of *TrkC*-miR1 knockdown on the neural cell differentiation, NT2 cells were first treated with RA to induce differentiation and after 10 days were transfected with the vector expressing anti-*TrkC*-miR1 sequence. RT-qPCR confirmed 60 % reduction of *TrkC*-miR1-5p expression in these cells at the end of the process (Fig. 5b). In these cells, *PAX6* (neural differentiation marker) was downregulated and *OCT4A* (a well-known stemness marker) was upregulated, compared to the control NT2 cells (Fig. 5c).

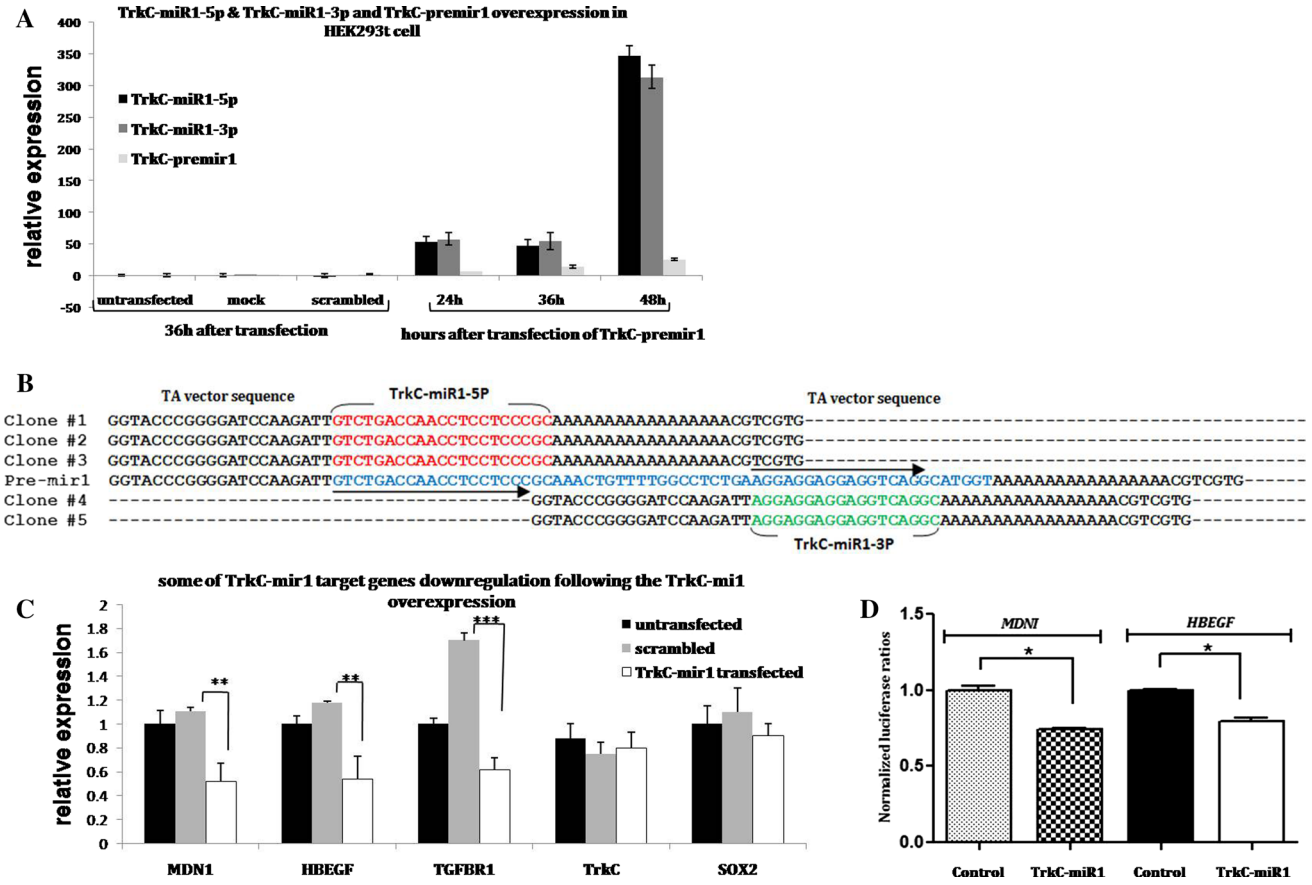

Fig. 2 Detection of *TrkC*-miR1 and downregulation of its predicted target genes. **a** Detection of *TrkC*-miR1 expression, 48 h after transfection of *TrkC*-premir1. *TrkC*-miR1-5p and *TrkC*-miR1-3p expression levels were 300 times more than controls, 48 h after transfection of HEK293t cells. **b** Sequencing result of three TA vector clones (numbered clone 1–3) containing mature *TrkC*-miR1-5p, and one clone (named precursor) containing *TrkC*-premir1, and two clones (clone 4–5) containing mature *TrkC*-miR1-3p are aligned. 3′-end of mature *TrkC*-miR1-5p and *TrkC*-miR1-3p was at least one nucleotide longer than the specific primer that was used for amplification of these miRNAs. The nucleotides downstream and upstream of miRNA sequence belong to the vector and anchored oligo-dT primer. **c** Downregulation of *MDN1*, *HBEGF* and *TGFβR1* target genes following *TrkC*-premir1 overexpression, compared to the related controls. In this figure, low-scored *TrkC* and non-target *SOX2* gene expression level has not been significantly altered. Data were normalized against *GAPDH* and error bars indicate standard deviation (SD) of duplicate experiments. **d** Luciferase assay shows interaction of *TrkC*-miR1-5p with its predicted target transcripts

Differential expression of endogenous *TrkC*-miR1-5p in rat tissues

RNA FOLD detected *TrkC*-premir1 orthologous within the rat *TrkC* gene (chr1:133,998,644–133,998,745). Spatial expression of *TrkC* and *TrkC*-miR1-5p was analyzed in various adult rat tissue samples using RT-qPCR. As expected, the highest level of *TrkC* expression was detected in the rat cerebrum and no expression was detected in lung, liver and heart. No *TrkC*-miR1-5p expression was detected in the cerebrum, lung and liver tissues of rat, but its highest level was detected in the cerebellum and was slightly detected in the heart tissues (Fig. 6a). Co-expression of the *TrkC* and *TrkC*-miR1-5p was also analyzed in the 16-day-old embryo and newborn rat brain tissues (Fig. 6b, c). The highest expression level of *TrkC* gene was detected in the hippocampus of embryo and its expression was reduced from embryo to newborn (Fig. 6b). On contrary, expression level of *TrkC*-miR1-5p was not significantly changed in different brain sections both in 16 days and newborn rat (Fig. 6c) suggesting the existence of an independent promoter for the miRNA in the rat genome.

Differential effect of 5-azacytidine treatment on *TrkC* and *TrkC*-miR1 expression

TrkC and *TrkC*-miR1 expression levels were compared following HCT116 cells treatment with 5-azacytidine epidrug. RT-qPCR result indicated that *TrkC* expression level was elevated up to 100 times in the 5-azacytidine-treated HCT116 cells while the *TrkC*-miR1-5p expression level was significantly decreased compared to the related

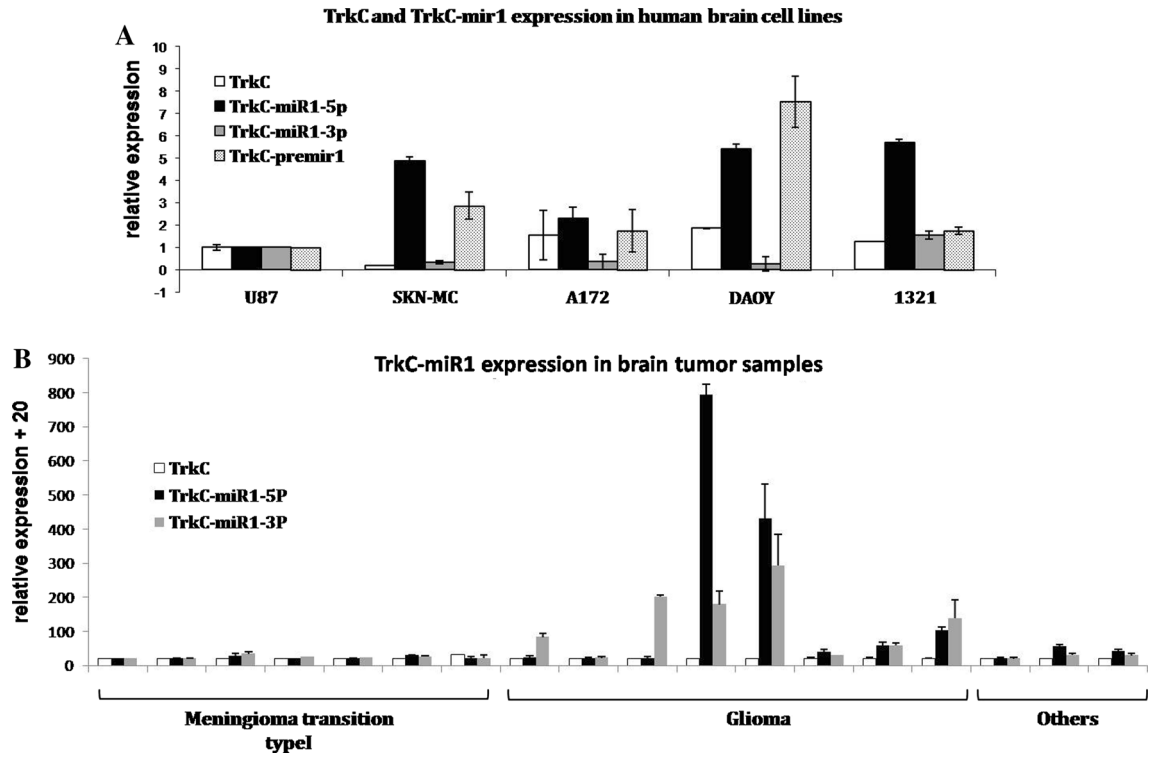

Fig. 3 Detection of *TrkC*-mir1 in the brain-derived cell lines and biopsies. **a** Expression of *TrkC*-mRNA, *TrkC*-premir1, *TrkC*-miR1-5p, and *TrkC*-miR1-3p in glioma and non-glioma cell lines is compared to the levels in U87-MG cell line. **b** *TrkC*-miR1 and *TrkC* relative expression levels in various human brain tumor samples were compared to the meningioma transition type I. The highest expression of *TrkC*-miR1-3p was detected in glioma tumor. *U48* RNA and *GAPDH* were used for normalizing the expression levels of *TrkC*-miR1 and *TrkC*, respectively. For easier comparison of the expression levels, 20 were added to the values. *Error bars* indicate SD of duplicate experiments

controls. Moreover, *TrkC*-miR1-3p expression was not detected in HCT116 cells after or before 5-azacytidine treatment (Fig. 7).

Discussion

Bioinformatic prediction of novel miRNAs

miRNAs are involved in many processes including differentiation, apoptosis and cancer [21]. miRNAs' small size, low and developmental expression and mutation tolerance make forward genetics relatively inefficient for their discovery [22]. Several bioinformatics tools are available for prediction of novel miRNAs [23]. SSC profiler predicted 150 hairpin structures in human *TrkC* gene that one of them (named *TrkC*-premir1) located in 14th intron (Fig. 1a) showed the most criteria for producing a real miRNA (Fig. 1b). Furthermore, microprocessor SVM predicted Drosha site in this sequence (Fig. 1c). Like most of the known miRNAs [14, 24], miREval and UCSC tools confirmed that both *TrkC*-premir1 (Fig. 1d) and *TrkC*-miR1-5p were strongly conserved in several mammalian organisms (Fig. 1e). However, *TrkC*-miR1-3p was only conserved at its seed sequence (Fig. 1f). Similar pattern of miRNA-5p and -3p conservation has been reported elsewhere [25, 26] both in plant (miR2111) [27] and animal (hsa-let-7) [24]. Here for the first time, *TrkC*-miR1-5p and *TrkC*-miR1-3p are introduced with no identical sequences reported in the mirbase database. DIANA microT v3.0 predicted *MDN1* and *HBEGF* as *TrkC*-miR1-5p target genes and DAVID and RNAHybrid tools predicted *TGFBR1* as a target for *TrkC*-miR1-3p. Overall, all of the applied bioinformatics tools strongly supported the presence of these miRNAs.

Efficient processing of *TrkC*-mir1 precursor

According to the Oncomine (http://www.oncomine.org/) and EMBL-EBI databases (http://www.ebi.ac.uk/gxa/), *TrkC* gene (and probably *TrkC*-premir1) expression level is very low in the HEK293t cell line. Therefore, HEK293t cells were used for exogenous expression of *TrkC*-mir1 precursor to test for the production of mature miRNA similar to the approach used by others [18, 28] (Fig. 2a). 48 h after transfection, specifically amplified RT-qPCR

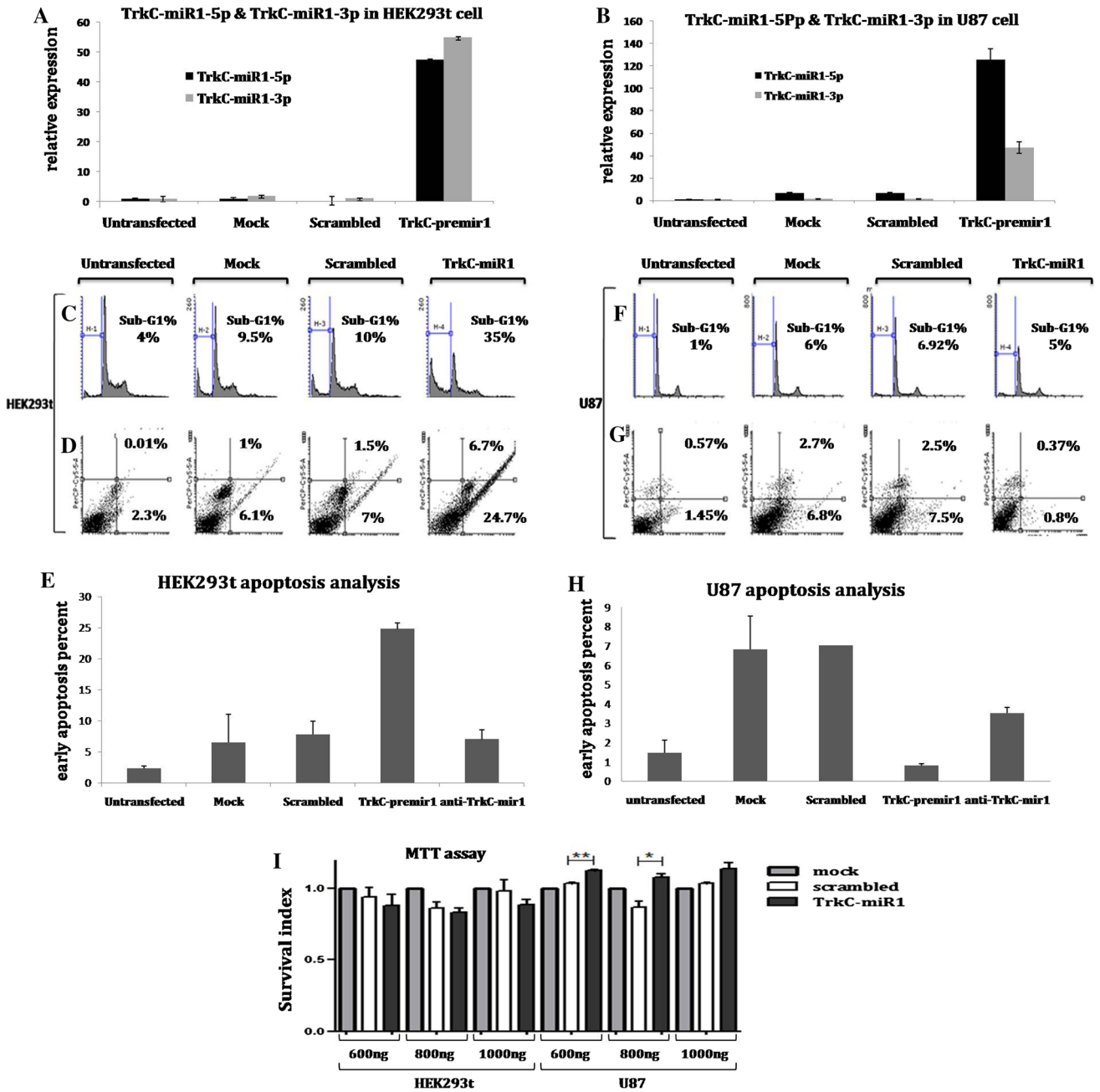

Fig. 4 Effect of *TrkC*-miR1 expression alteration on survival of HEK293t and U87 cell lines. **a**, **b** Differential accumulation of *TrkC*-miR1-5p and *TrkC*-miR1-3p in the cells overexpressing *TrkC*-premir1. *TrkC*-premir1 overexpression effect on HEK293t and U87 cell cycle distribution was measured by PI (**c**, **f**) and annexin–PI analysis (**d**, **g**), 36 h after transfection. In the cells overexpressing *TrkC*-mir1 compared to the related controls, about 20 % induction and 7 % reduction in sub-G1 (PI) and early apoptosis stage distribution (annexin) was calculated, in HEK293t (**c**, **d**) and U87 (**f**, **g**) cells, respectively. The percentage of early and late apoptosis is added to the panels for simplicity. **e**, **h** ANOVA analysis confirmed a significant change ($p < 0.05$) in the rate of apoptosis between negative controls and the cells overexpressing *TrkC*-miR1 (unlike knockdown situation). **i** MTT assay results in HEK293t and U87 cells overexpressing TrkC-miR1. Overexpression of *TrkC*-miR1 significantly increased survival rate of U87 cells. *Error bars* indicate SD of triplicate experiments

products with expected size on the acrylamide gel were cloned into TA vector for sequencing. Three independent *TrkC*-miR1-5p-TA clones and two independent *TrkC*-miR1-3p-TA clones had the expected nucleotide sequences as well as two or one extra nucleotides, respectively (Fig. 2b). That means, miRNA sequence and Drosha cleavage site [29] have been correctly predicted. miRNAs are reported to be 18–27 nucleotides long [30, 31] and here,

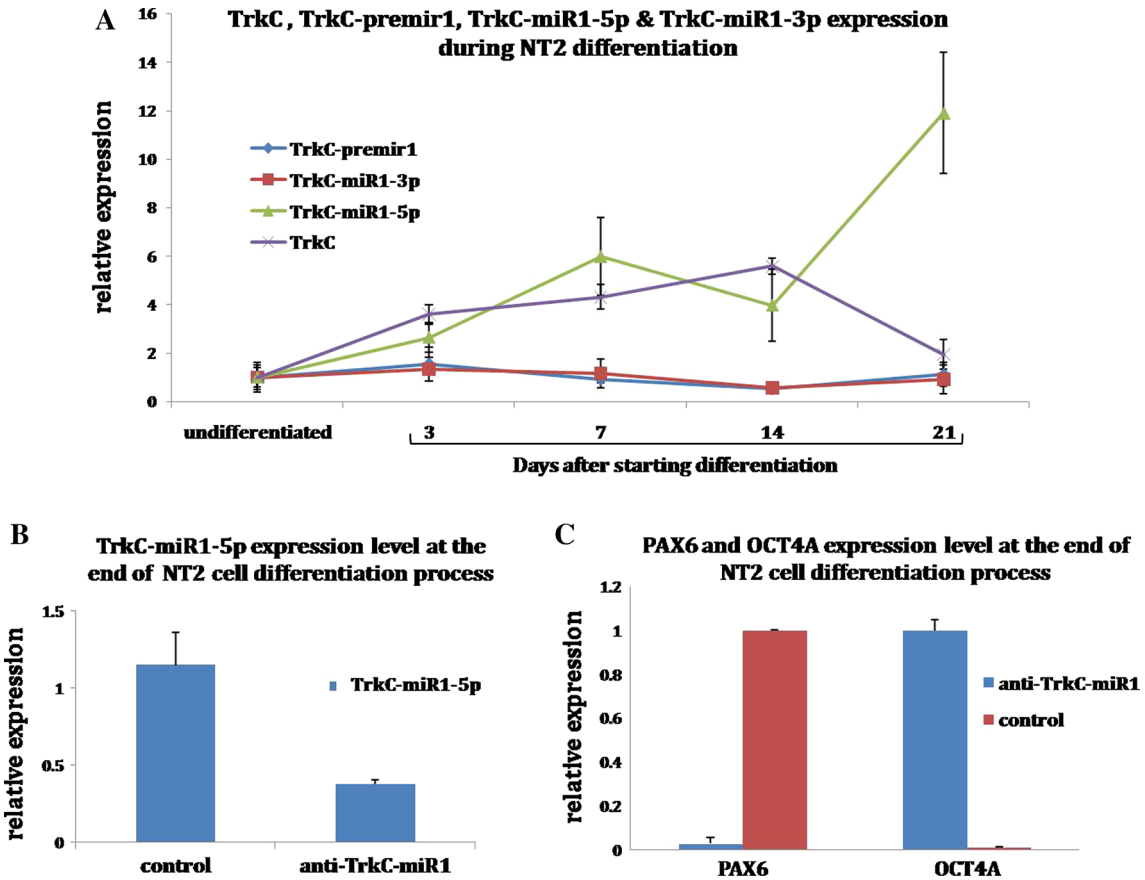

Fig. 5 *TrkC*, *TrkC*-premir1 and *TrkC*-miR1 expression alteration during the NT2 cell neural differentiation. **a** *TrkC* and *TrkC*-miR1-5P expression levels were similarly increased until 2 weeks. Then, *TrkC*-miR1-5p expression level continued to increase while, *TrkC* expression level was decreased. Meanwhile, *TrkC*-miR1-3p and its precursor expression level was not significantly changed. Data were compared with the levels in un-differentiated NT2 cells. *U48* RNA and *GAPDH* were used as controls. *Error bars* indicate SD of duplicate experiments. **b** RT-qPCR data shows overexpression of anti-*TrkC*-miR1 since 10th day of NT2 differentiation process, resulted in *TrkC*-miR1-5p knockdown at the end of process (21st day). NT2 cells that were only treated with RA were used as control. **c** RT-qPCR against neural and stemness markers showed that *PAX6* is downregulated while, *OCT4A* is upregulated as a result of *TrkC*-miR1 downregulation, respectively

TrkC-miR1-5p and *TrkC*-miR1-3p were at least 21 and 19 nucleotides, respectively.

Downregulation of predicted target genes following *TrkC*-miR1 overexpression

RT-qPCR results showed that *MDN1*, *HBEGF* and *TGβR1* highly scored predicted target genes were 50 % down-regulated while, low-scored *TrkC* or non-predicted Sox2 gene expression was not affected following *TrkC*-premir1 overexpression (Fig. 2c). *MDN1* that is expressed in tumor microenvironment [32] is involved in RNA metabolism [33]. *HBEGF* is a known growth factor that is potentially involved in cell cycle [34]. *TGβR1* as a *TrkC*-miR1-3p predicted target gene is involved in multiple opposite cellular processes such as apoptosis and proliferation [35, 36]. There are multiple highly conserved MREs for *TrkC*-miR1-5p within the 3′-UTR sequences of *MDN1* and *HBEGF* genes that may justify its strong effect on these target genes expression detected by qRT-PCR (Fig. 2c). This conclusion is consistently supported by a dual luciferase assay performed against *HBEGF* and *MDN1* 3′UTR sequences (Fig. 2d). Overall, overexpression effect of *TrkC*-miR1 on its predicted target genes supported the identity and functionality of these miRNAs.

Detection of endogenous *TrkC*-miR1 in human tissues and cell lines

TrkC gene is highly expressed both in normal [37] and tumors [38, 39] of neural and non-neural tissues [40] as well as medulloblastoma [41] and neuroblastomas [38] cell lines. Therefore, *TrkC*-miR1 expression was investigated in these samples. *TrkC*-miR1-5p, *TrkC*-miR1-3p and their precursor as well as *TrkC* host gene were detectable in all of the tested human cell lines. However, *TrkC*-miR1-5p

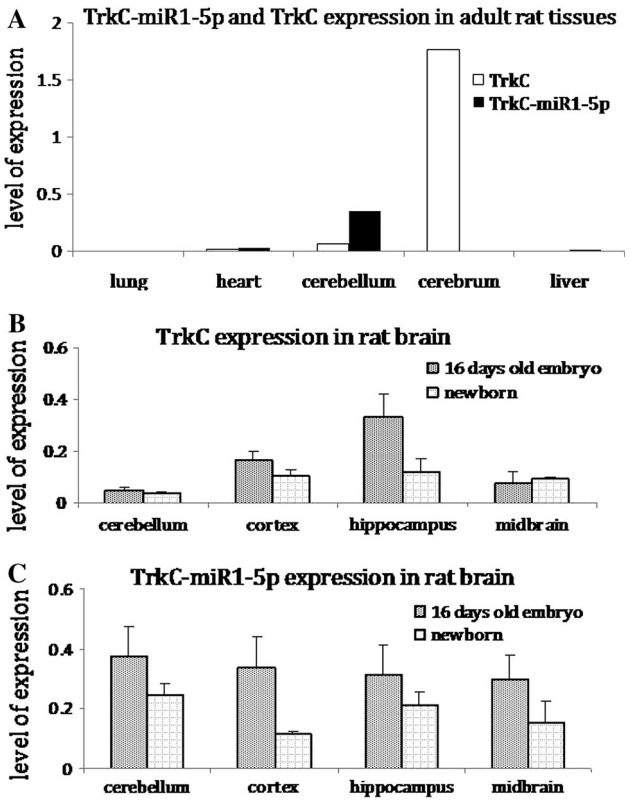

Fig. 6 Detection of *TrkC* and *TrkC*-miR1-5p in the rat tissues. **a** The highest level of *TrkC* and *TrkC*-miR1-5p expression was observed in the cerebrum and cerebellum of rat brain, respectively. **b**, **c** Expression levels of *TrkC* and *TrkC*-miR1-5p were compared in the brain tissues of 16-day-old embryo and new born rat. While, *TrkC* and *TrkC*-miR1-5p expression levels in the 16-day-old rat embryo brain tissues were higher than newborn rat brain, the differences were not significant. *Error bars* indicate SD of duplicate experiments. U6 RNA and *B2m* were used as internal controls for the amplifications

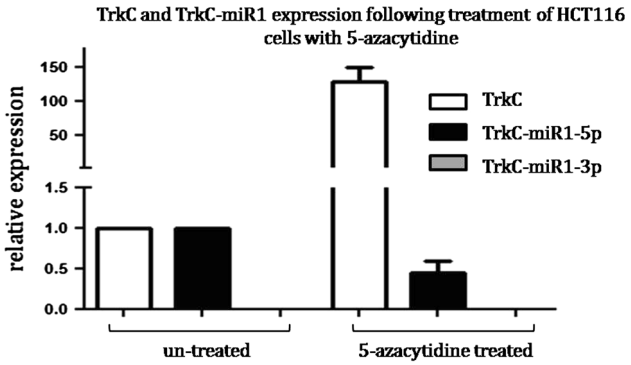

Fig. 7 *TrkC* and *TrkC*-miR1 expression alteration in HCT116 cells following 5-azacytidine treatment. *TrkC* expression level was increased up to 100 times after treatment of HCT116 cells with 5-azacytidine compared to the untreated cells. *TrkC*-miR1-5p expression level was reduced in 5-azacytidine-treated HCT116 cells compared to the control ones. However, no expression was detected for *TrkC*-miR1-3p before or after this treatment. *Error bars* indicate SD of duplicate experiments. *U48* RNA and *GAPDH* were used as internal controls for the amplifications

expression level was higher than *TrkC*-miR1-3p level in all of the tested cell lines (Fig. 3a) and most of the glioma tissue samples (Fig. 3b). This finding may reflect the stability of *TrkC*-miR1-5p versus its -3p counterpart which is consistent with similar report [42]. Relatively higher expression of *TrkC*-miR1 in glioma samples candidate it as a diagnostic marker against other brain tumors upon confirmation of its statistical significance. Over one-third of intronic-miRNAs have their own independent promoters from the host gene [43, 44]. Interestingly, no correlation was deduced between *TrkC* and *TrkC*-miR1 expression in the tested human tissues and cell lines (Fig. 3). This could be the result of independent promoter for *TrkC*-miR1 or a result of different miRNA stability in different tissue background [43, 44].

Apoptotic effect of *TrkC*-premir1 in cell lines

Similar to the method used for other miRNAs [45, 46], apoptosis induction of *TrkC*-miR1 was measured using flowcytometry and annexin V. HEK293t-transfected cells overexpressing *TrkC*-premir1 (Fig. 4a) showed more than 20 % sub-G1 cell population elevation compared to the controls (Fig. 4C). Consistently, annexin V test showed ~20 % of the transfected HEK293t cells were in early apoptosis (Fig. 4d, e). In similar experiments, significant sub-G1 and apoptosis reduction was detected in U87 cells (Fig. 4f–h). Later, MTT assay supported significant survival effect of *TrkC*-miR1 overexpression in U87 and cell survival reduction (non-significant) in HEK293t cell lines (Fig. 4i). *TrkC*-miR1 opposite effects in HEK293t and U87 cell lines are similar to the attributed *TrkC* gene contradictory functions including apoptosis, differentiation and survival [1, 2, 47]. Also, miRNAs originated from the same precursor as seen for hsa-miR-28 might show opposite functions [48]. Since, predicted targets of *TrkC*-miR1-5p and *TrkC*-miR1-3p (*HBEGF* and *TGβR1*, respectively) are suggested to be involved in apposite functions of apoptosis and survival [34–36], it remained to be tested if the contradictory function of *TrkC* is related to these miRNAs.

TrkC-miR1 was also suppressed using antisense strategy and results were in accordance with overexpression conclusion; since, *TrkC*-miR1 is slightly expressed in HEK293t cells (Fig. 2a) its suppression did not change the apoptosis rate (Fig. 4e). On the other hand, *TrkC*-miR1 induced survival of U87 cells (Fig. 4i). Therefore, its knockdown resulted in reduction of attributed survival rate compared to the untransfected control cells (Fig. 4h). Overall, it is generally accepted that miRNAs may target a cluster of hundred genes [49] and, different apoptotic effect of *TrkC*-miR1 in U87 and HEK293t cells may be due to the differences in genetic and epigenetic background of each cell line [2, 4, 49], cellular environment, context of the cell

or lack of partner(s) or target(s) of miRNA in each cell line [18, 50, 51].

Expression alteration of *TrkC*-mir1 during the NT2 cell differentiation

Since *TrkC* gene expression has been reported in neural cell differentiation [1], *TrkC*-miR1 expression pattern was also investigated during the course of neural differentiation of NT2 cell line to the neural-like cells [15]. While *TrkC*-miR1-5p was highly expressed at the third week of differentiation process, its precursor and *TrkC*-miR1-3p expression levels were not changed and *TrkC* gene expression was significantly reduced (Fig. 5). Again, this result suggests but is not evident yet that *TrkC*-miR1 is driven by an independent promoter. Accumulation of *TrkC*-miR1-5p in differentiated cells is similar to the accumulation of some miRNAs which are involved in prevention of return to the previous states [52, 53]. Consistently, suppressing *TrkC*-miR1-5p attenuated NT2 differentiation (Fig. 5c) that supports a part for the miRNA in neural differentiation process. *TrkC*-miR1-5p sharp expression alteration in these cells may establish sharp boundaries between "on" and "off" target genes expression during the advanced stages of development [54]. Consistently, several predicted target genes of *TrkC*-miR1-5p have been downregulated during the NT2 differentiation [55–57]. It remained to be tested if other predicted target genes of this miRNA are expressed in reverse correlation.

Conservation of *TrkC*-miR1-5p in rat tissues

Bioinformatics tools showed that *TrkC*-premir1 orthologous in rat genome is capable of producing stem loop structure and *TrkC*-miR1-5p is highly conserved in mammals. Therefore, human *TrkC*-miR1-5p-specific primer was capable of detecting this miRNA in rat tissues (Fig. 6). Trks like other related genes are primarily expressed in neural lineages [58–60] although the pattern is complex and includes non-neuronal cells [60]. Here, we detected *TrkC* gene expression in both 16-day-old embryo and newborn rat brain, particularly in the cerebrum (Fig. 6a, b). Although the gene expression alterations were not significant, results of current research (Fig. 6b) seemed consistent to the reports about downregulation of *TrkC* during the rat brain development [59]. Unlike *TrkC*, the highest *TrkC*-miR1-5p expression level was detected in the cerebellum again suggesting an independent promoter for this miRNA (Fig. 6a). Further evidence for the presence of independent promoter came from HCT116 cells treated with *5-azacytidine* epidrug. By default, *TrkC* gene is expressed in a very low level as a result of promoter methylation in HCT116 cells [2, 4]. *TrkC* gene expression level was increased 100 times in the HCT116 cells treated with *5-azacytidine* while, *TrkC*-miR1-5p expression level was significantly decreased (Fig. 7). Overall, accumulative evidences support the presence of an independent promoter for *TrkC*-miR1 novel miRNA.

In conclusion, here we have discovered a novel conserved microRNA, *TrkC*-miR1, located within the *TrkC* gene. Endogenous *TrkC*-miR1 was detectable in glioma tissue specimens, and its overexpression in U87 glioblastoma cell line had strong correlation with cell survival. The survival effect of this miRNA was in consistence with the effect of the *TrkC* host gene in the U87 cell line. It remains to be tested in more glioma cancerous samples if this miRNA has the suitability to be used as a tumor marker.

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Conflict of interest The authors declare that there are no conflicts of interest with any financial organization regarding the material discussed in the manuscript.

References

1. Reichardt LF (2006) Neurotrophin-regulated signalling pathways. Philos Trans R Soc B Biol Sci 361:1545–1564
2. Luo Y, Kaz AM, Kanngurn S, Welsch P, Morris SM et al (2013) NTRK3 is a potential tumor suppressor gene commonly inactivated by epigenetic mechanisms in colorectal cancer. PLoS Genet 9:e1003552
3. McGregor LM, McCune BK, Graff JR, McDowell PR, Romans KE et al (1999) Roles of trk family neurotrophin receptors in medullary thyroid carcinoma development and progression. Proc Natl Acad Sci 96:4540–4545
4. Genevois AL, Ichim G, Coissieux MM, Lambert MP, Lavial F et al (2013) Dependence receptor TrkC is a putative colon cancer tumor suppressor. Proc Natl Acad Sci USA 110:3017–3022
5. Jin W, Kim GM, Kim MS, Lim MH, Yun C et al (2010) TrkC plays an essential role in breast tumor growth and metastasis. Carcinogenesis 31:1939–1947
6. Aranha MM, Santos DM, Solá S, Steer CJ, Rodrigues CM (2011) miR-34a regulates mouse neural stem cell differentiation. PLoS ONE 6:e21396
7. Krol J, Loedige I, Filipowicz W (2010) The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet 11:597–610
8. Wang Z (2010) MicroRNA: a matter of life or death. World J Biol Chem 1:41
9. Wang Y, Lee CG (2009) MicroRNA and cancer–focus on apoptosis. J Cell Mol Med 13:12–23
10. Chen J, Zhang X, Lentz C, Abi-Daoud M, Paré GC et al (2011) miR-193b regulates Mcl-1 in melanoma. Am J Pathol 179:2162–2168
11. Lal A, Navarro F, Maher C, Maliszewski LE, Yan N et al (2009) miR-24 inhibits cell proliferation by suppressing expression of E2F2, MYC and other cell cycle regulatory genes by binding to "seedless" 3′ UTR microRNA recognition elements. Mol Cell 35:610

12. Miranda KC, Huynh T, Tay Y, Ang Y-S, Tam W-L et al (2006) A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes. Cell 126:1203–1217
13. Berezikov E, Cuppen E, Plasterk RH (2006) Approaches to microRNA discovery. Nat Genet 38:S2–S7
14. Berezikov E, Guryev V, van de Belt J, Wienholds E, Plasterk RH et al (2005) Phylogenetic shadowing and computational identification of human microRNA genes. Cell 120:21–24
15. Andrews PW (1984) Retinoic acid induces neuronal differentiation of a cloned human embryonal carcinoma cell line in vitro. Dev Biol 103:285–293
16. Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor
17. Xu N, Papagiannakopoulos T, Pan G, Thomson JA, Kosik KS (2009) MicroRNA-145 regulates OCT4, SOX2, and KLF4 and represses pluripotency in human embryonic stem cells. Cell 137:647–658
18. Parsi S, Soltani BM, Hosseini E, Tousi SE, Mowla SJ (2012) Experimental verification of a predicted intronic microRNA in human NGFR gene with a potential pro-apoptotic function. PLoS ONE 7:e35561
19. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408
20. Mestdagh P, Van Vlierberghe P, De Weer A, Muth D, Westermann F et al (2009) A novel and universal method for microRNA RT-qPCR data normalization. Genome Biol 10:R64
21. Dalmay T (2008) MicroRNAs and cancer. J Intern Med 263:366–375
22. Abbott AL, Alvarez-Saavedra E, Miska EA, Lau NC, Bartel DP et al (2005) The let-7 microRNA family members mir-48, mir-84, and mir-241 function together to regulate developmental timing in *Caenorhabditis elegans*. Dev Cell 9:403–414
23. Gomes CP, Cho JH, Hood L, Franco OL, Pereira RW et al (2013) A review of computational tools in microRNA discovery. Front Genet 4:81
24. Guo L, Lu Z (2010) The fate of miRNA* strand through evolutionary analysis: implication for degradation as merely carrier strand or potential regulatory molecule? PLoS ONE 5:e11387
25. Ellwanger DC, Büttner FA, Mewes H-W, Stümpflen V (2011) The sufficient minimal set of miRNA seed types. Bioinformatics 27:1346–1350
26. Shin C, Nam J-W, Farh KK-H, Chiang HR, Shkumatava A et al (2010) Expanding the microRNA targeting code: functional sites with centered pairing. Mol Cell 38:789–802
27. Hsieh L-C, Lin S-I, Shih AC-C, Chen J-W, Lin W-Y et al (2009) Uncovering small RNA-mediated responses to phosphate deficiency in Arabidopsis by deep sequencing. Plant Physiol 151:2120–2132
28. Li B, Duan H, Li J, Deng XW, Yin W et al (2013) Global identification of miRNAs and targets in *Populus euphratica* under salt stress. Plant Mol Biol 81:525–539
29. Winter J, Jung S, Keller S, Gregory RI, Diederichs S (2009) Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol 11:228–234
30. Dangwal S, Thum T (2013) MicroRNAs in platelet physiology and pathology. Hamostaseologie 33:17–20
31. Xiao-Chun W, Wei W, Zhu-Bo Z, Jing Z, Xiao-Gang T et al (2013) Overexpression of miRNA-21 promotes radiation-resistance of non-small cell lung cancer. Radiat Oncol 8:146
32. Linderoth J, Edén P, Ehinger M, Valcich J, Jerkeman M et al (2008) Genes associated with the tumour microenvironment are differentially expressed in cured versus primary chemotherapy-refractory diffuse large B-cell lymphoma. Br J Haematol 141:423–432
33. Aparicio O, Carnero E, Abad X, Razquin N, Guruceaga E et al (2010) Adenovirus VA RNA-derived miRNAs target cellular genes involved in cell growth, gene expression and DNA repair. Nucleic Acids Res 38:750–763
34. Koh YH, Suzuki K, Che W, Park YS, Miyamoto Y et al (2001) Inactivation of glutathione peroxidase by NO leads to the accumulation of H_2O_2 and the induction of HB-EGF via c-Jun NH2-terminal kinase in rat aortic smooth muscle cells. FASEB J 15:1472–1474
35. Korpal M, Kang Y (2010) Targeting the transforming growth factor-beta signalling pathway in metastatic cancer. Eur J Cancer 46:1232–1240
36. Moore-Smith L, Pasche B (2011) TGFBR1 signaling and breast cancer. J Mammary Gland Biol Neoplasia 16:89–95
37. Yamamoto M, Sobue G, Yamamoto K, Mitsuma T (1996) Expression of mRNAs for neurotrophic factors (NGF, BDNF, NT-3, and GDNF) and their receptors (p75 NGFR, TrkA, TrkB, and TrkC) in the adult human peripheral nervous system and non-neural tissues. Neurochem Res 21:929–938
38. Brodeur GM, Nakagawara A, Yamashiro DJ, Ikegaki N, Liu X-G et al (1997) Expression of TrkA, TrkB and TrkC in human neuroblastomas. J Neurooncol 31:49–56
39. Wang Y, Hagel C, Hamel W, Müller S, Kluwe L et al (1998) Trk A, B, and C are commonly expressed in human astrocytes and astrocytic gliomas but not by human oligodendrocytes and oligodendroglioma. Acta Neuropathol 96:357–364
40. Lomen-Hoerth C, Shooter EM (1995) Widespread neurotrophin receptor expression in the immune system and other nonneuronal rat tissues. J Neurochem 64:1780–1789
41. Segal RA, Goumnerova LC, Kwon YK, Stiles CD, Pomeroy SL (1994) Expression of the neurotrophin receptor TrkC is linked to a favorable outcome in medulloblastoma. Proc Natl Acad Sci 91:12867–12871
42. Vickers KC, Sethupathy P, Baran-Gale J, Remaley AT (2013) Complexity of microRNA function and the role of isomiRs in lipid homeostasis. J Lipid Res 54:1182–1191
43. Monteys AM, Spengler RM, Wan J, Tecedor L, Lennox KA et al (2010) Structure and activity of putative intronic miRNA promoters. RNA 16:495–505
44. Ozsolak F, Poling LL, Wang Z, Liu H, Liu XS et al (2008) Chromatin structure analyses identify miRNA promoters. Genes Dev 22:3172–3183
45. Qin Y, Yu Y, Dong H, Bian X, Guo X et al (2012) MicroRNA 21 inhibits left ventricular remodeling in the early phase of rat model with ischemia-reperfusion injury by suppressing cell apoptosis. Int J Med Sci 9:413
46. Rong M, Chen G, Dang Y (2013) Increased MiR-221 expression in hepatocellular carcinoma tissues and its role in enhancing cell growth and inhibiting apoptosis in vitro. BMC Cancer 13:21
47. Eguchi M, Eguchi-Ishimae M, Tojo A, Morishita K, Suzuki K et al (1999) Fusion of ETV6 to neurotrophin-3 receptor TRKC in acute myeloid leukemia with t (12; 15)(p13; q25). Blood 93:1355–1363
48. Almeida MI, Nicoloso MS, Zeng L, Ivan C, Spizzo R et al (2012) Strand-specific miR-28-5p and miR-28-3p have distinct effects in colorectal cancer cells. Gastroenterology 142(886–896):e889
49. Zhu S, Wu H, Wu F, Nie D, Sheng S et al (2008) MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. Cell Res 18:350–359
50. Chan JA, Krichevsky AM, Kosik KS (2005) MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. Cancer Res 65:6029–6033
51. Cheng AM, Byrom MW, Shelton J, Ford LP (2005) Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. Nucleic Acids Res 33:1290–1297

52. Ebert MS, Sharp PA (2012) Roles for microRNAs in conferring robustness to biological processes. Cell 149:515–524
53. Gardner TS, Cantor CR, Collins JJ (2000) Construction of a genetic toggle switch in *Escherichia coli*. Nature 403:339–342
54. Levine E, McHale P, Levine H (2007) Small regulatory RNAs may sharpen spatial expression patterns. PLoS Comput Biol 3:e233
55. Freemantle SJ, Kerley JS, Olsen SL, Gross RH, Spinella MJ (2002) Developmentally-related candidate retinoic acid target genes regulated early during neuronal differentiation of human embryonal carcinoma. Oncogene 21:2880–2889
56. Valerio A, Ferrario M, Martinez FO, Locati M, Ghisi V et al (2004) Gene expression profile activated by the chemokine CCL5/RANTES in human neuronal cells. J Neurosci Res 78:371–382
57. Wakamatsu A, J-i Imai, Watanabe S, Isogai T (2010) Alternative splicing of genes during neuronal differentiation of NT2 pluripotential human embryonal carcinoma cells. FEBS Lett 584:4041–4047
58. Lamballe F, Smeyne R, Barbacid M (1994) Developmental expression of trkC, the neurotrophin-3 receptor, in the mammalian nervous system. J Neurosci 14:14–28
59. Ringstedt T, Lagercrantz H, Persson H (1993) Expression of members of the trk family in the developing postnatal rat brain. Dev Brain Res 72:119–131
60. Tessarollo L, Tsoulfas P, Martin-Zanca D, Gilbert DJ, Jenkins NA et al (1993) trkC, a receptor for neurotrophin-3, is widely expressed in the developing nervous system and in non-neuronal tissues. Development 118:463–475