Supporting Information

Thymosin beta 4 up-regulates miR-200a expression and induces differentiation and survival of rat brain progenitor cells.

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Running Title: Thymosin beta 4 up-regulates miR-200a

Supporting Information

Supplementary Methods

Immunohistochemistry

Immuno-fluorescence staining was performed on primary rat embryonic OPCs, as previously described (Santra M 2014). These cells were fixed with 4% paraformaldehyde for 1h, washed with PBS, blocked with 1% serum for 1h and incubated with monoclonal antibodies of OPC specific marker O4, (1:1000, Chemicon, Billerica, MA, USA for one hour, rinsed with PBS and secondary antibodies labeled with fluorescein isothiocyanate (FITC- green fluorescence) for 1h. The slides were counterstained with 4', 6- diamidino-2-phenylindole (DAPI- blue fluorescence) and examined under Fluorescent Illumination Microscope (Olympus IX71/IX51, Tokyo, Japan). The O4 positive cells were quantified by counting in at least three slides per experiment. DAPI positive cells were considered as total number of cells. The OPCs which achieved greater than 90% O4 positive were utilized for all experiments.

For paraffin embedded slides, rats were anesthesized and sacrificed on day 58 after MCAO, as previously described (Santra M 2010). Perfusion of the animals, removal and fixation of brain tissue, sectioning of brain tissues into seven coronal blocks (1 mm thick), and processing for paraffin-embedded slides were performed, as previously described (Santra et al., 2006a, 2009). These paraffin-embedded slides were immunostained with DAB for FOG-2 (1:500; Santa Cruz Biotechnology, Dallas, Texas, USA) and rabbit polyclonal antibodies (1:200) for Grb2, Mig-6, Pten, p53 (Cell Signaling Technology, Danver, MA, USA), as previously described (Santra et al., 2006a, 2009).

Oxygen Glucose Deprivation (OGD) and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

The cells were cultured for 4 days. On day 2, the cells were rinsed twice with serum free and glucose free media. The cells were subjected to OGD by incubating for 4h with minimum volume of serum free and glucose free medium in hypoxia without oxygen in an airtight Plexiglas humidified chamber (Anaerobic Environment, Sheldon), as previously described (Santra M 2006a, Santra M 2006b). This anaerobic chamber was maintained at 37°C and continuously gassed with a mixture of 99% N2/1% CO2/0% O2. After OGD for 4h, the cells were returned to normal condition and Tβ4 treatment by replacing serum free and glucose free medium with the same medium as cultured on day 1, and by incubating in aerobic chamber at 37°C for 2 more days before apoptosis analysis. On day 5, the cells were fixed with 4.5% para-formaldedyde solution, andTUNEL assay was performed to examine apoptosis as follows.

TUNEL staining was performed by using the Apoptosis Detection Kit, ApopTag Peroxidase according to the manufacturer's protocol (EMD Millipore, Billerica, MA, USA), as previously described (Santra *et al.* 2006, Santra M 2012). Color was developed after addition of diaminobenzidine (DAB) peroxidase substrate. Apoptosis was quantified by counting DAB stained TUNEL positive cells.

Quantitative real time PCR (qrtPCR)

The extraction of total RNA and preparation of cDNA were performed, as previously described (Santra *et al.* 2011). The QrtPCR amplification was performed for 40 cycles in the following thermal cycle: 95^0C for 30 s, 60^0C. The QrtPCR amplification was done for 40 cycles in the following thermal cycle: 95^0C for 30s, 60^0C for 30s, and 72^0C for 45s using SYBR green (Life Technologies, Grand Island, NY). The sequences for each primer are used, as previously reported (Santra *et al.* 2012) and provided in Supplementary Table-1. Each sample was tested in triplicate and all values were normalized to GAPDH. Values obtained from three independent

experiments were analyzed relative to gene expression data using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001).

Preparation of lucifase constructs and assay

The oligonucleotide sequences of microRNA-200 (miR-200) target site in 3' untranslated region (3'UTR) of Mig-6 mRNA were selected based on TargetScan Analysis (http://www.mirbase.org), as follows.

1. Conserved oligo: 5'-CTAGCGGCCGCTGGTTCCTATTCAGTATTTCCTGGGGATTG-3' (100% homologous to human, rat and mouse);
2. Mutated oligo: 5'-CTAGCGGCCGCTGGTTCCTATTACTCGCGTCCTGGGGATTG-3'
   The sequence (ACTCGCG) in red font is mismatched/ mutated for miR-200 target site in 3'UTR of Mig-6 mRNA.

These oligonucleotide sequences contain 5'-Nhe I, 3'-Sal I, and Not I (next to Nhe I for clonal selection) restriction sites. These oligos ware sub-cloned in pmiRGLO vector (Promega Corporation, WI, USA), carrying the firefly luciferase gene (luc2) under the control of the PGK promoter and the internal control renilla luciferase (hRluc) under the control of the SV40 promoter. These sub-clones were confirmed by DNA sequencing in GENEWIZ, South Plainfield, NJ, USA. For the luciferase assay, 50 ng of plasmid were transfected in SVZ-neurospheres and primary OPCs in 100µl of electroporation solution (Mirus Bio, Madison, WI, USA). In some experiments, as reported in the Results section, these cells were also co-transfected with 50ng of control lentiviral vector and lentiviral vectors carrying miR-200 cluster genes (Gregory PA 2008) e.g. miR-200b-200a-429 and miR-200c-141 (addgene, Cambridge, MA, USA). The appropriate Nucleofector Program for Nucleofector I Device (O-03 for SVZ-neurospheres and X-01 for OPCs was selected. The transfected cells were suspended in appropriate growth medium, plated in 96 well plate and incubated in a humidified 37°C incubator. After 24 hours, Dual Luciferase assay was performed according to manufacturer's protocol (Promega

Corporation, WI, USA) to detect firefly luciferase and renilla luciferase activity in luminometer (Perkin Elmer, Walthan, MA, USA).

References

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Suppementary Table

Table-1: Sequences of oligonucleotide primers used in qrtPCR Analysis.

Primer	Forward sequence	Reverse sequence
EGFR	5′-ACCGTGGAGAGAATCCCTTT-3′	5′-TTGTTGCTAAATCGCACAGC-3′
Mig-6	5′-AAGCAGAAGAAGCAAACCCA-3′	5′-TCCATTTTCATTCTGGAGGC-3′
Grb2	5′-CGATGAGCTGAGCTTCAAGA-3′	5′-GAAGCCATCTTTCCCATTGA-3′
FOG-2	5′-TTGAAGACGAAGGCTCAGGT-3′	5′-TGCAGTTGTTTTTGCTGTCC-3′
Pten	5′-ACCAGGACCAGAGGAAACCT-3′	5′-CTTGTCATTATCCGCACGCT-3′
P53	5′-CTTCGAGATGTTCCGAGAGC-3′	5′-CTTCGGGTAGCTGGAGTGAG-3′
GAPDH	5′-AGAACATCATCCCTGCATCC-3′	5′-CACATTGGGGGTAGGAACAC-3′

Supplementary Figures

Fig. S1

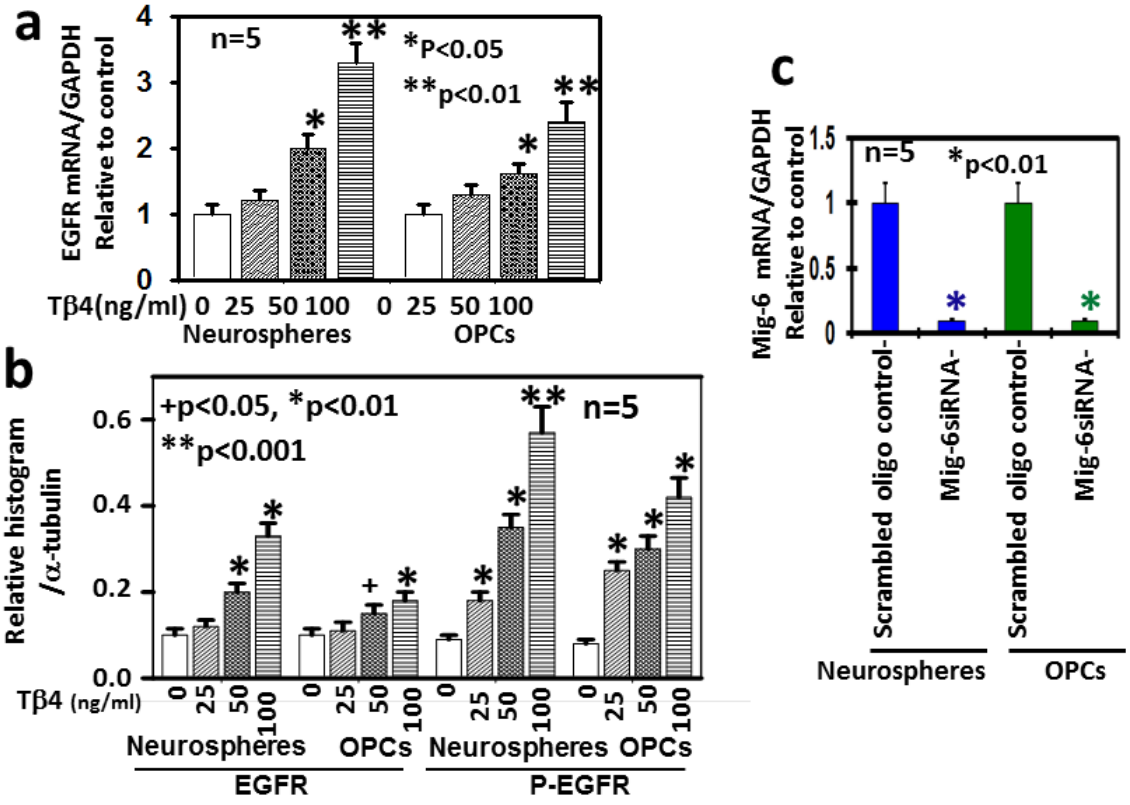


Fig. S2

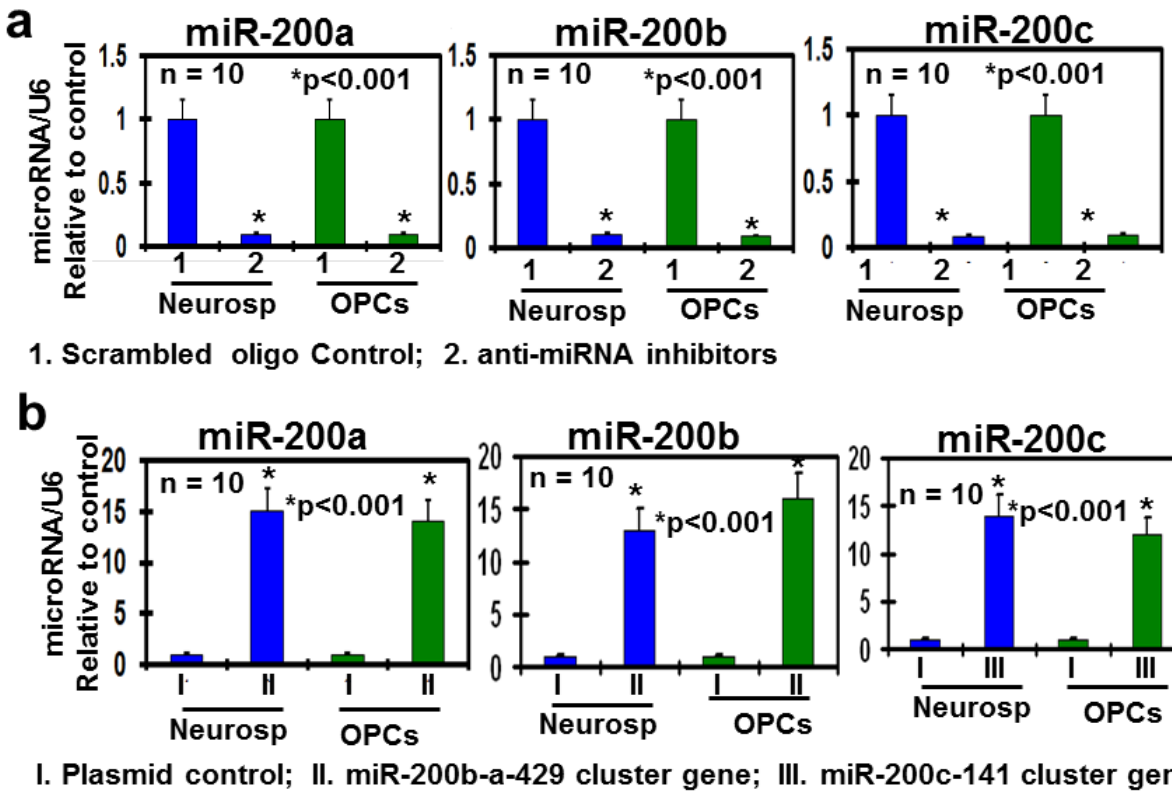

a
miR-200a
miR-200b
miR-200c
microRNA/U6
Relative to control
n = 10
*p<0.001
Neurosp
OPCs
1. Scrambled oligo Control; 2. anti-miRNA inhibitors
b
I. Plasmid control; II. miR-200b-a-429 cluster gene; III. miR-200c-141 cluster gene

Fig. S3

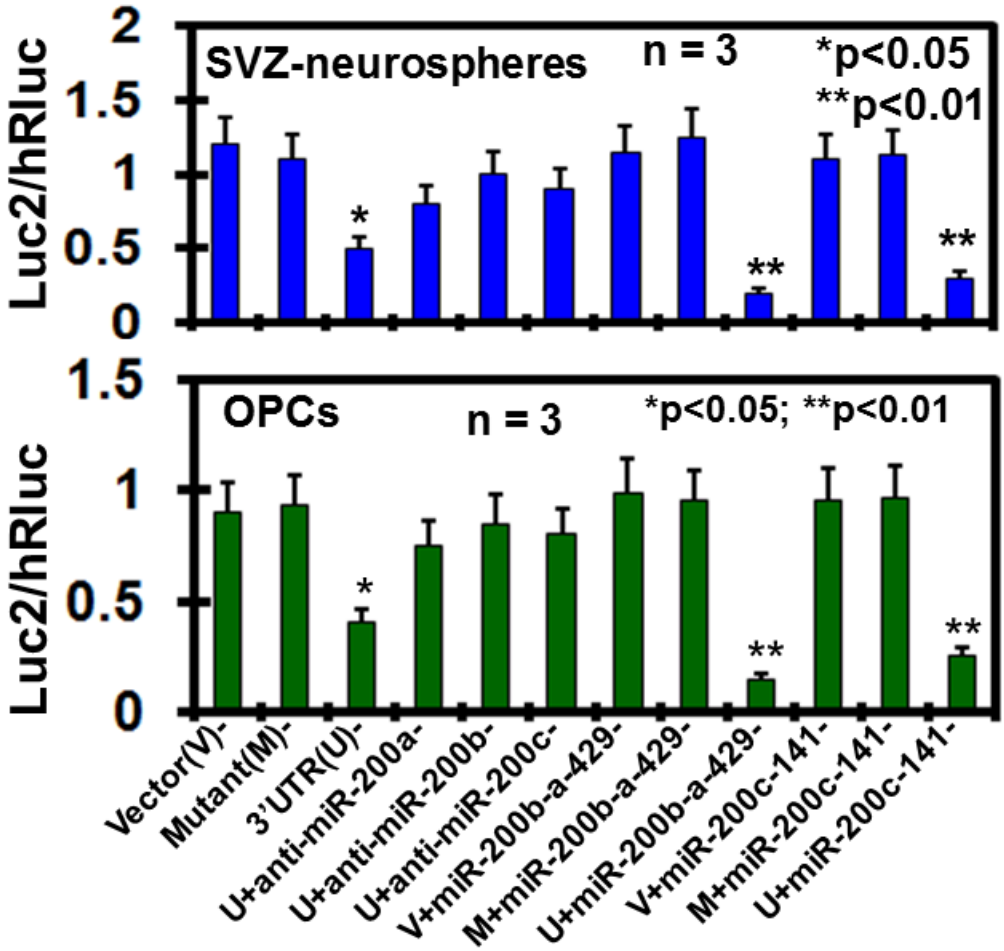

SVZ-neurospheres
n = 3
*p<0.05
**p<0.01
Luc2/hRluc
2
1.5
1
0.5
0
OPCs
n = 3
*p<0.05; **p<0.01
Luc2/hRluc
1.5
1
0.5
0
Vector(V)-
Mutant(M)-
3'UTR(U)-
U+anti-miR-200a-
U+anti-miR-200b-
U+anti-miR-200c-
V+miR-200b-a-429-
M+miR-200b-a-429-
U+miR-200b-a-429-
V+miR-200c-141-
M+miR-200c-141-
U+miR-200c-141-

Fig. S4

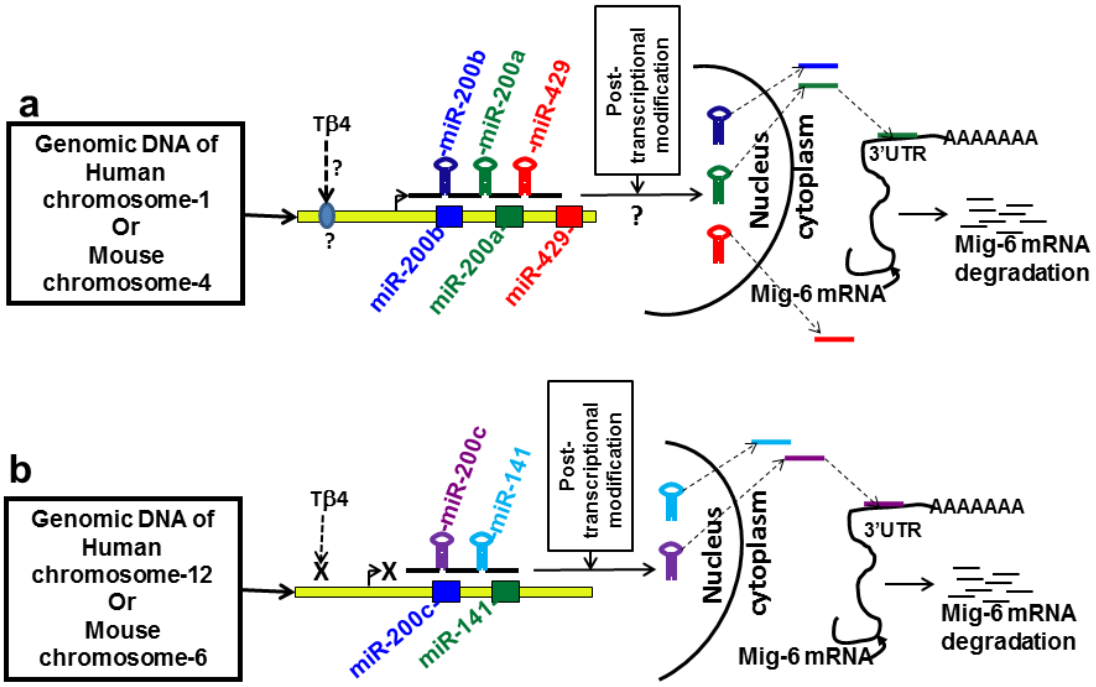

a
Genomic DNA of Human chromosome-1 Or Mouse chromosome-4
Tβ4
?
?
miR-200b
miR-200a
miR-429
miR-200b
miR-200a
miR-429
Post-transcriptional modification
?
Nucleus
cytoplasm
AAAAAAA
3'UTR
Mig-6 mRNA
Mig-6 mRNA degradation
b
Genomic DNA of Human chromosome-12 Or Mouse chromosome-6
Tβ4
X
X
miR-200c
miR-141
miR-200c
miR-141
Post-transcriptional modification
Nucleus
cytoplasm
AAAAAAA
3'UTR
Mig-6 mRNA
Mig-6 mRNA degradation

Fig. S5

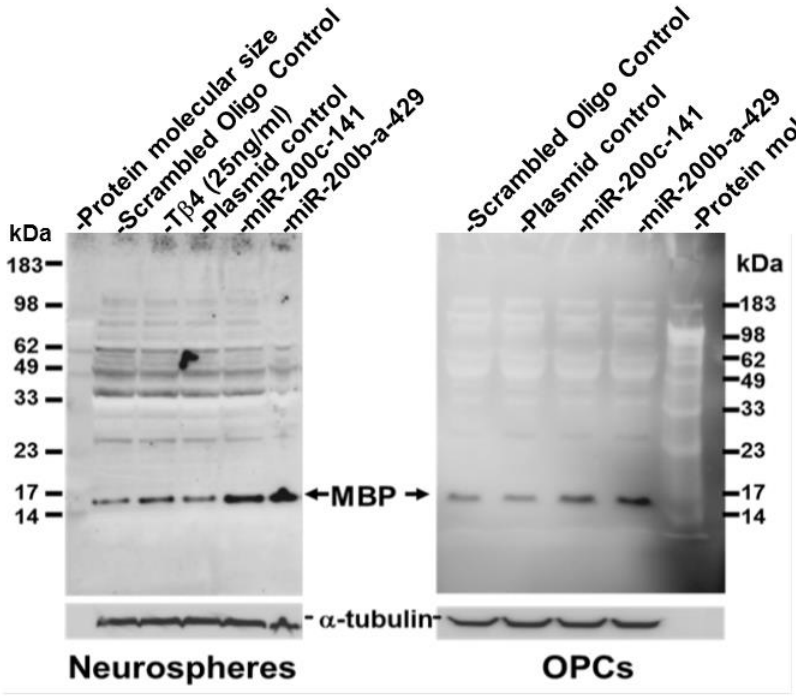

-Protein molecular size
-Scrambled Oligo Control
-Tβ4 (25ng/ml)
-Plasmid control
-miR-200c-141
-miR-200b-a-429
-Scrambled Oligo Control
-Plasmid control
-miR-200c-141
-miR-200b-a-429
-Protein molecular size
kDa
183
98
62
49
33
23
17
14
MBP
α-tubulin
kDa
183
98
62
49
33
23
17
14
Neurospheres
OPCs

Fig. 6

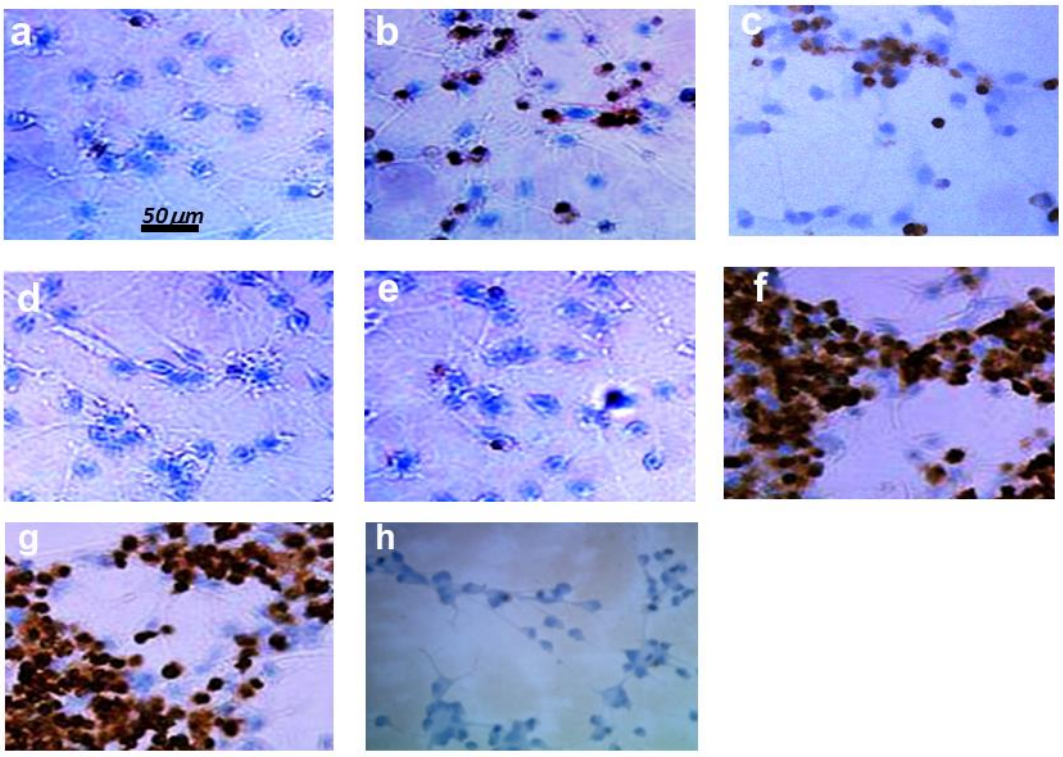


Fig. 7

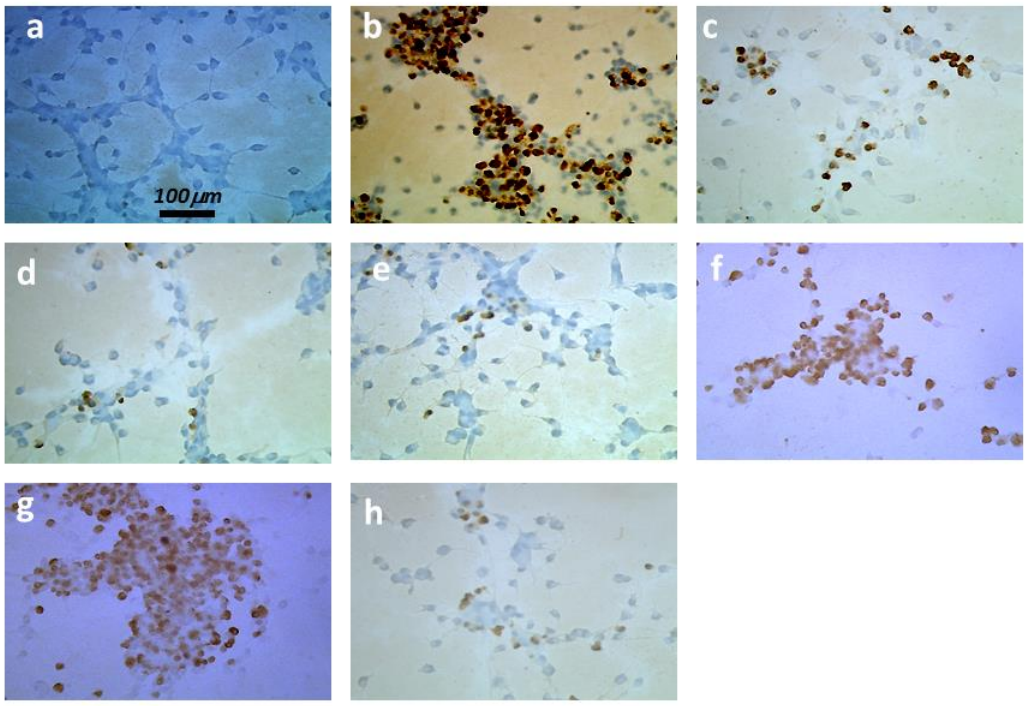
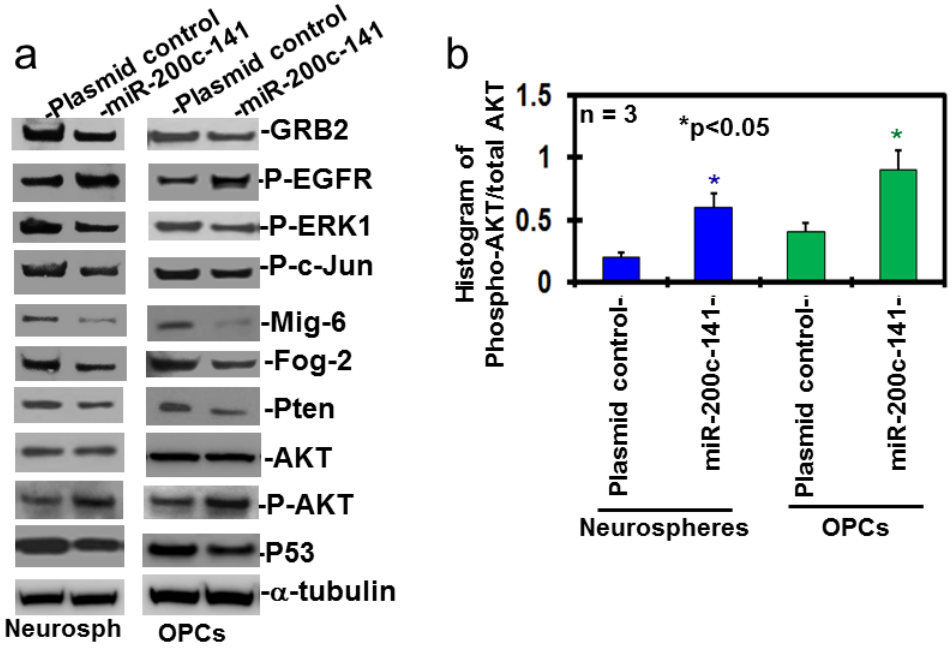


Fig. S8


a
-Plasmid control
-miR-200c-141
-Plasmid control
-miR-200c-141
-GRB2
-P-EGFR
-P-ERK1
-P-c-Jun
-Mig-6
-Fog-2
-Pten
-AKT
-P-AKT
-P53
-α-tubulin
Neurosph
OPCs
b
Histogram of Phospho-AKT/total AKT
1.5
1
0.5
0
n = 3
*p<0.05
*
*
Plasmid control-
miR-200c-141-
Plasmid control-
miR-200c-141-
Neurospheres
OPCs

Legends to supplementary figures

Fig. S1 Tβ4 treatment induces expression of EGFR in rat SVZ progenitor cells and rat embryonic primary OPCs in a dose dependent manner. The adult rat SVZ neurospheres/progenitor cells (Neurospheres) and rat embryonic primary OPCs (OPCs) were treated with Tβ4 at the dose of 0, 25, 50 and 100ng/ml in five independent experiments (n=5). After treatment, we analyzed EGFR mRNA expression by qrtPCR (a) and, expression of EGFR protein and phosphorylated EGFR (P-EGFR) by Western blot. Western blot data were quantified by histogram analysis (b) in Photoshop in five independent experiments (n=5). c) The qrtPCR was analyzed for Mig-6 in the adult rat SVZ neurospheres/progenitor cells (Neurospheres) and rat embryonic primary OPCs (OPCs) after transfection with scrambled oligo-nucleotide control (Oligo control) and Mig-6siRNA in five independent experiments (n=5). The p-value <0.05 was considered as significantly different.

Fig. S2 Measurement of knockdown and overexpression efficiency of miR-200 by qrtPCR. The SVZ-neurospheres (blue bars; Neurosp) and embryonic primary OPCs (green bars; OPCs) were transfected with scrambled oligo nucleotides, as scrambled oligo control (1) and anti-miR™ miRNA inhibitor corresponding to miR-200a, -200b and -200c as anti-miRNA inhibitor e.g. anti-miR-200a, -200c and -200c (2) in order to knockdown the expression of miR-200a, -200b and -200c, respectively. To determine knockdown efficiency after transfection of anti-miRNA inhibitors, individual microRNA (e.g. miR-200a, miR-200b and miR-200c as shown at the top of bar graph) was analyzed by qrtPCR in the top panel (a). In the bottom panel (b), the SVZ-neurospheres (blue bars; Neurosp) and embryonic primary OPCs (green bars; OPCs) were transfected with control lentiviral vector without insert as plasmid control (I), and lentiviral vector carrying miR-200b-a-429 cluster gene (II) and miR-200c-141cluster gene (III). To examine overexpression efficiency after cluster gene transfection, miR-200a and miR-200b

were measured after transfection of miR-200b-a-429 cluster gene (II) and, miR-200c was measured after transfection of miR-200c-141cluster gene (III) by qrtPCR .

Fig. S3 Luciferase assay for the reporter gene constructs of the pmirGLO vector carrying miR-200 target site in 3'UTR of Mig-6. The pmirGLO vector (Vector), the pmirGLO vector construct carrying miR-200 target sequence from 3'UTR of Mig-6 (3'UTR) and the pmirGLO vector construct containing mismatched miR-200 target sequence from 3'UTR of Mig-6 (Mutant) were transfected in SVZ-neurospheres (Top bar graph) and embryonic primary OPCs (Bottom bar graph) followed by luciferase (luc2)/ renilla luciferase (hRluc) analysis to quantify direct target of miR-200 in 3'UTR of Mig-6 in three independent experiments. These pmirGLO vector construct (3'UTR) transfected cells were co-transfected with anti-miR-200a or anti-miR-200b or anti-miR-200c followed by luciferase (luc2)/ renilla luciferase (hRluc) analysis to determine whether anti-miR-200 transfections reverse direct target of mir-200 in 3'UTR of Mig-6 in three independent experiments. The pmirGLO vector and the pmirGLO vector constructs (3'UTR and mutant) transfected cells were co-transfected with Lenti-miR-200b-a-429 cluster gene or Lenti-miR-200c-141 cluster gene followed by luciferase (luc2)/ renilla luciferase (hRluc) analysis to examine whether overexpression of miR-200 enhances the effect of direct target of mir-200 to 3'UTR of Mig-6 in three independent experiments. The p-value <0.05, was considered as significantly different.

Fig. S4 Schematic diagram of a single tricistronic primary miR-200b-a-429 cluster gene and a single bicistronic primary miR-200c-141 cluster gene. In the nucleus, genomic DNA of human chromosome-1 or mouse chromosome-4 transcripts a single tricistronic primary miR-200b-a-429 cluster mRNA encoding miR-200b, miR-200a and miR-429 and genomic DNA of human chromosome-12 or mouse chromosome-6 contains a single bicistronic primary miR-200c-141 cluster gene encoding miR-200c and miR-141. These polycistronic transcripts undergo post transcriptional modifications and are processed into pre-miRNAs which are then

exported to the cytoplasm and further processed into mature microRNAs. Tβ4 treatment may promote unknown (?) transcription factor to bind and activate promoter of tricistronic primary miR-200b-a-429 cluster gene by unknown mechanism (?) and drive transcription. In contrast, this Tβ4-induced transcription factor may fail to bind (X) and activate (X) promoter of bicistronic primary miR-200c-141 cluster gene, because specific sequences for binding of transcription factor may be missing in this promoter of bicistronic primary miR-200c-141 cluster gene.

Fig. S5 Western blot of MBP expression using whole blot in SVZ-neurosphere and embryonic primary OPCs. SVZ-neurosphere and embryonic primary OPCs were transfected with scrambled oligonucleotides (scrambled oligo control), control lentiviral vector without insert (Plasmid control), and lentiviral vectors carrying miR-200c-141 cluster gene (miR-200c-141) and miR-200b-a-429 cluster gene (miR-200b-a-429). Pre-stained protein molecular weight standard (protein molecular size) and the protein samples were separated in 10% SDS-poly-acrylamide gel for Western blot analysis. Note that the strongest protein band which ran at ~15 kDa (i.e. molecular weight of MBP) after blotting with MBP anti-body confirmed specificity of MBP antibody. Both controls- scrambled oligo and plasmid transfection showed the same amount of MBP expression. In contrast, MBP expression was elevated after miR-200c-141 and miR-200b-a-429 cluster gene transfection in SVZ-neurosphere and embryonic primary OPCs.

Fig. S6 TUNEL staining of neurospheres/progenitor cells from adult ischemic rat SVZ. TUNEL stainings with DAB were performed in the following samples. a) The adult rat SVZ neurospheres/progenitor cells were transfected with control plasmid and incubated for 4 days. b) The control cells (a) were subjected to OGD for 4h on day 2 during incubation for 4 days. c) The control cells (a) were treated with 50ng/ml Tβ4 for 4 days with exposure to OGD for 4h on day 2. d) The miR-200b-a-429 cluster transfected neurospheres/progenitor cells were subjected to OGD for 4h on day 2 during incubation for 4 days. e) The miR-200b-a-429 cluster transfected neurospheres/progenitor cells were treated with 50ng/ml Tβ4 for 4 days with

exposure to OGD for 4h on day 2. f) The anti-miR-200a transfected cells were subjected to OGD for 4h on day 2 during incubation for 4 days. g) The anti-miR-200a transfected cells were treated with 50ng/ml Tβ4 for 4 days with exposure to OGD for 4h on day 2. h) The miR-200c-141 cluster transfected neurospheres/progenitor cells were subjected to OGD for 4h on day 2 during incubation for 4 days.

Fig. S7 TUNEL staining of primary OPCs from the rat embryos on embryonic day 17. TUNEL staining of primary OPCs was performed in the following samples. The control plasmid transfected primary OPCs (a) and control cells (a) subjected to OGD for 4h on day 2 (b), were incubated for 4 days. The control cells (a) were treated with 50ng/ml Tβ4 for 4 days with exposure to OGD for 4h on day 2 (c). The miR-200b-a-429 cluster transfected OPCs (d) were treated with 50ng/ml Tβ4 for 4 days (e) subjected to OGD for 4h on day 2. The anti-miR-200a transfected cells (f) were treated with 50ng/ml Tβ4 for 4 days (g) subjected to OGD for 4h on day 2. h) The miR-200c-141 cluster transfected OPCs were subjected to OGD for 4h on day 2 during incubation for 4 days.

Fig. S8 Effect of miR-200c-141 cluster gene transfection on mediators of EGFR/ERK/c-Jun and EGFR/PI3K/AKT/p53 in SVZ-neurosphere and embryonic primary OPCs. Western blot analysis (a) was performed from total protein samples from SVZ-neurospheres (Neurosph) and embryonic primary OPCs (OPCs) after transfection with lentivival vector as control (plasmid control) and lentivival based miR-200c-141 cluster gene expression vector (miR-200c-141). Migration of proteins (mediators of EGFR signaling) was shown at right. Loading of the protein samples was normalized with α-tubulin. b) AKT activity was quanitified by ratio of phosphorylated AKT (P-AKT) and AKT in SVZ-neurospheres (Neurospheres) and embryonic primary OPCs (OPCs) from Western blot data in three independent experiments. The p-value <0.05, was considered as significantly different.