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Effects of Brain-Derived Neurotrophic Factor on MicroRNA Expression Profile in Human Endothelial Progenitor Cells

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Abstract

The mechanisms underlying proangiogenic function of brain-derived neurotrophic factor (BDNF) are not fully understood. The current study was designed to explore the microRNA (miRNA) profile in human early endothelial progenitor cells (EPCs, also referred to as CFU-Hill cells) treated with BDNF. Treatment of early EPCs with BDNF for 7 d significantly increased the colony formation of outgrowth endothelial cells. BDNF suppressed the expression of miR-4716-5p, miR-3928, miR-433, miR-1294, miR-1539, and miR-19b-1*. In contrast, BDNF significantly increased the levels of miR-432*, miR-4499, miR-3911, miR-1183, miR-4669, miR-636, miR-4717-3p, miR-4298, miR485-5p, and miR-181c. Since miR-433 has been reported to augment hematopoietic cells proliferation and differentiation, we examined the role of miR-433 in regenerative effects of BDNF. BDNF stimulated the protein expression of guanylate-binding protein 2 via the suppression of miR-433. However, the knockdown of miR-433 was not sufficient to significantly increase the number of outgrowth endothelial cell colonies, suggesting that modulation of miR-433 alone does not stimulate regenerative capacity of EPCs. In aggregate, our results also suggest that the effect of BDNF on regenerative function of EPCs may depend on complex changes in the expression of microRNAs.

Keywords

angiogenesis, endothelial progenitor cells, brain-derived neurotrophic factor, microRNA, miR-433

Circulating endothelial progenitor cells (EPCs[1]) play an important role in the recovery of cerebral vascular integrity after brain ischemia and exert stimulatory effects on neurogenesis[2]. Brain-derived neurotrophic factor (BDNF) has been shown to mobilize hematopoietic progenitor cells, enhance endothelial survival, and stimulate angiogenesis[3,4]. Our previous study has also demonstrated that BDNF increases MnSOD expression in EPCs[5]. Notably, BDNF production is increased in both hemispheres after unilateral ischemic stroke[6]. In addition, platelets release a large amount of BDNF upon activation at the site of injury, which may further increase BDNF content on the surface of blood vessels with damaged endothelium[7]. Therefore, it is conceivable that elevated local concentration of BDNF may exert beneficial effects on regenerative function of EPCs. To date, the effect of BDNF on microRNA (miRNA) profile in EPCs has not been studied. The present study was designed to determine the effects of BDNF on expression profile of miRNA in human EPCs.

Materials and Methods

Human EPC Isolation and Cell Culture

Peripheral blood mononuclear cells-containing apheresis donor cones (the blood components retained in the leukoreduction system chamber after plateletpheresis[8]) derived from 45 male subjects (47.1 $\pm$ 1.5 years old) were provided by Mayo Clinic, Division of Transfusion Medicine. The study was approved as an exempt from review by Institutional Review Board at the Mayo Clinic. Early EPCs (CFU-Hill cells[1]) were cultured and collected as described in a previous study[9]. Briefly, after the isolation of peripheral blood mononuclear cells from apheresis donor cones by density gradient centrifugation with Ficoll-Paque Plus

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(Amersham Biosciences Corp., Piscataway, NJ, USA), red blood cells were lysed, mononuclear cells were cultured on 6-well plates coated with human fibronectin (R&D systems Inc., Minneapolis, MN, USA) in endothelial growth medium 2 (EGM2, Lonza, Walkersville, MD, USA). Early EPCs were obtained at day 7 of cell culturing. Outgrowth endothelial colonies (Supplemental Fig. 1) were cultured and obtained as described in our previous study[10]. Early EPCs were cultured in EGM2 containing 100 ng/mL BDNF (Alomone Labs, Jerusalem, Israel; 6 wells per subject) or EGM2 alone (6 wells per subject) for 7 d. Culturing of cells in EGM2 was continued for another 16 d. Then the number of colonies was counted by an investigator (who was blinded) to identity of control and BDNF-treated cells.

miRNA Microarray and Analysis

Total RNA was isolated and purified from EPCs (of 4 subjects) treated with BDNF (100 ng/mL) for 7 h or EBM2 culture medium alone (control), using miRNeasy mini kit (Qiagen, Germantown, MD, USA). All RNA samples met the quality control standards for miRNA microarray conducted at Medical Genome Facility of Mayo Clinic at Rochester MN, USA. Affymetrix GeneChip miRNA-3.0 array (containing 1733 human miRNA probe sets, Affymetrix, Santa Clara, CA, USA) was used to examine miRNA expression in 8 samples, with 4 samples as the control group and 4 samples treated with BDNF. We used Partek Genomics Suite (Partek Incorporated, St. Louis, MO, USA) to perform the normalization (Robust Multi-array Analysis) and paired *t* test analysis (Log_2 scale). Differential expression between the 2 groups was determined by using filtering criteria of paired *t* test *P* values lower than 0.05 and fold changes equal and greater than 1.5. Additional information can be found in data repository in GEO PUBMED (GSE107667).

Small Interfering RNA and miRNA Mimic Treatment

EPCs were transfected with 80 nM small interfering RNA (siRNA) against miR-433 (miR-433-siR; target sequence 5′-TACGGTGAGCCTGTCATTATT-3′; Qiagen), AllStarts negative control siRNA (Ct-siR; target sequence 5′-CAGGGTATCGACGATTACAAA-3′; Qiagen), or 80 nM miR-433 mimic (human miR-433-3p, 5′- AUCAUGAUGGGCUCCUCGGUGU-3′; Qiagen), using HiPerfect transfection reagent (Qiagen), according to the manufacturer's protocol.

Quantitative Real Time (RT)-PCR

Total RNA was isolated using miRNeasy mini kit (Qiagen), and reverse transcribed using miScript II RT Kit (Qiagen). Hs_MiR-433_1 miScript Primer Assay (target sequence 5′-AUCAUGAUGGGCUCCUCGGUGU-3′) and miScript SYBR Green PCR Kit (Qiagen) were employed to determine mature human miR-433-3p levels. The amount of miR-433-3p to internal control human RNU6-2 (Hs_RNU6-2_11 miScript Primer Assay, Qiagen) was then calculated as described previously[11].

Western Blot

Western blot was conducted as previously described[11]. Rabbit anti-guanylate-binding protein 2 (GBP2) antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-cyclic adenosine monophosphate response element-binding protein-1 (CREB1) (48H2), rabbit anti-growth factor receptor-binding protein 2 (GRB2), and mouse anti-cyclin-dependent kinase 6 (CDK6) (DCS83) were obtained from Cell Signaling Technology (Danvers, MA, USA). Rabbit anti-γ glutamylcysteine synthetase (GCS) was purchased from NeoMarkers (Fremont, CA, USA). β-actin (Sigma-Aldrich) was used as a loading control.

Statistical analysis

For colony formation assay, quantitative RT-PCR, and Western blot, data are presented as mean ± standard error of the mean; "n" denotes the number of subjects from which cell samples were collected. Differences between mean values of 2 groups were compared using unpaired student *t* test. $P < 0.05$ was considered statistically significant.

Results

Treatment of human early EPCs with BDNF for 7 d significantly increased outgrowth endothelial colony formation (colony number: control group 2 ± 0.5; BDNF-treated group 4 ± 0.6, $n = 12$, $P < 0.05$; Fig. 1A). We next examined the miRNA profile in response to BDNF treatment. By using miRNA 3.0 array, we detected 1,716 mature miRNAs in human early EPCs. After running statistical analysis, we detected that 6 miRNAs were significantly decreased, and 10 miRNAs were significantly increased by at least 1.5-fold after BDNF treatment (100 ng/mL for 7 h; Table 1). Among them, miR-3928, miR-433, miR-636, miR-485-5p, and miR-181c have been reported to be involved in cell proliferation and/or differentiation (Supplemental Table 1). Since miR-433 has been shown to play an important role in angiogenesis and hematopoietic cells proliferation and differentiation (Supplemental Table 1), we next focused on the function of miR-433 in response to BDNF.

In human early EPCs, knockdown of miR-433 caused an upregulation in protein expression of GBP2 (Fig. 1B, C). In contrast, treatment with miR-433 mimic suppressed GBP2 expression (Fig. 1D). BDNF treatment increased GBP2 protein expression in early EPCs (Fig. 1E). These results indicated that BDNF stimulated GBP2 expression via suppression of miR-433. Treatment of human early EPCs with miR-433-siRNA or miR-433 mimic did not change protein expressions of other target genes including CDK6,

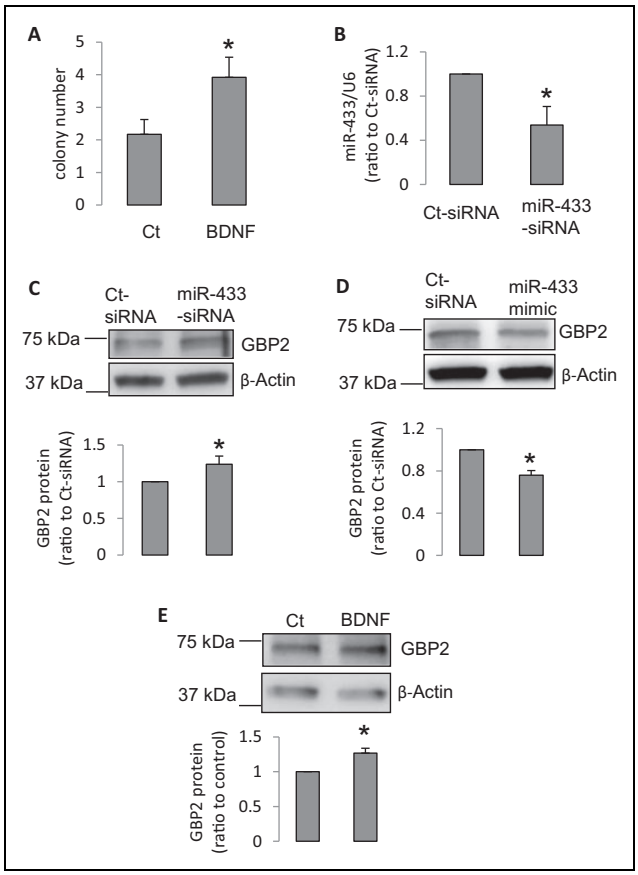

Fig 1. Brain-derived neurotrophic factor (BDNF) increased endothelial colony formation and induced guanylate-binding protein (GBP2) expression mediated by miR-433. A, Early endothelial progenitor cells (EPCs) were treated with BDNF 100 ng/mL for 7 d. Following the treatment, the cells were continued to be cultured in endothelial growth medium 2 (EGM2) for 16 d, and outgrowth colonies were counted ($n = 12$, $^*P < 0.05$). B, Early EPCs were treated with control-small interfering RNA (siRNA) or miR-433 siRNA (80 nM) for 30 h ($n = 6$, $^*P < 0.05$). C and D, Early endothelial progenitor cells (EPCs) were treated with 80 nM Ct-siRNA, miR-433-siRNA (C), or miR-433 mimic (D) for 48 h. $n = 6$ (C), $n = 4$ (D), $^*P < 0.05$. E, Early EPCs were treated with 100ng/mL BDNF for 24 h ($n = 4$, $^*P < 0.05$).

GRB2, GCS, and CREB1 (Supplemental Fig. 2). We further determined the role of miR-433 in endothelial colony formation. Knockdown of miR-433 by siRNA treatment had a tendency to increase endothelial colony formation but did not reach statistically significant difference (colony number: Ct-siRNA, 3 $\pm$ 0.5; miR-433-siRNA, 4 $\pm$ 0.7; $n = 26$; $P > 0.05$). These results indicate that inactivation of miR-433 alone is not sufficient to affect BDNF-induced endothelial colony formation.

Discussion

Our study is the first to demonstrate the stimulatory effect of BDNF on outgrowth endothelial colony formation that involves processes of differentiation and proliferation. Previous studies have shown that BDNF mobilizes hematopoietic progenitors and endothelial progenitor cells[4,12] and enhances the homing of stem cells[13]. We propose that miRNA may contribute to the regenerative function of BDNF. This mechanism may play an important role in the neurovascular regeneration during recovery from stroke when local concentration of BDNF is augmented[6]. Relevant to the current study, we have previously reported that early EPCs produce endogenous BDNF[14], thereby emphasizing an important role of autocrine effects of BDNF in the regenerative function of EPCs.

BDNF-induced alterations in miRNA profile have not been defined in EPCs. The present study reveals the alterations of 16 miRNAs in EPCs treated with BDNF. Five of them have been previously implicated in cell proliferation and differentiation of various cell types (Supplemental Table 1). For example, miR-3928 has been shown to stimulate osteosarcoma cell proliferation, and miR-636 and miR-485-5p have been shown to inhibit cancer cell proliferation (Supplemental Table 1). Although in our study BDNF negatively regulated miR-3928 and positively regulated miR-636 and miR-485-5p, the effects of these miRNAs on endothelial colony formation remain to be determined. Importantly, we also found that BDNF significantly suppressed miR-433 and promoted miR-181c levels. It has been shown that miR-433

Table 1. Identification of microRNA (miRNA) change in response to brain-derived neurotrophic factor (BDNF) treatment.[a]

miRNA	*P* Value	Fold change (BDNF/Control)	miRNA	*P* Value	Fold change (BDNF/Control)
miR-4716-5p	0.013118	−1.78412	miR-432*	0.011767	1.506591
miR-3928	0.027364	−1.75844	miR-4499	0.004593	1.537733
miR-433	0.012369	−1.60691	miR-3911	0.02734	1.548567
miR-1294	0.038649	−1.57833	miR-1183	0.027124	1.568415
miR-1539	0.039771	−1.56272	miR-4669	0.033849	1.585457
miR-19b-1*	0.044215	−1.52937	miR-636	0.036284	1.632052
			miR-4717-3p	0.022632	1.6608
			miR-4298	0.040393	1.690261
			miR-485-5p	0.028276	1.870173
			miR-181c	0.008333	1.899249

Abbreviations: BDNF, brain-derived neurotrophic factor; miRNA, microRNA.[a]Day 7 endothelial progenitor cells (EPCs) were treated with BDNF (100ng/mL) for 7 h. Six miRNAs were significantly downregulated ($n = 4$), 10 miRNAs were significantly upregulated ($n = 4$).
Note: * is part of denotation of this miRNA. It is not a indication of statistical significance.

inhibits proliferation of CD34+ cells, endothelial cells, and various cancer cells, as well as hematopoietic cell differentiation (Supplemental Table 1), whereas miR-181c stimulates proliferation and differentiation of stem cells or progenitor cells (Supplemental Table 1). Based on these observations, it appears likely that the suppression of miR-433 and stimulation of miR-181c expression, induced by BDNF, may be beneficial to the regenerative function of EPCs.

Since miR-433 has abilities to inhibit human umbilical vein endothelial cell proliferation (Supplemental Table 1), and to negatively regulate CD34+ cell growth and differentiation by targeting GBP2[15], we next focused on the possibility that miR-433-GBP2 pathway is activated in response to BDNF. GBP2, an interferon-induced GTPase (an enzyme that can hydrolyze guanosine triphosphate), has been shown to mediate hematopoietic cell proliferation and erythropoiesis[15] and to induce fibroblast proliferation[16]. However, the function and regulation of GBP2 in EPCs and endothelial cells have not been studied. In the present study, knockdown of miR-433 significantly increased GBP2 expression, while miR-433 mimic suppressed GBP2 protein levels, suggesting that in human early EPCs miR-433 targets GBP2. BDNF decreased miR-433 expression and augmented GBP2 protein levels. Thus, ours is the first report to demonstrate that BDNF stimulates the expression of GBP2 by suppressing miR-433 level. We also observed that in human early EPCs miR-433 did not regulate other targets such as CDK6, GRB2, GCS, and CREB1[17–20], thereby demonstrating the selectivity of miR-433 for GBP2. However, the knockdown of miR-433 did not significantly increase the endothelial colony formation. One of the reasons for this observation may be that BDNF targeting of multiple genes is required in order to orchestrate the process of differentiation from early EPCs to outgrowth endothelial colonies. It is possible that the alteration of miR-433 by BDNF may have synergistic effects with other factor(s) changed by BDNF (such as miR-181c or other target genes). This possibility remains to be investigated in further studies.

The change of the miRNA profile by BDNF also indicates other possible mechanisms responsible for pro-angiogenic effects of BDNF. For example, existing literature suggests that miR-181c downregulates the expression of tumor necrosis factor-α [21]. Stimulation of miR-181c by BDNF may therefore have anti-inflammatory effects under pathological conditions such as diabetes and atherosclerosis.

In conclusion, we have demonstrated that BDNF stimulates the generation of endothelial cell colonies and changes the expression profile of miRNA. BDNF also enhances GBP2 expression in a miR-433-dependent manner. However, it appears that multiple miRNAs and their target genes are involved in the stimulatory effect of BDNF on outgrowth endothelial colony formation.

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Ethical Approval

This study was approved as an exempt from review by Institutional Review Board at the Mayo Clinic.

Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplementary material for this article is available online.

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