Ectopic Expression of Rat TrkB Genes in HEK293T Cells

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Abstract

Aim: To construct prTrkB-FL-EGFP, prTrkB-T1-EGFP and prTrkB-T2-EGFP which are able to be expressed in eukaryotic cells and to achieve ectopic expressions for these three vectors in HEK293T cell. Methods: The EGFP encoding sequence was linked in downstream of encoding sequences for TrkB-FL, TrkB-T1 and TrkB-T2 respectively, and then subcloned into pEGFP-N1 vector. The accuracy of individual sequence was confirmed by endonuclease cutting with agarose electrophoresis and DNA sequencing. Three constructed expression vectors were transfected into the HEK293T cells respectively. The green fluorescence positive under fluorescence microscopy was applied to confirm the ectopic membrane expression for these three vectors. In addition, the immunofluorescence for anti-phospho-TrkB was applied to verify the phosphorylation of transfected TrkB- FL-EGFP on cell membrane. Results: The sequences of prTrkB-FL-EGFP，prTrkB- T1- EGFP and prTrkB-T2-EGFP were correct. When three constructed vectors were transfected into the HEK293T cells respectively, they were able to achieve ectopic expression on the cell membrane. The ectopic expression of TrkB-FL-EGFP on cell membrane was able to be phosphorylated. Conclusion: The expression vectors which were constructed in present study were able to be expressed on the eukaryotic cell membrane. The ectopic expression of transfected prTrkB-FL-EGFP on cell membrane was able to be phosphorylated. The constructed vectors and the ectopic expression system which were established in study were useful tool for use to investigate the mechanism of BDNF/TrkB pathway.

Keywords

TrkB, TrkB/BDNF pathway, Eukaryotic expression vector.

1. Introduction

It is demonstrated that Brain derived neurotrophic factor (BDNF) plays an important role in neuronal survival, differentiation, growth and development, preventing the death of injured neurons, improving the neuronal pathological state and promoting regeneration and differentiation of neuronal injury[1]. Except nervous system, BDNF and its receptor TrkB are also expressed in the endothelial cells of the coronary arteries[2], and are known to be associated with the development of capillaries and cardiac endothelium formation in the heart tissue during the late gestation period [3,4,5]. Recently, it was

reported that BDNF can promote young CMECs to migrate via the activation of the BDNF-TrKB-FL-PI3K/Akt pathway, which may benefit angiogenesis after MI. However, the aging of CMECs led to changes in the expression of receptor TrkB isoforms in that among the three isoforms (TrkB-FL, TrkB-T1 and TrkB-T2), only one of its truncated isoforms, TrkB-T1, continued to be expressed, which leads to a decrease in the migration of CMECs in aging hearts. While although the potency of promoting migration from BDNF-TrkB-T1 pathway in old CMECs is significantly decreased comparing with young CMECs via BDNF-TrkB-FL pathway, BDNF-TrkB-T1 pathway in old CMECs was still able to promote migration of aged CMECs[6].

Tyrosine Receptor Kinase B (TrkB) is the high affinity receptor of BDNF. There are three isoforms of TrkB in rats: full-length isoform TrkB-FL and two truncated isoforms TrkB-T1 and TrkB-T2, which lack the intracellular tyrosine kinase area. Thus far, it is generally recognized that the BDNF signal transduction is mainly completed by TrkB-FL. BDNF binding can induce the dimerization of TrkB-FL and phosphorylation of specific intracellular tyrosine residues to transducer the BDNF signaling. While TrkB-T1 is a dominant negative inhibitor of TrkB-FL by forming non-function heterodimer with TrkB-FL[7,8]. So far, the mechanism regarding to BDNF intracellular signal transduction via three isoforms of TrkB is not yet fully demonstrated. Therefore, a cellular model which is established by ectopic expression of TrkB-FL, or TrkB-T1, or TrkB-T2 in a eukaryotic cell will benefit us to conduct related studies for the BDNF-TrKB pathway. HEK293T cell is such cell which does not express all isoforms of TrkB receptor, but express BDNF. In this study, HEK293T cell was applied to conduct the ectopic expression of three isoforms of TrKB receptors.

2. Methods

2.1 Construction of prTrkB(FL, T1, T2)-EGFP Eukaryotic Expression Vector

According to the Rat TrkB mRNA sequence searched in Genebank (FL:NM_012731.2, T1:NM_001163168.1, T2:NM_001163169.1), PCR amplification primers are designed by Primer5.0. The primers are shown in Table 1. Total RNA from mice brain is extracted using Trizol and reverse transcribed to cDNA using SuperScript^{TM}III First-Strand Synthesis System kit. Then, using the primers designed before and pfuUltra II Fusion HS DNA polymerase, the cds sequences of TrkB-FL, TrkB-T1 and TrkB-T2 are obtained by PCR amplification. PCR reaction system: 10xBuffer 5μl, $MgSO_4$(50 mmol/L) 2μl，dNTP(10 mmol/L) 1μl，pfuUltra II Fusion HS DNA Polymerase 0.5μl，Primer F(10 mmol/L) 1μl，Primer R(10 mmol/L) 1μl，cDNA 1μl, add ddH_2O to 25μl. PCR reaction:

Predenature:	94℃	2min	
Denature:	98℃	10sec	
Extension:	68℃	1min30s	25cycles
	16℃	pause	

pEGFP-N1 vector and PCR product were respectively cut using 5units BamHI/EcoRI and the FastDigest Green Buffer(10x) according to manufacturer's protocol. Restriction digest products (Linear pEGFP-N1 and TrkB-FL/T1/T2) were run on an agarose gel for analysis and extraction. pEGFP-TrkB-FL/T1/T2 were constructed using T4 DNA ligase.

2.2 Transfection of pEGFP-TrkB-FL/T1/T2 into HEK293T Cells

HEK293T cells were transfected with DNA using LipoFiterTM Liposomal Transfection Reagent (Hanbio) according to the manufacturer's manual. HEK293T cells were harvested and the appropriate cell density was added to 24-well culture plate were 50%-70% confluent on the day of transfection. 24 hours after the transfection, fluorescence results are able to be observed by fluorescence microscope.

Table 1 The primers used for sequencing

Primer	Primer sequence (5'→3')
rTrkB-VF	TCGAATTCTGGCCACCATGTCGCCC TGGCCGAGG
rTrkB-FLVR	GTGGATCCCGGCCTAGGATGTCCAGGTAGA
rTrkB-T1VR	GTGGATCCCGCCCATCCAGGGGGATCTTA
rTrkB-T2VR	GTGGATCCCGAGAAGCAAAATAAGCACACTTCTG

2.3 Immunofluorescence Staining

HEK293T were fixed with 4% paraformaldehyde-PBS for 15 min. Following permeabilization and blocking, cells were incubated with primary antibodies overnight at 4℃. Secondary antibodies used were Anti-rabbit IgG (CY3; red). Samples were mounted using ProLong Gold antifade reagent with DAPI, and immunofluorescence was detected using Olympus confocal microscopy.

3. Results

In present study, the vectors of prTrkB-FL-EGFP, prTrkB-T1-EGFP and prTrkB-T2-EGFP were constructed (Figure 1 and 2) to investigate whether HEK293T cell is ideal cell model to ectopic expression of the above constructed vectors. The prTrkB-FL-EGFP, prTrkB-T1-EGFP and prTrkB-T2-EGFP were transfected into HEK293T cells. The result of fluorescent confocal imaging showed that prTrkB-FL-EGFP, prTrkB-T1-EGFP and prTrkB-T2-EGFP were expressed and located on cell membrane mainly (Figure 3). In addition, the phosphorylation of TrkB-FL-EGFP after transfection in HEK293T cells was further observed. The result of immunofluorescence staining of anti-phospho-TrkB for phospho-TrkB in transfected HEK293T cells documented that the phosphor-TrkB/TrkB-FL-EGFP positive cells were found after 24 hours (Figure 4). The results suggest that after transfection, TrkB (FL, T1, T2)-EGFP fusion proteins are able to be expressed on membrane of HEK293T cells. In addition, the ectopic expressed TrkB-FL is able to phosphorylate and dimerize on the cell membrane.

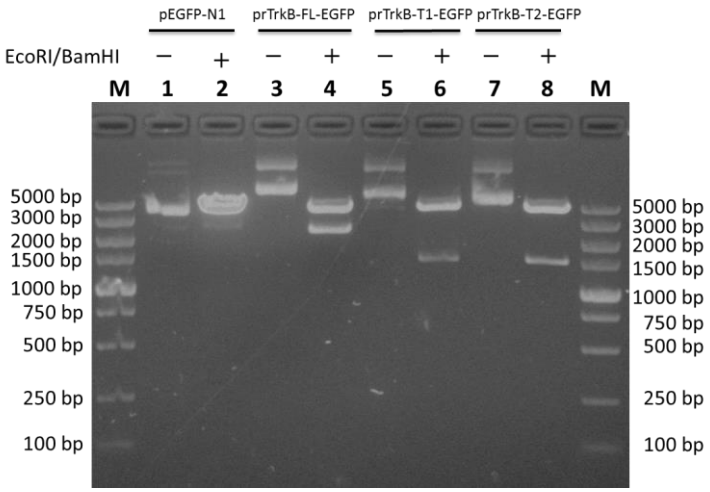

Figure 1: Restriction enzyme digestion for prTrkB-FL-EGFP, prTrkB-T1-EGFP and prTrkB -T2 -EGFP.

Lane 1, 3, 5 and 7: pEGFP-N1-, prTrkB-FL-EGFP-, prTrkB-T1-EGFP- and prTrkB- T2-EGFP-vectors. Lane 2, 4, 6 and 8: pEGFP-N1-, prTrkB-FL-EGFP-, prTrkB-T1-EGFP- and prTrkB-T2-EGFP-vectors were digested by EcoRI and BamHI. M: DNA marker. The digested fragments of prTrkB-FL-EGFP, prTrkB-T1-EGFP and prTrkB-T2-EGFP vectors by EcoRI and BamHI were well

matched the length cloned fragment which were 7181bp, 6168bp and 6162bp, while the length of digested and non-digested pEGFP-N1 vector was same.

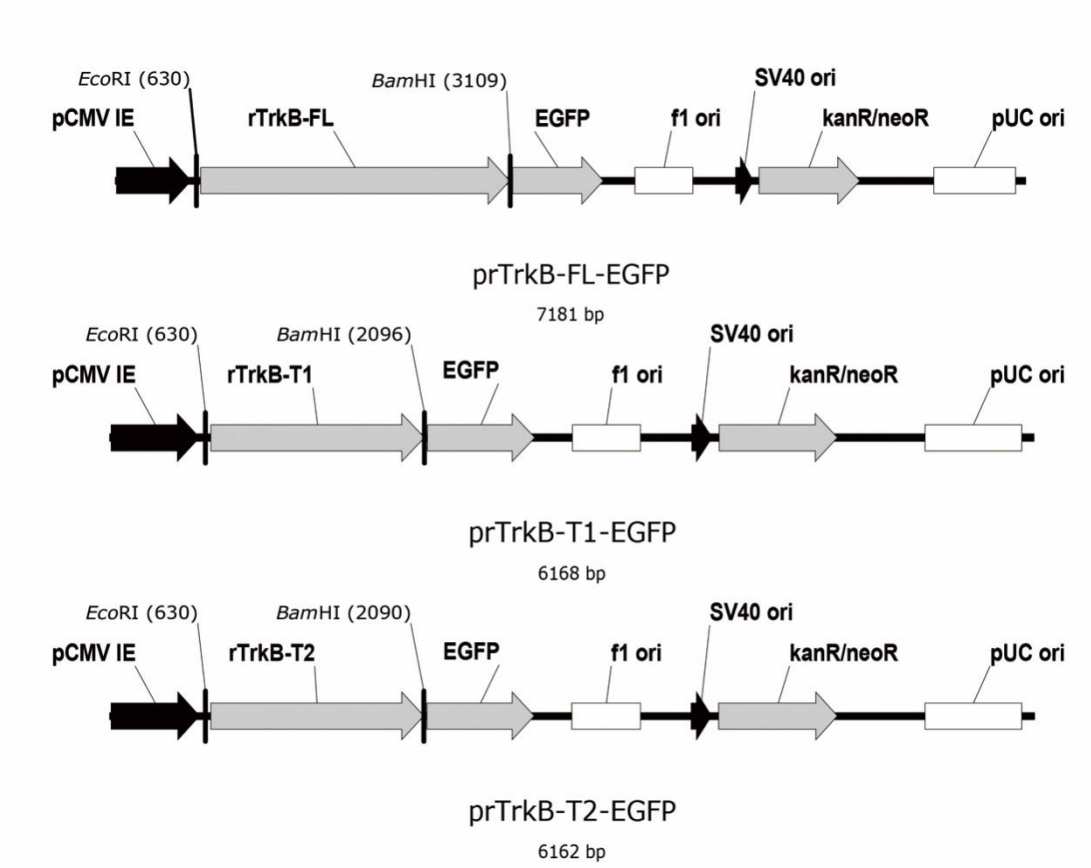

Figure 2：Schematic of constructed vectors, prTrkB-FL-EGFP, prTrkB-T1-EGFP and prTrkB-T2-EGFP

The length of of rTrkB-FL, rTrkB-T1 and rTrkB-T2 were 2479bp, 1466bp and 1460bp.

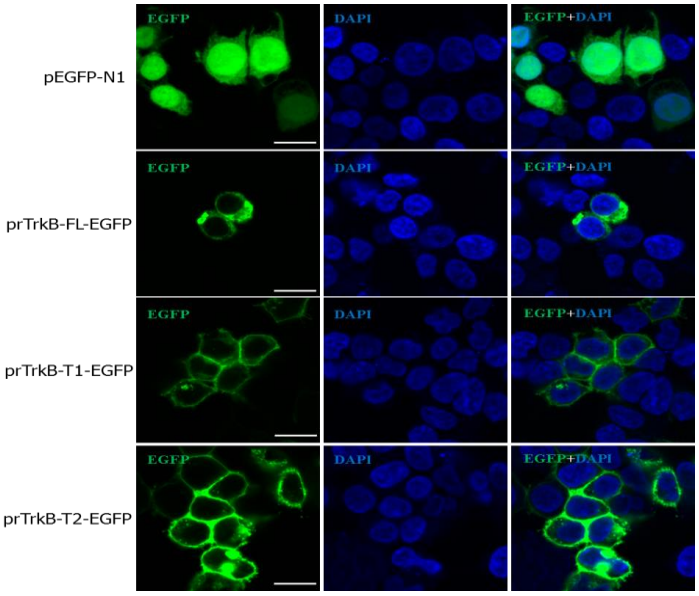

Figure 3: The transfected prTrkB-FL-EGFP, prTrkB-T1-EGFP and prTrkB-T2-EGFP were expressed and located on cell membrane of HEK293T cells.

pEGFP-N1, prTrkB-FL-EGFP, prTrkB-T1-EGFP and prTrkB-T2-EGFP were transfected into HEK293T cells. EGFP positive cells were found 24 hours after transfection. The EGFP positive

signals of prTrkB-FL-EGFP, prTrkB-T1-EGFP and prTrkB-T2-EGFP transfected cells were mainly expressed and located on cell membrane, while it was not in pEGFP-N1 transfected cells (control). Bar=20 μm.

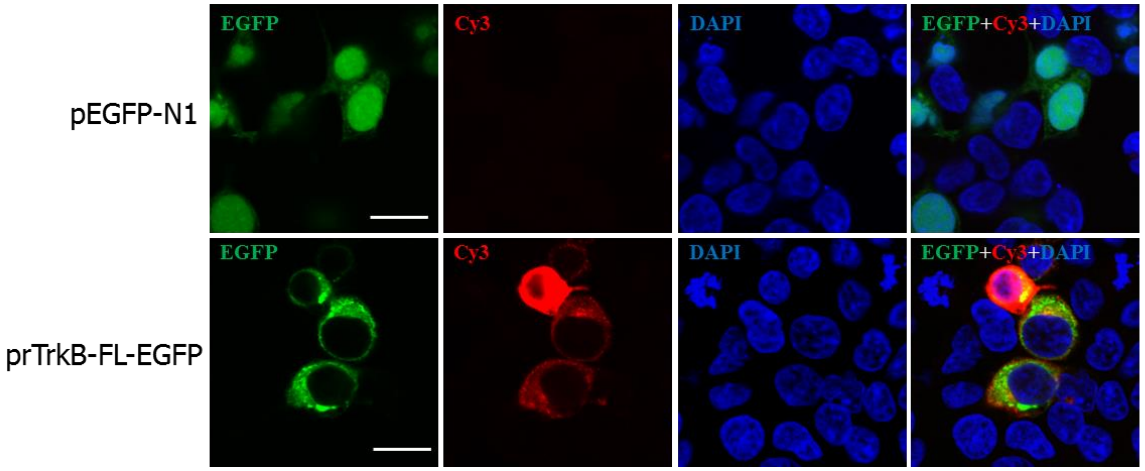

Figure 4: Auto Phosphorylation of transfected TrkB-FL-EGFP

pEGFP-N1 and prTrkB-FL-EGFP were transfected into HEK293T cells. The immunofluorescence staining for rabbit anti-rat phospho-TrkB (red) and Anti-rabbit IgG (CY3; red) was conducted. The red fluorescence positive cells were found over lapped with EGFP (green). It suggests that the transfected TrkB-FL was expressed on membrane and is able to be autophosphorylated. Bar＝20 μm.

4. Discussion

The construction and transfection of prTrkB-EGFP proved that HEK293T cells could be an ideal model to study theTrkB signaling activation. HEK293T is a human cell line, however, TrkB from rat, in which we are interested, could do the normal cell trafficking, dimerization and phosphorylation in HEK293T cells. As a cell line which is very commonly used in biology for protein expression, HEK293T cells could be more convenient and easier for the follow-up research. The constructed vectors and the ectopic expression system which were established successfully in this study are useful for us to investigate the mechanism of BDNF/TrkB pathway. In conclusion, the ectopic expression of TrkB in HEK293T cell model could be useful for studying the role of TrkB in eukaryotic cells.

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