Ectopic Expression of Rat TrkB Genes in HEK293T Cells

Lilin Li

Key laboratory for Regenerative Medicine, Ministry of Education, Jinan University, Guangzhou 510632, China

Abstract

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

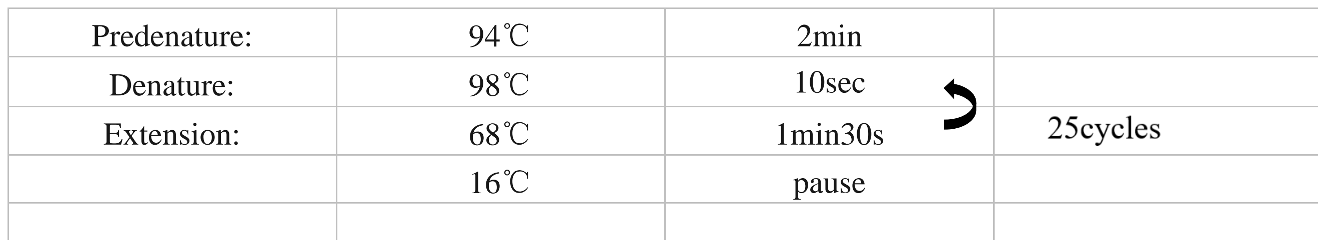
Brain derived neurotrophic factor (BDNF) is a type of protein, which is widely distributed in the central nervous system. In the process of the central nervous system development, BDNF plays an important role in neuronal survival, differentiation, growth and development, preventing the death of injured neurons, improving the neuronal pathological state and promoting regeneration and differentiation of neuronal injury. Furthermore, BDNF is also necessary for the survival and normal physiological function of mature central and peripheral nervous system. Tyrosine Receptor Kinase B (TrkB) is the high affinity receptor of BDNF[1]. (There are three isoforms of TrkB: Full-length isoform TrkB-FL and two truncated isoforms TrkB-T1 and TrkB-T2, which lack the intracellular tyrosine kinase area. Therefore, it is generally believed that the BDNF signal transduction is mainly completed by TrkB-FL. However, the intracellular signal transduction mechanism of the three isoforms of TrkB is unclear. The study of the formation and intracellular signal transduction of TrkB may provides new strategies for the treatment of nervous system diseases. It is generally known that the signal transduction of BDNF-TrkB is mainly completed by TrkB-FL, which causes the study of TrkB-FL signal pathway is much more established than truncated isoforms[9-13].TrkB-FL receptor has resistance to cell apoptosis, cell hypertrophy, dementia and emotional disease. BDNF binding can

induce the dimerization of TrkB-FL and phosphorylation of specific intracellular tyrosine residues[14]. Therefore, Phosphorylated tyrosine residues are able to activate different signaling pathways to change gene expression of neural cell and produce different biological response[15].BDNF could be able to regulate the angiogenesis of cardiac microvascular endothelial cells by activating TrkB during the development[2,3]. However, there is few studies about the mechanism of BDNF function in adult and aging mice, and the mechanism of BDNF-TrkB pathway in cardiac microvascular endothelial cells is still not clear[8]. With the increase of age, the structure and function of tissues and organs showed degenerative changes, and the ability of self-renewal and regeneration is declining[4]. Due to the aging could affect the function of cardiovascular system, we want to find out a signal pathway relating to both aging and cardiovascular system function, which could explain why the phenotype and function of cardiovascular system changes with age. It’s reported that the expression of TrkB in the rat pituitary is changed with age, in which the expression of TrkB-FL and TrkB-Tl decreased in middle age, furthermore, BDNF could increase the inflammatory response in the old rat model of heart damage.

2. Methods

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

According to the RatTrkB 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™III First-Strand Synthesis Systemkit. 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 $ddH_20$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.

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

2.2 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.

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 3.1 and 3.2). To investigate whether HEK293T cells are ideal cell model to ectopic expression of 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 3.3). In addition, the phosphorylation of TrkB-FL-EGFP after transfection in HEK293T cells was further observed. The result of immunefluorescence staining of anti-phospho-TrkB for phospho-TrkB transfected HEK293T cells documented that the phosphor-TrkB/TrkB-FL-EGFP positive cells were found in 24 hours after (Figure 3.4). 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.

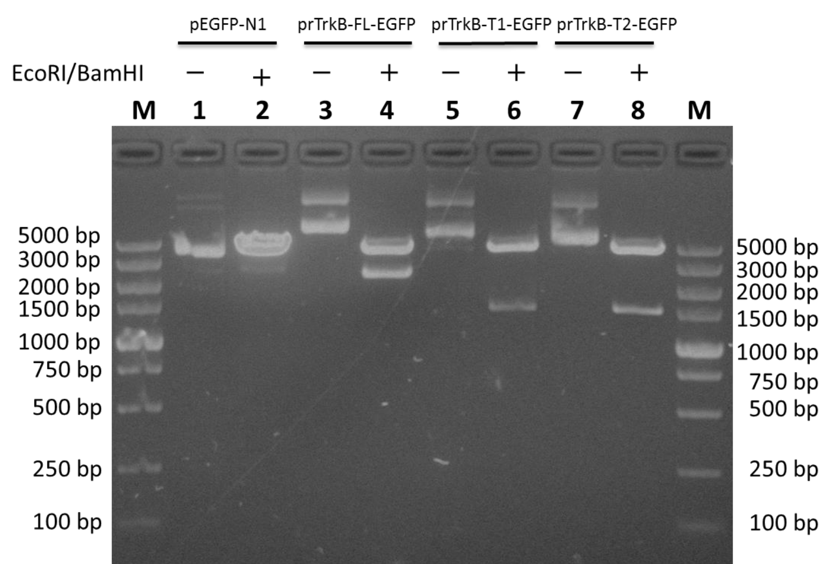


Figure 3.1: Restriction enzyme digestion for prTrkB-FL-EGFP, prTrkB-T1-EGFP and prTrkB-T2-EGFP.

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.

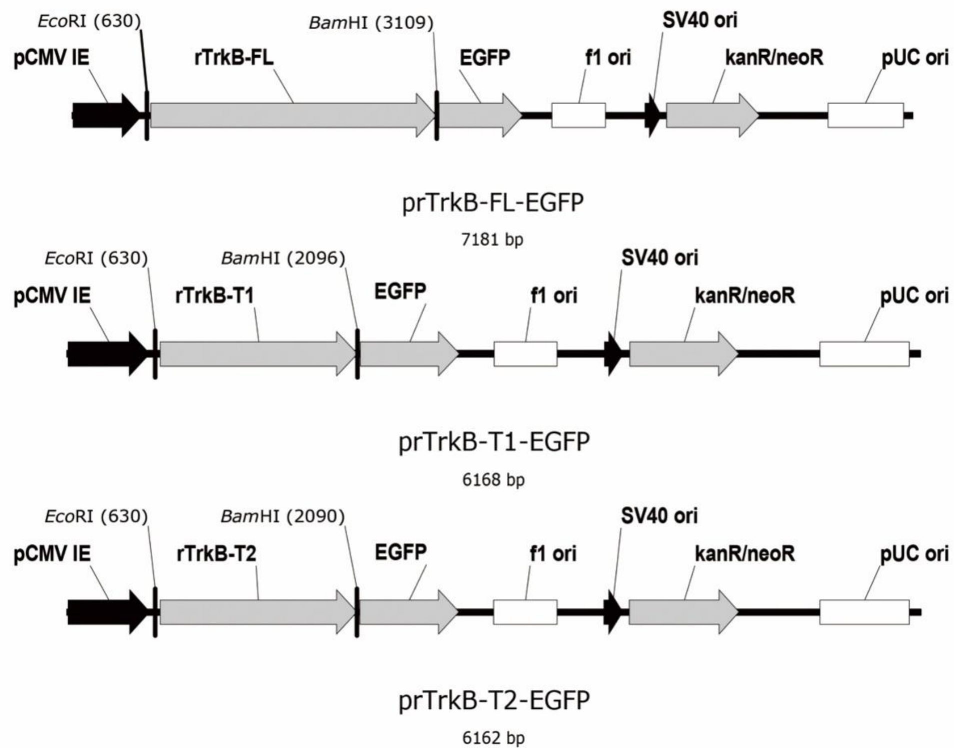


Figure 3.2：Schematic of constructed vectors, prTrkB-FL-EGFP, prTrkB-T1-EGFP and prTrkB-T2-EGFP

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

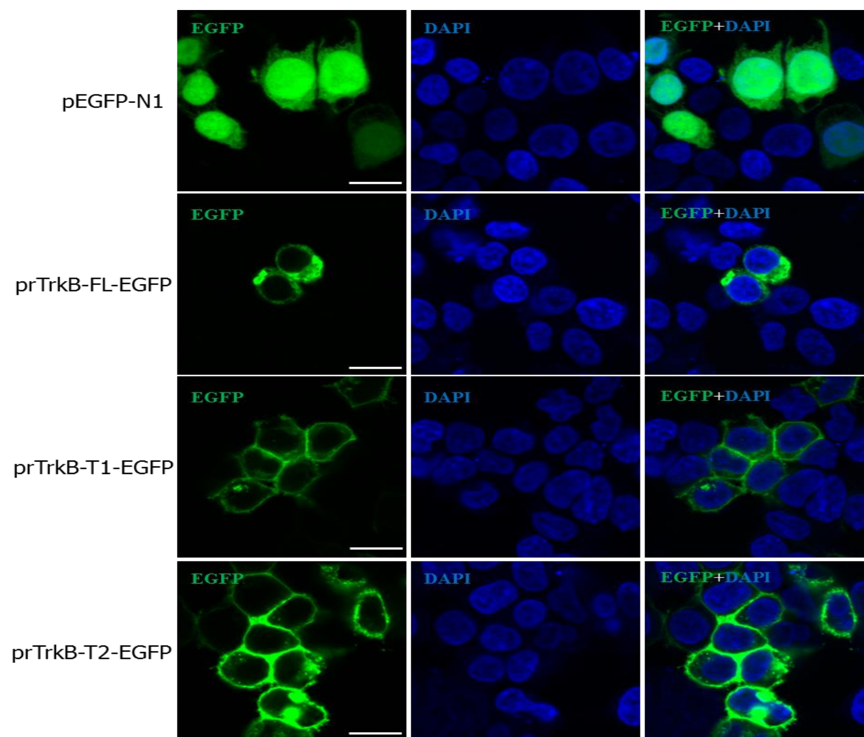


Figure 3.3: The transfected prTrkB-FL-EGFP, prTrkB-T1-EGFP and prTrkB-T2-EGFP were expressed and located on cell membrane of HEK293T cells.

pEGFP-N1, prTrkB-FL-EGFP, prTrkB-T1-EGFP and prTrkB-T2-EGFP were transfected into HEK293T cells. EGFP positive cells were found in 24 hours after transfection. The EGFP positive signal of prTrkB-FL-EGFP, prTrkB-T1-EGFP and prTrkB-T2-EGFP transfected cells was mainly expressed and located on cell membrane, while it was not in pEGFP-N1 transfected cells (control). Bar=20 μm.

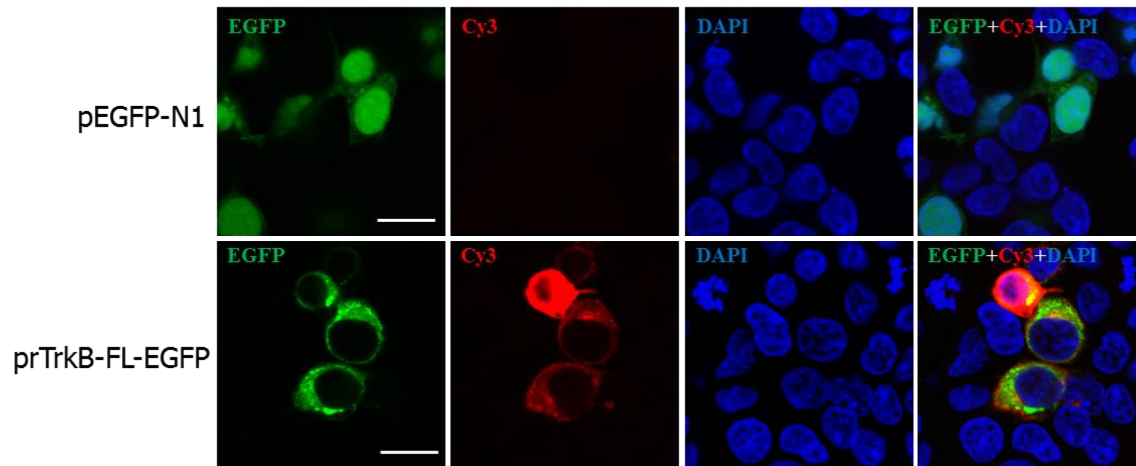


Figure 2.5: Auto Phosphorylation of transfected TrkB-FL-EGFP

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. Conclusion

The construction and transfection of prTrkB-EGFP proved that HEK293T cells could be an ideal model to study theTrkBsignaling 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 moreconvenient 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.

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