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Single-cell analysis for BDNF and TrkB receptors in cardiac microvascular endothelial cells

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Abstract. Recent studies revealed that *BDNF-TrkB* pathway plays an important role in cardiac microvascular endothelial cells (CMECs) mediated myocardial angiogenesis. Single-cell analysis is a powerful tool for studying gene expression in individuals since cellular heterogeneity and dynamic microenvironments which individual cell will experience. Little is currently known about the expression of *BDNF* and *TrkB receptors* at the single CMEC level. Our single-cell analysis of seven randomly selected CMECs for *BDNF* and *TrkB receptors* (*FL, T1, T2*) showed that under an in vitro culture environment, *BDNF* was expressed in two of the seven selected CMECs. None of the single CMEC expressed *TrkB-FL*. *TrkB-T1* was expressed in all seven selected CMECs, while, *TrkB-T2* was expressed in three of these. In addition, none of single CMEC was found to express both *BDNF* and three *TrkB receptors or BDNF* and *TrkB-FL* simultaneously. These results suggest that a stochastic or random expression pattern for *BDNF* and their receptors might be set in each of the CMEC to response requirement of the time and spatial change, regulation or pathophysiological change.

Keywords: Single cell, BDNF, TrkB receptor, cardiac microvascular endothelial cells

1. Introduction

Recent studies have shown that brain-derived neurotrophic factor (*BDNF*) and its receptor, *TrkB*, are expressed in endothelial cells and play an important role in angiogenesis [1,2]. There are three isoforms of the *TrkB receptors* in mammalian cells. The full-length isoform (*TrkB-FL*) is a tyrosine kinase receptor that transduces the *BDNF* signal [3–5]. The two truncated isoforms, *TrkB-T1* and *TrkB-T2*, possess the same extracellular domain, transmembrane domain and the first 12 intracellular amino acids in sequence as does *TrkB-FL*. However, the C-terminal sequences are isoform-specific:

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T1 has 11 amino acids, and *T2* has 9 amino acids [3]. Previously we reported that the age-related increase of truncated *TrkB* in cardiac microvascular endothelial cells (CMECs) appears to be linked to an age-associated increase in inflammatory response and a significant increase in myocardial injury following coronary artery occlusion [6]. More recently, we found that *BDNF* promotes CMECs to migrate via the activation of the *BDNF-TrkB-FL-PI3K/Akt* pathway, which may benefit angiogenesis after myocardial infarction (MI) [7].

Traditionally, gene expression is studied in experiments that measure the average gene expression level in populations containing millions of cells [8]. However, this approach has a major shortcoming in which averaging over populations masks differences in gene expression that may occur between individual cells. It is believed that within a single cell gene expression is inherently stochastic or random and its expression profile adequately fits the time and spatial environment experienced by the individual cell. Therefore, the differences may, in turn, have consequences for the whole multicellular community or organism, which makes it important to understand gene expression in single cells [9]. However, little is known regarding to the expression of *BDNF* and *TrkB receptors* at the single CMEC level. This study was undertaken to investigate this intriguing issue.

2. Material and method

2.1. Isolation and culture of CMECs

Isolation and culture of rat CMECs was accomplished according to a previous report [7]. The primary culture of isolated CMECs was set as passage 0 (P0) and the first subculture was set as passage 1 (P1). CMECs used in this study experienced 5 to 10 passages.

2.2. Collection of single CMECs

CMECs were digested using 0.25% trypsin-EDTA at 37°C under approximately 80% confluence. After washing three times with PBS (pH=7.4), the cell pellet was resuspended with PBS-BSA. A maximum of 5×10^3 cells were added to a sterile 35mm Petri dish. The PBS-BSA medium is crucial to avoid cells attaching to the dish bottom which facilitates extraction of an individual cell.

A glass capillaries (Sutter instrument OD: 1.0 mm; ID: 0.75 mm; length: 10 cm B100-75-100) was prepared using micropipette puller (Sutter Instrument P-97) using the following parameters: Heat=508, Pull=30, Velocity=120, Time=200 and Pressure=200. Single cell was captured using micromanipulator (Eppendorf, Transferman NK2) with a manual microinjector (Eppendorf 5176 000.033), and immediately transferred into a 0.2ml PCR tube (QSP 11280494) containing 15μl pre-chilled TRI reagent (Sigma-Aldrich T9424) and then kept in ice until used. It was critical to complete this process in 2 minutes to avoid RNA degradation [10]. To trace the captured cell, the CMECs prepared as above were dyed with DiI at 37°C for 2 minutes before manipulation. After the single-cell was transferred into PCR tube, the collected cell existence was confirmed using a fluorescence microscopy (Figure 1). Non-cell controls containing an equivalent TRI reagent and PBS-BSA medium without CMECs were prepared to exclude possible contamination from non-CMECs.

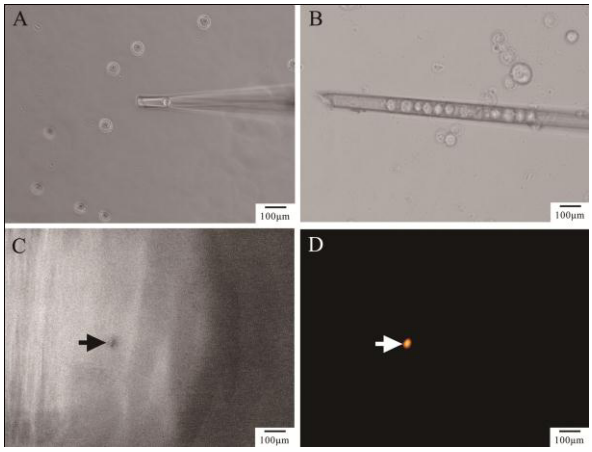

Fig. 1. One and ten cardiac microvascular endothelial cells (CMECs) were captured using micromanipulator under microscope (A, B). For confirmation of the cell was transfered into the collected tube, CMECs were dyed with DiI before digestion. The single cell was confirmed in the bottom of collected tube under light (C), and the fluorescence (D). Bar=100 μm.

2.3. RNA isolation from single CMEC

This process was performed according to a recent report [11] with some modifications. All the reagents used were pre-chilled on ice. After 5 μl of chloroform was added into TRI reagent with the collected cell, the mixture was kept on ice for 5 minutes, then centrifuged at 14000 g for 20 minutes at 4°C. The supernatant (top layer) was collected and precisely transferred into a new PCR tube. Next isopropanol (Sigma-Aldrich I9516) was added and the mixture was kept at -80°C for 10 minutes. After being centrifuged at 14000 g for 20 minutes at 4°C, the supernatant was discarded and the collected RNA precipitation was dissolved with 3.3 μl nuclease-free water (Invitrogen 10977-015).

2.4. Reverse transcription of mRNA from single CMEC

Reverse transcription of mRNA was performed using the High capacity cDNA reverse transcription kit with RNase inhibitor (Applied Biosystems 4368814). The detailed procedure was described in the user manuals. Reverse transcription was carried out by Tgradient 96-Gradient thermocycler (Biometra 050-811) at 25°C for 10 min, 37°C for 2h, 85°C for 5 min and then at 4°C. A non-transcriptase control containing all the reagents needed but no reverse transcriptase was prepared to exclude possible contamination from DNA.

2.5. Real-time PCR Using SYBR Green

Real-time PCR was conducted using SYBR Green® Realtime PCR Master Mix (TOYOBO QPK-201) and run on an CFX96 real-time PCR detection system (Bio-rad 184-5096&185-5096). The non-template control and positive sample containing total RNA as the initial template were applied. *β-actin* and *GAPDH* were chosen as internal controls. All primer sequences were provided in Table 1. The PCR was run under following parameters: 95°C for 2 min, then 45 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 30 s. Melt curve: 55°C to 95°C, read plate at each 0.5°C increase for 2 seconds. Specific amplification was initially identified by the melt curve. The positive control containing normal cDNA

template showed the correct Tm value of each target gene's PCR product, and non-template control enabled exclusion of the interference by primer dimers. All the PCR products were validated by 2% agarose gel electrophoresis.

Table 1

Primers used for Real-time PCR

Gene Symbol		Sequence (5'>3')	Length	Product length
GAPDH	Forward	AGACAGCCGCATCTTCTTGT	20	207
	Reverse	CTTGCCGTGGGTAGAGTCAT	20	
β-actin	Forward	TCATGAAGTGTGACGTTGACATCCGT	26	285
	Reverse	CCTAGAAGCATTTGCGGTGCACGATGG	27	
BDNF	Forward	CAGGGGCATAGACAAAA	17	153
	Reverse	CTTCCCCTTTTAATGGTC	18	
TrkB-T1	Forward	CAACCTAACGACTAACAGAGCC	22	196
	Reverse	TTGGTTCAAGTCCACACTCC	20	
TrkB-T2	Forward	TTGGCATGAAAGGTAAGCAG	20	204
	Reverse	AGTGGGCAAGGCTGAGTAAT	20	
TrkB-FL	Forward	GATCTTCACCTACGGCAAGC	20	200
	Reverse	TCGCCAAGTTCTGAAGGAGT	20	

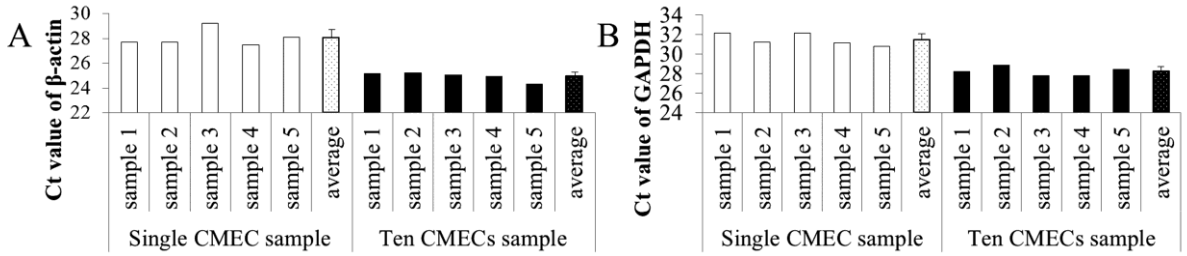

Fig. 2. *β-actin* (A) and *GAPDH* (B) expression in single CMEC and ten-CMECs level. The expression level of both genes in single and ten cell level was quite similar respectively. The Ct values for *β-actin* and *GAPDH* in single cell were around 3 cycles higher than that of ten cells which is consistent with the theoretical values.

Table 2

Ct value of β-actin and GAPDH in one or ten CMECs

Gene	β-actin		GAPDH	
	single CMEC sample	Ten CMECs sample	single CMEC sample	Ten CMECs sample
Sample 1	27.68	25.17	32.18	28.22
Sample 2	27.71	25.19	31.21	28.9
Sample 3	29.19	25.02	32.15	27.82
Sample 4	27.48	24.94	31.15	27.83
Sample 5	28.06	24.33	30.79	28.45
Mean	28.02	24.93	31.50	28.24
ΔCt	3.09		3.25	

Note: ΔCt represents difference between one-cell level and ten-cell level.

Table 3

Tm value of melt curve for β-actin and GAPDH in each CMEC sample, non-cell control, non-transcriptase control, non-template control and positive control

Gene		β-actin	GAPDH
Single CMEC sample	Sample 1	86	85
	Sample 2	86	85
	Sample 3	86	85
	Sample 4	86	85
	Sample 5	86	85
Ten CMECs sample	Sample 1	86	85
	Sample 2	86	85
	Sample 3	86	85
	Sample 4	86	85
	Sample 5	86	85
Non-cell control		None	None
Non-transcriptase control		None	None
Non- template control		None	None
Positive control		86	85

3. Results

3.1. Gene expression in a single cell

To confirm the reliable of methodology established in this study, the housekeeping genes, *β-actin* and *GAPDH* were detected both in one and ten CMECs. It was found that the expression levels of both genes were detectable with at the level in one CMEC- and ten CMEC-level, respectively. The Ct value for *β-actin* and *GAPDH* in the ten-cell level was around 3 cycles higher than the one-cell level (Figure 2; Table 2). This matched the computational value in which a 10-fold increase in the cell number results in an approximately 3.3 cycles increase of theoretical Ct value [11]. Based on the value of the melt curve, in this study, the Ct value of each gene under 40 was considered to be a positive expression. In Parallel, the Tm value of melt curve of each sample was identical with positive control, the non-cell control, non-transcriptase control and non-template control was zero (Table 3). These results revealed that gene expression level was measured precisely in a single CMEC quantitatively.

3.2. Expression of BDNF and TrkB receptors at the single-cell level

BDNF and *TrkB receptors*, *TrkB-FL*, *TrkB-T1* and *TrkB-T2*, were detected in the seven randomly selected CMECs. The *GAPDH* was applied as an internal control. It was found that *BDNF* expressed in two of the seven selected CMECs. None of the single CMEC expressed *TrkB-FL*. *TrkB-T1* was expressed in all seven selected individual CMEC, while, *TrkB-T2* was expressed in three of these. None of the single CMEC was found to express both *BDNF* and three *TrkB receptors* or *BDNF* and *TrkB-FL* simultaneously (Figure 3). All positive expressions of the genes in each sample were confirmed in duplicate by gel electrophoresis (Figure 4).

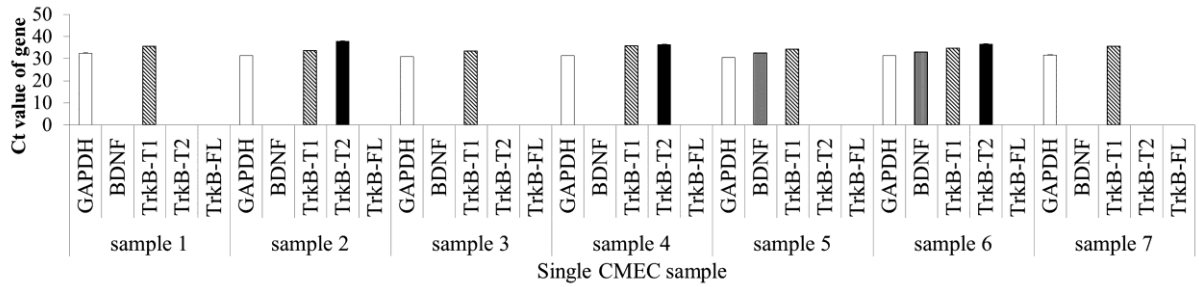

Fig. 3. The expressions of BDNF and TrkB receptors (FL, T1, T2) in single CMEC. The GAPDH was applied as internal control. It was found that BDNF expressed in two of seven selected CEMEs. None of the single CMEC expressed TrkB-FL. TrkB-T1 was expressed in all seven selected individual CMEC, while TrkB-T2 was expressed in three of them. None of single CMEC was found to express BDNF and three TrkB receptors or BDNF and TrkB-FL simultaneously.

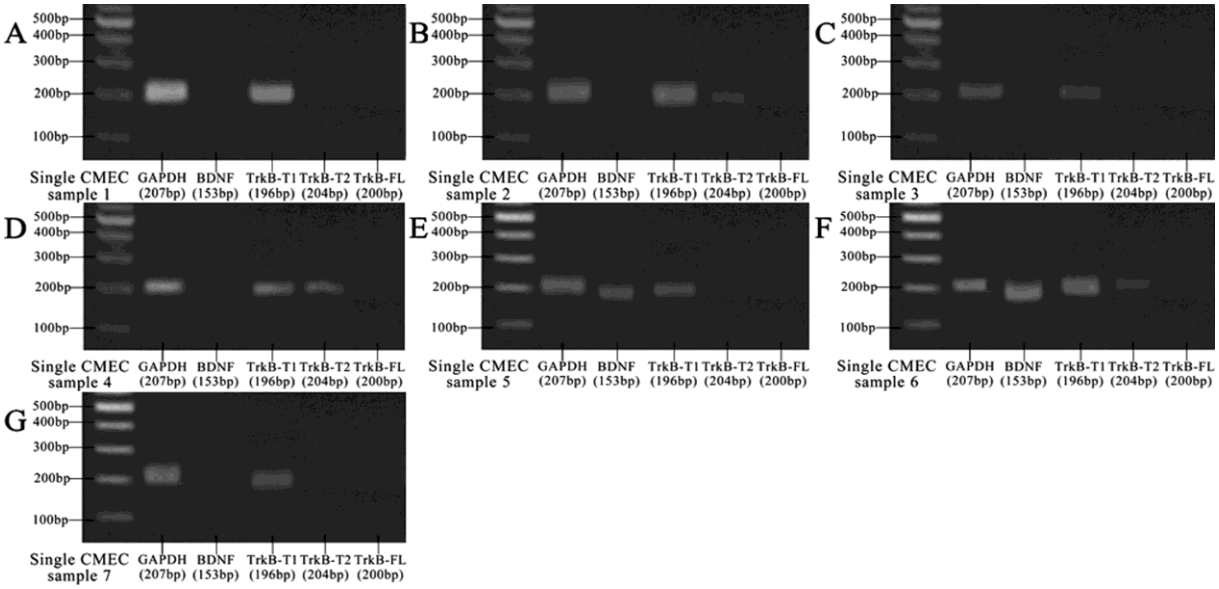

Fig. 4. Agarose gel electrophoresis confirmed positive gene expressions in each individual CMEC. The positive bands were well matched the results of Figure 3.

4. Discussion

Current progress reveals that Real-time PCR using SYBR Green is able to measure a single copy of a gene expression. This unique characteristic coupled with the melt curve, gel-e verification analysis and high primer specificity endorse the advantages of this technique for single-cell expression analysis [12,13]. Since the copy number of RNA was extremely limited at the single-cell level, the efficiency for both reverse transcription and avoiding the bias of amplification in the Real-time PCR process are critical. Thus the primers used in this study are designed according to the rule for free energy of the formation of the double-stranded DNA which is lower than 10.0 [kcals/mol] in the 3' end to avoid incorrect priming. Indeed, our results for the housekeeping genes, *β-actin* and *GAPDH*, which were

measured with SYBR Green Real-time PCR, showed a convincing quantitative result. This revealed that the methodology for gene expression of single cell used in this study is reliable.

It is generally believed that a gene expressed stably in cell population, but in a single cell exhibits random fluctuations, the expression may be higher, lower or nonexistent [14]. Our single-cell level study for CMEC demonstrated that none of the single CMEC expressed *TrkB-FL*. This suggested that in an isolated in vitro culture environment, after several passages, *TrkB-FL* receptor was not expressed in individual CMEC as the individual cell was captured from the population between passage 5-10. In support of this conclusion, in the CMEC population (10^6) analysis for the *TrkB-FL* receptor following passage-5 revealed that the expression was negative (data not shown). In addition, it was found that *TrkB-T1* receptor was expressed in all seven selected individual CMEC. This suggested that *TrkB-T1* receptor might be expressed in each of CMECs under in vivo and in vitro environments. The single-cell analysis also documented that *TrkB-T2* receptor was expressed in three of selected seven CMECs. It appeared from these results that the expression of *TrkB-T2* receptor in each of CMECs was stochastic or random.

Even though, the endothelial cell population demonstrated expression of *BDNF* [1,2], but, the single-cell analysis for CMEC revealed that *BDNF* was expressed in two of seven selected CMECs. In addition, none of single CMEC was found to express both *BDNF* and three *TrkB receptors* or *BDNF* and *TrkB-FL* simultaneously. This suggested that a stochastic or random expression pattern for *BDNF* and their receptors might be established in individual cells of CMEC to a response requirement of the time and spatial change, regulation or pathophysiological change. The underlying cellular and molecular mechanism of diversity for *BDNF* and *TrkB receptors* expressions in individual CMEC need further study. In addition, the possible subsets which express *BDNF* and three *TrkB receptors*, or barely or randomly express respectively in CMEC population is also an intriguing phenomenon for study.

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