Original Article
Downregulation of microRNA-222 facilitates endothelial progenitor cell function in patients with coronary artery disease through targeting STAT5A

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Abstract: Background and aim: Previous findings have indicated that abnormal expression level of microRNA in endothelial progenitor cells (EPCs) is correlated with dysfunction of EPCs. EPCs have a beneficial effect on endothelial repair and integrity which fight against coronary artery disease (CAD). This study aimed to determine the role that microRNA-222 plays on human EPC functions and investigate the underlying mechanisms.

Methods: EPCs were separately collected from peripheral blood mononuclear cells of patients with CAD and healthy donors. EPC functions (proliferation, adhesion and migration) were performed by CCK-8 assay, cell counting, and Transwell migration assay. Identification of the target gene of miR-222 was studied by bioinformatics and Western blot analysis. Expression of miR-222 was measured by quantitative real-time polymerase chain reaction. Protein levels were analyzed by Western blot. Furthermore, vascular endothelial growth factor (VEGF) production was quantified by enzyme-linked immunosorbent assay. Results: miR-222 was significantly higher in CAD EPCs than in healthy donor-derived EPCs. Overexpression of miR-222 in healthy donor-derived EPCs contributed to decreases in proliferation, adhesion, and migration in vitro. Conversely, downregulation of miR-222 effectively reverted CAD EPC functions. STAT5A is a target of miR-222 in EPCs. The overexpression or inhibitory level of miR-222 regulated VEGF production and p38 mitogen-activated protein kinase (MAPK) activation in human EPCs. Conclusions: Downregulation of miR-222 might facilitate CAD EPC function through the activation of p38 MAPK/VEGF signal pathway. Thus, inhibition of miR-222 might have important medical applications in EPC-based therapy for CAD.

Keywords: Endothelial progenitor cell, microRNA-222, coronary artery disease, STAT5A, p38 MAPK/VEGF pathway

Introduction
Coronary artery disease (CAD) is among the main causes of morbidity and mortality around the world [1]. Endothelial function and integrity play a key role in maintaining vascular homeostasis which leads to control and delay in CAD progression [2-4]. Since the initial discovery of EPCs deriving from human peripheral blood in 1997, circulating EPCs have been considered important in maintaining endothelial function and mediating postnatal vasculogenesis. Circulating EPCs originate from bone marrow. These cells can directly contribute to regenerating vasculature and possess the capacity to secrete various paracrine cytokines including vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase to promote the function of pre-existing ECs [5-8]. Accumulating evidence has indicated that CAD can diminish the number and function of EPCs, leading to impaired vascular homeostasis and associated angiogenesis [9-11]. Thus, enhancing the quality and quantity of EPCs is of clinical significance and is important in making progress against CAD.

microRNAs (miRNAs) are endogenous, short, and highly conserved endogenous oligonucleotides (18-23 nucleotides) that downregulate target gene expression through mRNA degradation and translational inhibition [12, 13]. miRNAs are well known to affect key functions of human cells including proliferation, adhe-
miR-222 modulates EPC function

The signal transducer and activator of transcription 5A (STAT5A) are members of the STAT family of transcription factors which act as targets of various growth factors and cytokines [23]. The STAT protein family plays a crucial role in normal cell decision, modulating cell growth, and homeostasis [24]. STAT5A is linked to modulation of the various aspects of cellular transformation, cell proliferation, hematopoiesis, anti-apoptosis, differentiation, cell cycle, and cell survival. Moreover, STAT5A is especially associated with the maintenance, engraftment, and enlargement of human stem/progenitor cells [25-27]. One previous study revealed that STAT5A acted as an important regulator of inflammation-mediated neovascularization in human umbilical vein endothelial cells [28]. Thus, this study suggested that STAT5A might play a major role in inflammation and CAD.

The members (mainly including ERK, SAPK/JNK, and p38 MAPK) of the mitogen-activated protein kinase (MAPK) superfamily play an essential role in cellular signaling pathways. Several reports have shown that p38 MAPK signaling pathway is involved in endothelial progenitor cell function in response to activation by high glucose or pro-inflammatory cytokines [29]. Recently, one finding has confirmed that the role of miR-26a modulates EPC function through p38 MAPK/VEGF signaling pathway [30]. Moreover, other reports have revealed that VEGF is a crucial mitogen of EPCs which promotes EPC functions (proliferation, migration, adhesion, and tube formation) [31, 32]. In light of these studies, we hypothesized that activation of the p38 MAPK/VEGF pathway might play an important role in the effect of miR-222 on EPC functions.

In our present study, we demonstrate that -222 is upregulated in EPCs in patients with CAD. We also examined the regulatory relationship between miR-222 and STAT5A in human EPCs. In addition, a mechanism involving p38 MAPK/VEGF signaling pathways regulated the effect of miR-222 on EPC functions. Thus, our findings show critical roles for miR-222 in dysregulation of EPCs and demonstrate its potential application in clinical transplantation for CAD treatment and early diagnosis.

Materials and methods

Patient recruitment and blood collection

Fifteen patients with stable CAD, conforming to the criteria of the American College of Cardiology (ACC)/American Heart Association (AHA), and 15 healthy controls were recruited into this study. Study participants were excluded if they had a history of acute coronary syndrome, heart failure, heart transplantation, immunosuppressant use, treatment with statins, malignancy, sepsis, large surgical operation in the previous two months, or other severe diseases. Clinical characteristics of the two groups are presented in Table 1. This study conformed to the principles drafted in the Declaration of Helsinki for the use of human blood. All experiments were performed in accordance with the Ethics Committee of the Affiliated Hospital of Jiangsu University, Jiangsu University. Informed consent was acquired from all patients before blood collection.

Isolation and cultivation of EPCs

Isolation and culture of the EPCs were executed as described previously [33, 34]. Concisely, peripheral blood mononuclear cells were iso-

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<th>Table 1. Demographic and clinical characteristics of CAD and healthy groups</th>
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<td>Sex, male/female</td>
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Note: Calculated using Fisher’s exact test for categorical covariates. \( P \) values < 0.05 were considered statistically significant.
miR-222 modulates EPC function

Table 2. Sequences of primers used in qRT-PCR as follows

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<th>Forward (5' - to 3')</th>
<th>Reverse (5' - to 3')</th>
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<tr>
<td>miR-222</td>
<td>GCGGCACGTACATCTGGC</td>
<td>AGTGCGTGTCCGGAGTGC</td>
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<tr>
<td>RNU6B</td>
<td>ACTCAAGACAGTGGTATAAGTTT</td>
<td>TAAAGAAGAAGGATCGGAGC</td>
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<tr>
<td>STAT5A</td>
<td>GATGAGGTGTTGGAAGGCA</td>
<td>TGATGACGGTCTGAGG</td>
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<tr>
<td>GAPDH</td>
<td>TCAACGGATTGGCTGTATTG</td>
<td>TGGGTGGAATCATATGGCAA</td>
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lated from patients with CAD and healthy controls by Ficoll-Isopaque Plus (Histopaque-1077, Sigma-Aldrich, USA) centrifugation. Cells were then seeded into human fibronectin-coated (Sigma-Aldrich, Saint Louis, USA) six-well plates in endothelial basal medium-2 (EBM-2) (Clonetics, Lonza, MD, USA) supplemented with EGM-2-MV single aliquots containing hydrocortisone, insulin-like growth factor-1, VEGF, human fibroblast growth factor-2, human epidermal growth factor, ascorbic acid, and 20% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) at 37°C in a 5% CO₂ incubator. After 4 hours, non-adherent cells were removed from fresh medium and EBM-2 was refreshed every 3 days. These cells were cultured for 7 days and subjected to subsequent experiments (cell viability assay, adhesion assay, cell migration assay, and transfection).

Identification of EPCs

After 7 days of cultivation, these adherent cells gradually turn to elongated spindle-shaped morphology. They could gradually develop some clusters with spindle-shaped cells sprouting from the central core, which we called colony forming units. The characterization and surface marker phenotype of EPCs were analyzed by fluorescence microscopy and flow cytometry, respectively. Expression of the surface marker of EPCs was assessed by FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, USA) after staining with anti-CD31, anti-CD34, and anti-CD45 (all antibodies conjugated with PE were from eBioscience, CA, USA). The data were performed using CellQuest software. To determine the early EPC phenotype, adherent cells cultured for 7 days were incubated with 30 μg/mL 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate-acetylated low-density lipoprotein (Di-I-Ac-LDL; Yiyuan Biotechnologies, Guangzhou, China) for 4 hours at 37°C. After washing with probe-free medium and fixation with 2% paraformaldehyde, cells were counterstained with 10 μg/mL FITC-labeled Ulex europeus agglutinin-I (FITC-UEA-I, Sigma) for 1 hour. Cells were observed with fluorescence microscopy (Olympus BX51 Fluorescence Microscope, Tokyo, Japan). Adherent cells, dual positive for both Dil-Ac-LDL and FITC-UEA-I, were identified as EPCs.

Cell transfection

miR-222 was overexpressed or inhibited in human EPCs using 50 nM miR-222 mimic (miR-222), negative control mimic (miR-NC), 50 nM miR-222 inhibitor (anti-miR-222), or its negative control (anti-NC) for miR-222 inhibitor (Genepharma, Shanghai, China). EPCs were cultured to 40% confluence and Lipofectamine 2000 reagent (Life Technologies, CA, USA) for each transfection. EPCs were collected for 24 hours after transfection and the level of miR-222 was verified by qRT-PCR. All of the transfections were stable in EPCs for at least 7 days, allowing for further study. Sequences used were as follows (5' to 3'): miR-222 (AGCUACAUCUGGCUACUGGGU), miR-NC (CAGUACGUAGCAGAUGUACUU), anti-miR-222 (ACCCAGGCAAGUGACU), and anti-NC (CA-GUACUUCUGUGUACACAA).

Target gene prediction

To identify the potential targets of miR-222 that mediated its antiangiogenic role in EPCs, in-silico data analysis was performed by TargetScan (www.targetscan.org), miRTarBase (mirtarbase.mbc.nctu.edu.tw), miRTargetLink (ccb-web.cs.uni-saarland.de/mirtargetlink), RNA22 (cm.jefferson.edu/rna22), and miRPathDB (mpd.bioinf.uni-sb.de).

RNA isolation and qRT-PCR

Total RNA was extracted from EPCs using RNAiso plus (Takara, Tokyo, Japan) according to the manufacturer’s directions. Subsequently, RNA was diluted with RNase-free water and stored at -80°C. RNA concentrations were analyzed by NanoDrop spectrophotometry (Thermo Fisher Scientific, Wilmington, USA). Then, cDNA was synthesized using 3 μg of total RNA in 20 μL volume, oligo (dT) primers, and reverse transcriptase. The qRT-PCR was performed using SYBR® Premix Ex Taq™ Perfect Real Time (Takara, Tokyo, Japan) in the Real-time PCR Mx3000PTM System (Genetices Technology, Shanghai, China). PCR conditions comprised an
miR-222 modulates EPC function

initial holding at 95°C for 10 minutes, then 40 cycles at 95°C for 10 seconds and 60°C for 20 seconds (for miRNAs), and at 95°C for 15 seconds and 60°C for 20 seconds (for mRNAs). Data analyses were performed by the comparative threshold cycle (Ct) method, as described previously [35]. All reactions were executed in triplicate. U6 small nucleolar RNA and GAPDH were used as an endogenous control for sample normalization. The primer sequences used for cDNA are shown in Table 2.

Enzyme-linked immunosorbent assay (ELISA)

The total concentrations of VEGF after transfection were determined by 96-well enzyme immunoassay ELISA kits (MultiSciences, Zhejiang, China) with a sensitivity of at least 3.66 pg mL, according to the manufacturer’s instructions. Each experiment was independently repeated in triplicate.

Western blotting analysis

For total cellular protein, EPCs were lysed with 300 µL lysis buffer (Beyotime, Shanghai, China) and supplemented with phenylmethanesulfonyl fluoride (Sigma) and protein phosphatase inhibitors (Sigma). After centrifugation for 15 minutes at 12,000 g (4°C), the protein concentration was detected using a Biomate 3s (Thermo Fisher Scientific, USA). Protein lysates were electrophoresed in 10% SDS-PAGE gels and then transferred onto polyvinylidene difluoride membranes (Millipore, MA, USA) by electrophoresis. After blocking for 1 hour in 5% BSA at room temperature, the membranes were incubated with primary antibodies against β-actin (β-actin, 1:1000, Cell Signaling Technology, CA, USA), STAT5A (1:500, Abcam, USA), phospho-p38 MAPK and p38 MAPK (1:1000, Cell Signaling Technology, CA, USA), and secondary goat anti-rabbit antibodies (1:5000, PerkinElmer, USA). Finally, immunoblot signals were visualized using Pierce ECL plus substrate (Thermo Fisher Scientific, Rockford, USA) and then imaged and quantitated using a Fluor ChemFC3 camera system (Protein Simple, CA, USA).

Cell viability assay

Viability of EPCs was evaluated by using the Cell Counting Kit-8 assay (CCK-8, Sigma). EPCs
miR-222 modulates EPC function

were seeded in each well of 96-well plates at a density of 5000 cells in 100 µL culture medium and were cultured at 37°C in a 5% CO₂ incubator for 24 hours. Cells were then detected using Cell Counting Kit-8 for 2 hours. The absorbance (OD 450 nm) of the medium value in each well was determined using a microplate reader which assessed the cell viability.

Cell adhesion assay

EPC adhesion was done as previously reported [36, 37]. Human EPCs were briefly washed with phosphate-buffered saline (PBS) and gently detached with 0.25% trypsin after treatment overnight with transfection. An equal number of EPCs (2×10⁴ cell/well in 100 µL) was seeded into fibronectin-coated 96-well plates. Then, EPCs were incubated for 30 minutes at 37°C in 5% humidified CO₂. Adhesion was quantified by independently counting adherent cells in five randomly selected fields per well (×200).

Cell migration assay

The migration ability of EPCs was assessed using Transwell cell migration assays. A total of 5×10⁴ EPCs in 100 µL of serum-free medium was loaded into the upper chambers of a 24-well Transwell plate with 8.0 µm pore size (Corning, NY, USA). The lower chamber was filled with the medium containing 20% FBS, which served as a chemotactrant. Then, EPCs were incubated for 24 hours at 37°C in a 5% CO₂ incubator. After 24 hours, the non-migrated cells from the upper chamber of the membrane were washed with PBS. Migrated cells were fixed with 4% paraformaldehyde for 20 minutes and stained with hematoxylin for 30 minutes. Migration activity was evaluated as the mean number of migrated cells in five random high-power fields (magnification ×100) in each chamber.

Statistical analysis

All data from three independent experiments were expressed as mean ± standard deviation. Statistical comparisons were carried out using the t-test for two groups, Mann-Whitney test for non-normally distributed data, and one-way ANOVA for multiple groups. P < 0.05 was deemed statistically significant. GraphPad Prism version 6.01 (GraphPad, San Diego, CA, USA) was used for statistical analyses.

Results

Baseline characteristics of subjects

CAD and healthy groups were mainly matched by age, gender, smoking history, hypertension, diabetes mellitus, and lipid profile (Table 1). No statistically significant differences were observed between CAD and unhealthy groups for baseline characteristics.

Characterization of EPCs and expression of miR-222 in EPCs from CAD patients

EPCs were isolated from the blood of patients with CAD and healthy groups. After 6 hours of initial culture, the human peripheral blood total mononuclear cells showed a round and small morphology (Figure 1Aa). After 7 days, so-
miR-222 modulates EPC function

Overexpression of miR-222 decreases proliferation, adhesion, and migration of EPCs in vitro

Cell proliferation, adhesion, and migration are the key functions of EPCs and play crucial roles in angiogenesis and neovascularization. Thus, we conducted the overexpression of miR-222 in healthy donor-derived EPCs to investigate its effect on EPC functions. miR-222 negative control mimic served as negative control. Then, cell viability assay, adhesion assay, and Transwell migration assay were carried out. Expressions of miR-222 in both healthy and CAD groups were assessed by qRT-PCR. The value of nontransfected control cells was normalized to 1 in Transwell migration assays. miR-222 showed a significant increase in miR-222 mimic-transfected EPCs (P < 0.01, compared with the nontransfected group) (Figure 3A). We observed that transfection with miR-222 significantly inhibited EPC proliferation (P <
miR-222 modulates EPC function

Figure 3. miR-222 modulates EPC function in patients with CAD in vitro.

Inhibition of miR-222 reverses the function of EPC from patients with CAD in vitro

CAD EPC functions are known to be much feeble than healthy EPCs [32]. To further explore the effect of miR-222 on CAD EPC functions, we decreased levels of endogenous miR-222 in CAD EPCs. miR-222 inhibitor with corresponding irrelevant sequences served as negative control. We conducted the same assays to detect the effect of miR-222 inhibitor-transfected CAD EPCs. The value of CAD control cells was normalized to 1 in Transwell migration assays. miR-222 showed a significant decrease in miR-222 inhibitor-transfected EPCs (P < 0.01, compared with the control group; Figure 3E). We observed that CAD EPCs transfected with a miR-222 inhibitor-rescued proliferation (P < 0.05, compared to the control group; Figure 3F), adhesion (P < 0.01, compared with the control group; Figure 3G), and migration (P < 0.01, compared with the control group; Figure 3H). These findings reveal that inhibition of miR-222 might reverse CAD EPC functions, which are deemed to promote vascular homeostasis and endothelium.

miR-222 regulates EPC functions by targeting STAT5A expression

STAT5A was identified as a potential target of miR-222 through bioinformatics approach (Figure 4A). Notably, miRBase showed that the sequence of miR-222 is significantly conserved in humans, rats, and mice. They have the same sequence as 5' to 3': AGCUACAUCCGUACUGGU. Then, we conducted healthy EPCs with no transfection (nontrans), miR-222 mimic (miR-222), negative control mimic (miR-NC), miR-222 inhibitor (anti-miR-222), and corresponding negative control of inhibitor (anti-NC) and transfected for 24 hours to confirm the hypothesis on miR-222 target STAT5A in human EPCs. Expression levels of STAT5A
miR-222 modulates EPC function through p38 MAPK/VEGF signaling pathway

We also investigated whether miR-222 affected p38 MAPK/VEGF signal pathway in human EPCs, which is reported to be a key regulator of EPC function [30]. miR-222 mimic was transfected into healthy EPCs to increase miR-222 expression. miR-222 inhibitor was transfected into CAD EPCs to knock down miR-222 expression. In the present study, we found that miR-222 overexpression significantly decreased the levels of phospho-p38 MAPK and total-p38 MAPK and downregulated VEGF expression (Figure 5A-D). Meanwhile, downregulated miR-222 in CAD EPCs had a reverse result (Figure 5E-H) (p < 0.01 for all).

Discussion

In this study, we elucidated the role of miR-222 in human EPCs and investigated the underlying regulatory mechanisms. The following are the main findings of the study: 1) miR-222 was upregulated in EPCs derived from patients with CAD, which is in accordance with previous findings [19, 20]. 2) miR-222 also displays antiangiogenic mRNA
miR-222 modulates EPC function

characteristics in EPCs as in endothelial cells. 3) The novel target gene of miR-222 in EPCs was STAT5A. 4) The role of miR-222 effects on EPC functions is through p38 MAPK/VEGF signal pathway.

The role of miR-222 in cell proliferation, migration, and survival seems to be highly cell specific but the mechanisms remain poorly defined. miR-222 has been proven to exhibit anti-angiogenic properties in vascular endothelial cells by targeting c-kit, encode cyclin-dependent kinase inhibitor 1B (P27KIP1), and cyclin-dependent kinase inhibitor 1C (P57KIP2) [21, 39]. A recent study has demonstrated that overexpression of miR-222 induces heart failure through the downregulation of p27 and the activation of mTOR pathway [40]. Nardelli et al. reported that miR-222 impairs engraftment capacity and the stem cell activity of cord blood CD34+ progenitor cells coupled with the downmodulation of kit protein [41]. Downregulation of miR-222 enhances angiogenesis in the rat refractory model [42]. Conversely, miR-222 promotes the tumorigenicity of human breast cancer stem cells by targeting PTEN/Akt signal pathway [43]. miR-222 has a pro-proliferative, pro-migration, and anti-apoptosis effect on vascular smooth muscle cells [44]. Another study has shown that downregulation of miR-222 promoted VEGF expression and enhanced vascular density in rat brain after bilateral carotid artery ligation [45]. In our present study, we showed that overexpression of miR-222 in healthy EPCs inhibited angiogenic activity and VEGF expression whereas its downregulation promoted angiogenic capability and VEGF expression in CAD EPCs.

We identified STAT5A as a target of miR-222 in EPCs and showed, for the first time, that miR-222 overexpression downregulated STAT5A protein levels in healthy EPCs whereas its downregulation significantly had an opposite effect. STAT5A, one of the direct targets of miR-222, has been testified in two previous studies [28, 46]. Dentelli et al. showed that miR-222 modulated inflammation-mediated neoangiogenesis through targeting STAT5A, regarded as an important regulator of neovascularization. The group also reported that STAT5A rescued the angiogenic capability of miR-222-overexpressing EPCs [28]. STAT5A, a transcription factor of the family of signal transducers and activators of transcription, is modulated by Rh-oA and decreased p38 MAPK expression levels by SB203580 that resulted in the activation of STAT5A [47]. Hu et al. recently revealed that miR-211 modulated mesenchymal stem cell (MSC) migration through its target STAT5A partly through MAPK signal pathway [48]. These findings support the main findings of our present study, namely that miR-222 inhibits the expression level of its target gene STAT5A in human EPCs and is the likely correlation among miR-222, STAT5A, and p38 MAPK signaling pathway.

Cumulative data are available showing that p38 MAPK signaling pathway and VEGF play a crucial role in governing EC and EPC functions. Moreover, Zuo et al. recently reported that miR-26a impairs EPC functions through inhibiting the p38 MAPK/VEGF signaling pathway [30]. To further verify miR-222-modulating EPC functions by p38 MAPK signaling pathway, we conducted loss-of-function and gain-of-function studies. Furthermore, we used ELISA to detect the level of VEGF. Our findings clearly show that overexpression of miR-222 in healthy EPCs by miR-222 mimics inhibited the activation of p38 MAPK and downregulated VEGF expression. Meanwhile, silencing of miR-222 in CAD EPCs had opposite effects. Previous studies have shown that p38 MAPK is a crucial mediator that results in secreting various cytokines [49, 50]. The secretion of VEGF in fibroblast cell lines is mediated by the activation of p38 MAPK [51]. In addition, activation of p38 MAPK can promote the secretion of VEGF, HGF, and IGF-I in human mesenchymal stem cells or human adipose progenitor cells. Moreover, inhibition of p38 MAPK results in decreased secretion of cytokines [52]. These results also support miR-222 modulating EPC functions by p38 MAPK/VEGF signaling pathway.

A limitation of our present study is that we failed to further investigate the accurate mechanism between p38 MAPK and STAT5A. Therefore, in our next study we will be focusing on elucidating the intrinsic mechanism between p38 MAPK and STAT5A in EPCs to ensure their completion.

Taken together, this study demonstrates that overexpression of miR-222 induces EPC dysfunction and the down-regulation of miR-222 can reverse this effect. We first identified STAT5A as a target of miR-222 in human EPCs.
and confirmed that the effect of miR-222 on EPCs is mediated by p38 MAPK/VEGF signal pathway.

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Disclosure of conflict of interest

None.

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miR-222 modulates EPC function


miR-222 modulates EPC function


