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Chemokine-Like Receptor 1 Regulates the Proliferation and Migration of Vascular Smooth Muscle Cells

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: We aimed to explore how chemokine-like receptor 1 (CMKLR1) influences the proliferation and migration of vascular smooth muscle cells (VSMCs).

Material/Methods: Normal VSMCs, negative control VSMCs interfered by CMKLR1 gene, and VSMCs with stable knockdown of CMKLR1 gene were divided into the control group, PDGF group, negative-shRNA group, and CMKLR1-shRNA group. Both cell number counting and BrdU incorporation assays were employed to investigate the proliferation status of VSMCs. Transwell migration assay was used to measure the migration status of VSMCs. Inflammation markers, including cytokines IL-1β, IL-6, TNF-α, and chemokines MCP-1 in VSMCs, were detected by real-time quantitative RT-PCR. Western blotting assay was used to detect protein expressions of the MAPK pathway in VSMCs.

Results: The number of VSMCs and the OD value of BrdU in PDGF group were significantly higher than those in the control group (both $P<0.05$). Compared with the control and negative-shRNA group, the CMKLR1-shRNA group exhibited significantly reduced VSMCs number and BrdU OD value (both $P<0.05$). Transwell migration assay indicated that PDGF-BB promoted whereas CMKLR1-shRNA inhibited the migration of VSMCs. The expression of IL-1β, IL-6, TNF-α, and MCP-1 were up-regulated in the PDGF group but down-regulated in the CMKLR1-shRNA group. Compared with normal VSMCs, the protein level of p-ERK1/2 was up-regulated in VSMCs treated with PDGF-BB, while it was down-regulated in the CMKLR1-shRNA group.

Conclusions: CMKLR1 exacerbated the proliferation and migration of VSMCs by activating ERK1/2.

MeSH Keywords: Cell Migration Inhibition • Cell Proliferation • Mitogen-Activated Protein Kinase Phosphatases • Muscle, Smooth, Vascular • Receptors, CCR1

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Background

Coronary heart disease results in many deaths all over the world and it involves the process of pathological atherosclerosis, which is closely associated with hypertension, diabetes, and obesity [1]. Restenosis occurs when local damage to the coronary artery contributes to local vascular lumen narrowing as a "healing" reaction after surgical intervention [2]. In general, robust artery membranes contain tunica intima, outer membrane, and medial membrane, which is the main part of the normal vascular smooth muscle cells (VSMCs) [3]. Since VSMCs exhibit small responses to growth-stimulating factors, the proliferation and migration rate is low [4]. Once pathological changes occur in the body, abnormal VSMCs generate chemokines and secrete large amounts of collagen fibers, elastin, proteoglycans, and inflammatory factors together with enormous proliferation and migration factors into the intima, which further contributes to cell proliferation and progression of vascular remodeling [5–7]. Previous reports suggested that the above mechanism was correlated with a series of processes, including atherosclerosis, restenosis, hypertension, and coronary heart disease, as well as multi-aspects process such as plaque rupture and apoptosis [3,5,8,9].

Chemerin is an adipokine secreted by adipocytes and are usually presented in white adipose and hepar tissues [10]. CMKLR1 (chemokine-like receptor 1) or ChemR23 (chemerin receptor 23) is a G protein-coupled receptor (GPCR) and it is expressed in macrophages, dendritic cells, adipocytes, vascular cells, natural killer (NK) cells, and microglia [10,11]. Chemerin acts through CMKLR and exhibits both pro-inflammatory and anti-inflammatory features [12]. Chemerin also influences proliferation and migration of VSMCs, which are crucial to the development of hypertension [10,13]. As a leukocyte attractant, adipokine, and antimicrobial protein, chemerin is associated with obesity, insulin resistance, metabolic syndrome, and multiple sclerosis [10,11,14,15].

The mitogen-activated protein kinase (MAPK) cascade is a vital signaling pathway which comprises 4 parts: MAPK/ERK (extracellular signal regulated kinases) system or classic pathway, Big MAP kinase-1 (BMK-1), c-Jun N-terminal kinase (JNK), and p38 signaling system [16]. These pathways share a fundamental system composed of 2 serine/threonine kinases and 1 double-specificity threonine/tyrosine kinase 14 [17]. These kinases from the upstream to the downstream are referred to as MAPK kinase-kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK [18]. The canonical MAPK/ERK pathway is composed of 3 types of MAPKKK: A-RAF, B-RAF, and RAF-1 or C-RAF kinases [19]. It is widely accepted that the MAPK signaling pathway is related to cell dissemination, survival, and resistance to therapeutic drugs of human cancer [17,20].

In the present study, the lentiviral shRNA vector was used to construct VSMC strains with deficient human CMKLR1 genes in order to observe VSMCs proliferation and migration status after silencing human CMKLR1 gene and to explore the association between CMKLR1 and the proliferation or migration status of VSMCs.

Material and Methods

Materials

SPF (specific pathogen free) NIH mice (6 weeks old) were purchased from Guangdong Medical Laboratory Animal Center (China). All animal treatment and care procedures complied with the US National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. This study was approved by Research Ethics Committee of Shenzhen People's Hospital and Shenzhen Institutes of Advanced Technology Chinese Academy of Science.

Cell culture

Primary mouse vascular smooth muscle cells (VSMCs) were isolated from NIH mouse thoracic aortas as previously described [21]. VSMCs were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) containing 10% fetal bovine serum (FBS; Gibco), streptomycin (100 mg/ml; Gibco), and penicillin (100 units/ml; Gibco) at 37°C in an incubator with 5% CO_2. Subsequent experiments were conducted on cells located between the third and sixth passage.

Construction and identification of cells with knockdown of CMKLR1

Stable knockdown of gene CMKLR1 in VSMCs was carried out using lentiviral shRNA vectors. The CMKLR1 shRNA sequence used in this experiment was 5'-GATCCGACACCCAAGGATATGTCT TTCAAGAGAAGACATATCCTTGGGTGTCTTTTTT-3'. CMKLR1 shRNA plasmid was constructed using the Lenti-X Lentiviral Expression Systems (Takara, Dalian, China). VSMCs without any treatment were set as the blank control group. Plasmid pLVX-CMKLR1 shRNA containing CMKLR1 shRNA and plasmid pLVX-CMKLR1 negative shRNA containing unspecific shRNA (Takara) were considered as the CMKLR1-shRNA and negative shRNA group. Cells were transfected with the above plasmids using Lipofectamine 2000 (Invitrogen, USA) and cultured at 37°C in an incubator with 5% CO_2. Transfected cells were selected with puromycin (2 μg/ml, 7 days) (Takara). Silencing of the CMKLR1 gene was identified through detecting CMKLR1 mRNA and protein expression levels.

RNA extraction and RT-PCR

Total RNA was extracted from VSMCs using the TRIzol reagent kit (Invitrogen) following the manufacturer's specific

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instructions. Complementary DNA (cDNA) was acquired using the Omniscript reverse transcription kit (Qiagen, Germany). Real-time quantitative RT-PCR assay was conducted with ABI7500 quantitative PCR instrument (Applied Biosystems) for detecting the relative expression level of CMKLR1 mRNA. Primers of CMKLR1 (purchased from Invitrogen) were: CMKLR1 forward, 5'-ATGGACTACCACTGGGTTTTCGGG-3' and reverse, 5'-GAAGACGAGAGATGGGGAAACTCAAG-3'. The relative expression quantity of CMKLR1 mRNA were calculated by the $2^{-\Delta\Delta Ct}$ method and normalized to the expression of U6 snRNA. All the above assays were repeated 3 times.

Cell proliferation analysis

VSMCs proliferation status was tested by cell counting and bromodeoxyuridine (BrdU) incorporation assay using a commercial kit (Cell Proliferation ELISA; Roche, Switzerland). Briefly, VSMCs were inoculated into 96-well plates with 1×10^4 cells/well for 24 h and a total of 6 wells were repeated. Then cells were transfected with CMKLR1-shRNA or negative shRNA. Cells added to phosphate-buffered solution (PBS) were used as the blank control, whereas cells with platelet-derived growth factor-BB (PDGF-BB, 10 ng/ml, PeproTech, USA) were considered as the positive control. DMEM medium was added as a zero well in unseeded cell wells. Cells were detected after treatment was applied for 12, 24, 48 and 72 h. For cell counting assay, VSMCs were resuspended in PBS after they were trypsinized, then the number of VSMCs was counted under a microscope with a hemocytometer. BrdU incorporation assay was carried out following the manufacturer's instructions. The absorbance value was detected at a wavelength of 450 nm using a microplate reader (SpectraMAX Plus, Molecular Devices, CA) in which the wavelength of 690 nm was set as the reference.

Transwell migration assay

Cell migration assay was conducted using 24-well transwell plates (8-μm pores) (BD Biosciences, USA) to measure the migration status of VSMCs. Briefly, cells were trypsinized and resuspended in serum-free medium after 48-h transfection or the addition of PDGF-BB. Then, 2×10^4 cells were placed in the upper transwell chamber and 0.5 ml complete DMEM medium was added into the lower chamber as the chemoattractant. Cotton swabs were used to remove any cells that were not migrated from the top chambers after 24-h incubation at 37°C. By contrast, migrated cells at the bottom of the membrane were fixed with methanol, stained with crystal violet, and counted. The number of cells that migrated to the lower membrane in each group was considered as an index which reflected the migration status and ability of cells in each group. This experiment was replicated 6 times.

Analysis of inflammation markers

Several cytokines, including IL-1β, IL-6, TNF-α, and chemokines MCP-1 in VSMCs, were detected by real-time quantitative RT-PCR until cells were treated for 48 h. Primer sequences used for IL-1β, IL-6, TNF-α and MCP-1 genes were: IL-1β, 5'-ACAGATGAAGTGCTCCTTCCA-3' (F), 5'-GTCGGAGATTCGTAGCTGGAT-3' (R); IL-6, 5'-AACAGGTA AAGGCCCACTATGC-3' (F), 5'-TGACTTGTCCTGAGACCTGATGT-3' (R); TNF-α, 5'-CCCAGGGACCTCTCTCTAATC-3' (F), 5'-ATGGGCTACAG GCTTGTCACT-3' (R); MCP-1, 5'-GCTGTGATCTT CAAGACCATTGTG-3' (F), 5'-GAA TCCTGAACCCACTTCTGCTT-3' (R). The relative mRNA expression quantity of IL-1β, IL-6, TNF-α and MCP-1 were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to the expression of U6 snRNA. All the above assays were replicated 6 times.

Western blotting assay

The expression levels of p-ERK1/2, ERK1/2, p-p38 MAPK, p38 MAPK, p-JNK, and JNK were examined by Western blotting assay. Cellular proteins were extracted after 48-h treatment. The BCA method was used for protein density detection. Equal amounts of proteins in each group were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene fluoride membranes, and blocked with 5% skim milk. Membranes were separately incubated with primary antibodies (p-ERK1/2, ERK1/2, p-p38 MAPK, p38 MAPK, p-JNK and JNK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (CST, American) at 4°C overnight. Membranes were washed with TBST 3 times (10 min each) and incubated with horseradish-peroxidase-linked secondary antibodies for 1 h at room temperature. Finally, membranes were washed with TBST for another 3 times (10 min each) and signal detection was carried out using Super ECL Plus Detection Reagent (Applygen Technologies Inc., China).

Statistical analysis

All statistical analyses were performed by SPSS 19.0 software. Significant differences in numerical data (mean ±SD) were estimated and tested by the analysis of variance (ANOVA), and *P*-value of less than 0.05 provided strong evidence for statistical significance.

Results

CMKLR1 mRNA and protein expression was down-regulated by shRNA in VSMCs

CMKLR1 mRNA and protein expression in VSMCs were detected using RT-PCR and Western blotting, respectively. The results showed that CMKLR1 mRNA (Figure 1A) and protein (Figure 1B)

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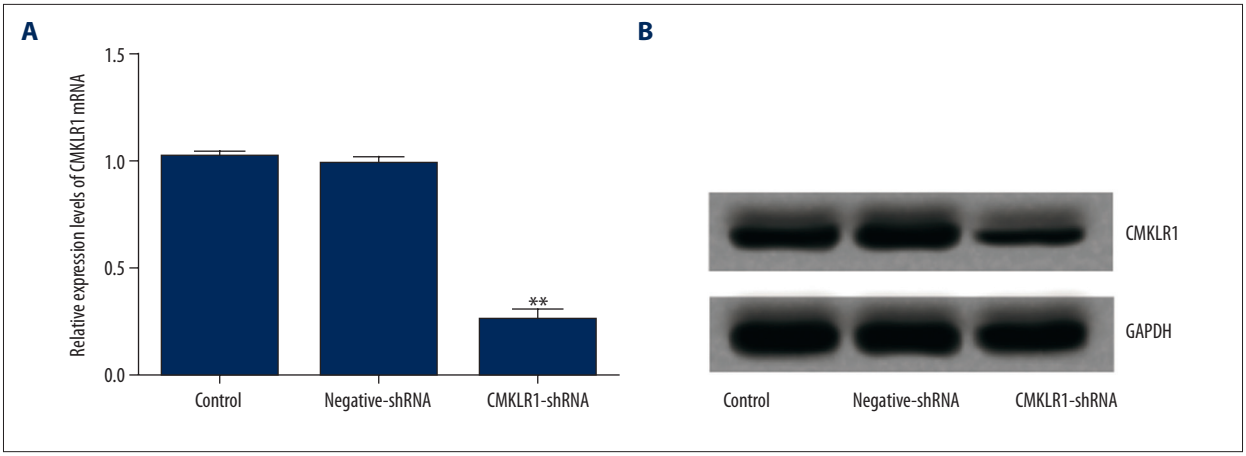

Figure 1. Knockdown of CMKLR1 by shRNA in VSMCs. VSMCs were stably transfected with pLVX-CMKLR1 shRNA plasmid. (**A**) CMKLR1 mRNA expression detected by RT-PCR with U6 snRNA as an internal control. (**B**) CMKLR1 protein expression detected by Western blot assay and normalized to that of GAPDH. Results are from 3 independent experiments. Data are presented as mean ±SD. ** *P*<0.01, compared with the control and negative-shRNA group. shRNA – short hairpin RNA; VSMCs – vascular smooth muscle cells; RT-PCR – reverse transcription-polymerase chain reaction; GAPDH – glyceraldehyde 3-phosphate dehydrogenase.

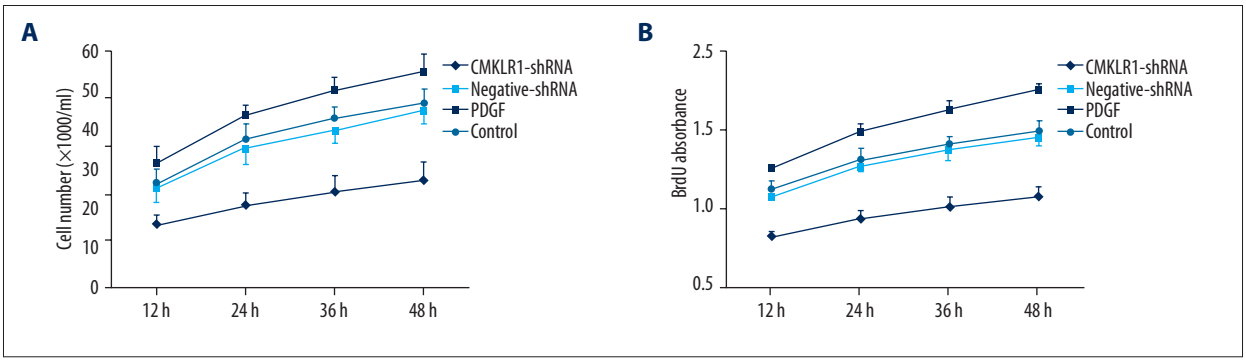

Figure 2. Effects of CMKLR1-shRNA and PDGF-BB on VSMCs proliferation. (**A**) Cell counting assay. (**B**) BrdU incorporation assay. Results (mean ±SD) were from 6 independent experiments. shRNA – short hairpin RNA; PDGF-BB – platelet-derived growth factor-BB; VSMCs – vascular smooth muscle cells; BrdU – bromodeoxyuridine.

expression in the CMKLR1-shRNA group were significantly reduced compared with the negative shRNA and control group (*P*<0.01). However, VSMCs showed insignificant changes in CMKLR1 mRNA and protein expression levels in the control and negative shRNA groups (*P*>0.05).

Effects of CMKLR1 knockdown on cell proliferation

Both cell counting assay and BrdU incorporation assay were used to evaluate the proliferation status of VSMCs. The results demonstrated that PDGF-BB could exacerbate VSMCs proliferation compared to the control group (all *P*<0.05) and difference in VSMCs proliferation between the PDGF-BB and control group appeared to be more significant as time of treatment extended. Compared with the control and negative-shRNA group, knockdown of CMKLR1 significantly inhibited VSMCs proliferation over the treatment period (all *P*<0.05). No significant difference in cell proliferation was observed between the control and negative-shRNA group (*P*>0.05) (Figure 2). All these data revealed that activation of the CMKLR1 signaling pathway could stimulate VSMCs proliferation.

Effects of CMKLR1 knockdown on cell migration

Results of transwell migration assay showed that the migrated cell number of VSMCs which were transfected with CMKLR1-shRNA (60.33±8.05) were significantly lower than those of the control group (98.05±11.04) and negative shRNA group (95.36±15.52) (both *P*<0.05), while there was no significant difference in these figures between the control and negative-shRNA group (*P*>0.05). Compared with the control and negative shRNA group, PDGF-BB significantly increased the number of migrated VSMCs (171.15±12.08) (both *P*<0.05) (Figure 3). These findings demonstrated that PDGF-BB and activation of

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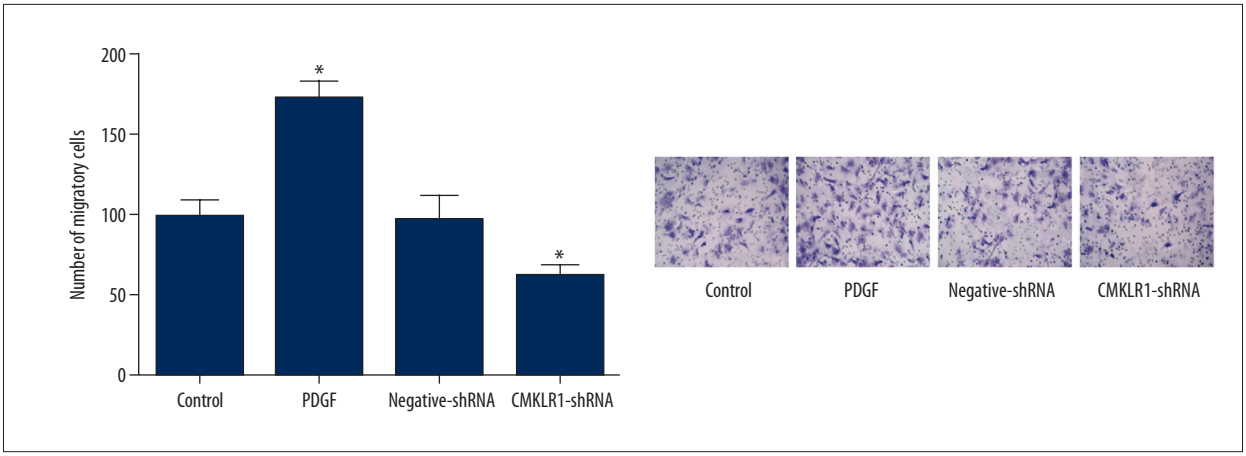

Figure 3. VSMCs migration was inhibited by CMKLR1-shRNA and exacerbated by PDGF-BB. The migratory cells after 48-h treatment was detected by a transwell migration assay. Results (mean ±SD) were from 6 independent experiments. * $P<0.05$ versus the control and negative shRNA group. VSMCs – vascular smooth muscle cells; shRNA – short hairpin RNA; PDGF-BB – platelet-derived growth factor-BB.

CMKLR1 signaling pathway could stimulate the migration status of VSMCs.

Effects of CMKLR1 knockdown on inflammation

Cytokines, including IL-1β, IL-6, and TNF-α, and chemokines MCP-1 in VSMCs were detected by real-time quantitative RT-PCR. The results indicated that CMKLR1-shRNA significantly decreased IL-1β, IL-6, TNF-α, and MCP-1 gene expressions compared with the control and negative shRNA group (both $P<0.05$). No significant differences in IL-1β, IL-6, TNF-α, and MCP-1 gene expressions were demonstrated between the control and negative shRNA group ($P>0.05$). Compared with the control and negative shRNA group, PDGF-BB could significantly elevate IL-1β, IL-6, TNF-α, and MCP-1 gene expressions (both $P<0.05$) (Figure 4).

CMKLR1 knockdown inhibited phosphorylation of ERK1/2 in VSMCs

The effect of CMKLR1-shRNA and PDGF-BB on MAPK pathway activities in human VSMCs was detected by Western blotting. As shown in Figure 5, CMKLR1-shRNA suppressed the phosphorylation of ERK1/2 whereas PDGF-BB increased the phosphorylation of ERK1/2. CMKLR1-shRNA and PDGF-BB did not have significant impact on the total protein levels. Furthermore, CMKLR1-shRNA or PDGF-BB did not have a significant effect on the total and phosphorylated p38 MAPK and JNK activity. These findings indicate that CMKLR1 is able to activate ERK1/2 pathways in human VSMCs.

Discussion

Chemerin has been considered as a new adipokine which promotes adipose cell differentiation because it is relevant to both BMI and insulin level [22]. Chemerin is leukocyte attractant ligands extensively expressed for G protein-associated receptors [23,24]. Among the 3 receptors that bind with chemerin, chemokine-like receptor 1, which is also referred to as chemR23, is the natural receptor that is related to chemerin-associated signal transduction in macrophages, dendritic cells, adipocytes, and vascular cells [25–27]. As suggested by our study, shRNA CMKLR1 suppressed VSMCs proliferation and migration in comparison to negative shRNA. After lentivirus vectors were constructed by CMKLR1 shRNA, shRNA exhibited more effective and durable transduction in target issues [28,29]. It is also known that CMKLR1 is able to stimulate VSMCs proliferation and migration [13]. A prior study concluded that chemerin stimulated the proliferation of mouse myoblast C2C12 cells via activating ERK [30]. Another study also suggested that chemerin/CMKLR1 regulated the activation of both Akt and ERK and this mechanism might maintain the activity of endothelial cells [31]. In addition, Carlino et al. has demonstrated that chemerin stimulated the migration of human natural killer cells through activating ERK [32]. All these results suggested that CMKLR1 may have positive effects on cell proliferation and migration via activating both Akt and ERK signals. Moreover, results from RT-PCR indicated that CMKLR1 shRNA restrained expressions of inflammatory factors in VSMCs. Previous studies have found that CMKLR1 was associated with inflammation and CMKLR1 cracked into different fragments with pro-inflammatory or anti-inflammatory actions [33–36]. Apart from that, CMKLR1 is up-regulated in chronic inflammatory diseases and CMKLR1 expression level is related to pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β, MCP-1, and C reactive protein [37,38]. Interestingly, CMKLR1 has been suggested to exhibit anti-inflammatory effects on vascular cells through several processes, including protein kinase B (Akt) signaling and nitric oxide production [39].

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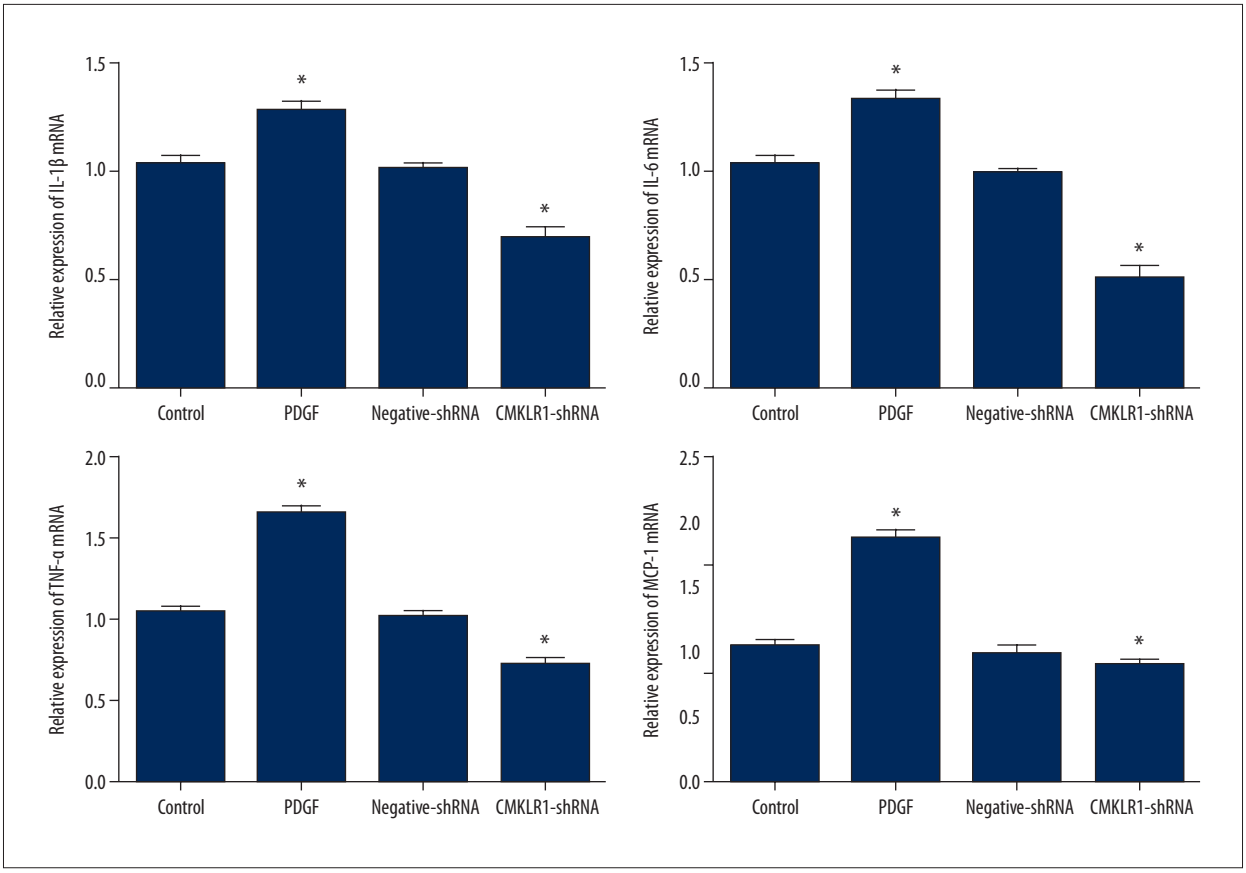

Figure 4. The relative expression of inflammation markers (IL-1β, IL-6, TNF-α, MCP-1) in VSMCs were measured by real-time quantitative RT-PCR. Results (mean ±SD) were from 6 independent experiments. * $P<0.05$ versus the control and negative-shRNA group. IL-1β – interleukin-1β; IL-6 – interleukin-6; TNF-α – tumor necrosis factor-alpha; MCP-1 – monocyte chemoattractant protein-1; VSMCs – vascular smooth muscle cells; RT-PCR – reverse transcription-polymerase chain reaction.

The effect of CMKLR1 on inflammation depends on the inflammation site [15].

Our research also discovered that PDGF could stimulate VAMCs proliferation and migration, which is similar to the conclusion that PDGF-BB stimulated the migration of rat mesenteric arterial SMC [40]. A series of studies confirmed that more than 2 small GTPases of Rho family proteins (CDC42-GTP pathway or Rac-GTP pathway) act on the migration process induced by PDGF-BB [41]. Moreover, it has been suggested that Akt and ERK signals exist in the downstream of Rac-GTP pathway [42]. The data in our study showed that PDGF could significantly increase expressions of inflammation factors and this conclusion was consistent with those from prior studies. Wang et al. found that PDGF-BB contributed to the production of cystolic reactive oxygen species and up-regulation of inflammation factors such as TNF-α, TNFR1, MCP-1, and IL-6. PDGF-BB exhibited synergistic effects when it was integrated with MAPK/ERK promoters [43].

The mitogen-activated protein kinase (MAPK) cascade is vital to cancer cell survival, distribution, and resistance to medication [18]. Main members of MAPK include ERK and JNK, and the p38 MAPK and ERK pathway is the final effector, which is located in the downstream of the MARPK pathway [16]. As suggested by Western blotting analysis, both CMKLR1 shRNA and PDGF activated ERK1/2, while their effects on JNK and p38 MAPK levels were not significant. ERK is a contractive signal joint consisting of ERK1 and ERK2. Furthermore, ERK receives various stimulus afferents, including internal metabolic stress and DNA damage signals [17]. ERK phosphorylation contributes to the activation of multiple basal plates responsible for stimulating cellular proliferation [19]. Activatory ERK inside the karyon triggers phosphorylation and activation of assorted transcription factors including carbamoyl phosphate synthetase II. These effects of MEK/ERK contribute to the stimulation of cell proliferation [44,45]. In immunocytes, activatory ERK plays an important role in responding to every inflammatory cascade procedure and in stimulating the TNF-α expression [46].

This study enabled us to verify that CMKLR1 could stimulate the proliferation and migration of mouse VSMCs by activating ERK1/2. Since only mouse VSMCs was used in this *in vitro*

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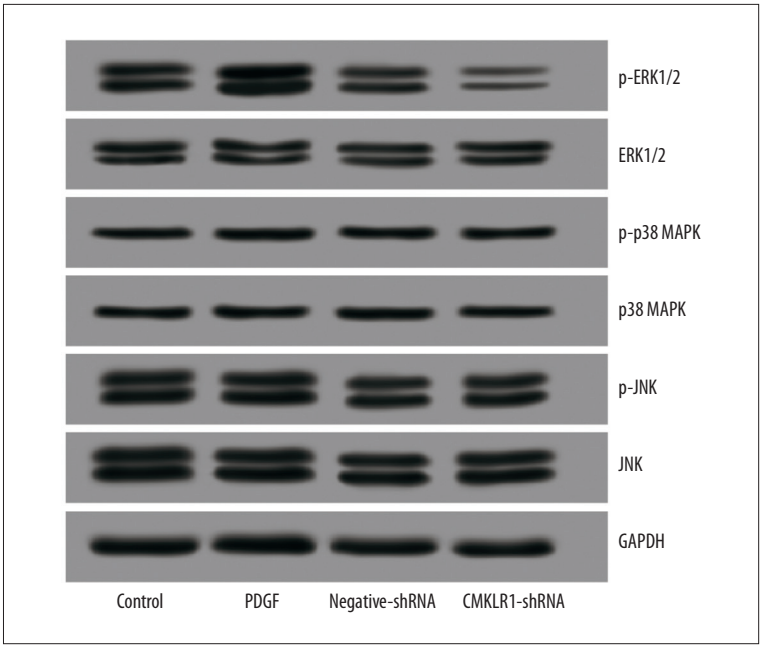

Figure 5. CMKLR1-shRNA inhibited ERK1/2 pathway in human VSMCs. Total and phosphorylated ERK1/2, p38 MAPK, and JNK were measured by Western blot analysis.

study, it is recommended that experiments on other types of cells should be conducted to confirm these conclusions. Ongoing research on the molecular mechanism of CMKLR1 and ERK1/2 pathway in VSMCs is encouraged to address the limitations contained in our study.

Conclusions

Our study provides evidence that CMKLR1 not only exacerbated the proliferation and migration of VSMCs but also stimulated expressions of several inflammation factors. The effect of CMKLR1 on the proliferation and migration of VSMCs, as well as on the expression of inflammation factors, was enlarged by the addition of PDGF. Therefore, this study may provide additional information for developing new therapeutic targets of coronary heart disease.

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