의학박사 학위논문

Mitochondrial abnormalities are related to the dysfunction of circulating endothelial-colony-forming cells in moyamoya disease

모야모야병에서 혈관내피전구세포와 미토콘드리아의 이상에 관한 연구

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의학과 중개의학 (뇌신경과학) 전공

최 정 원

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국 문 초 록

모야모야병에서 혈관내피전구세포와 미토콘드리아의 이상에 관한 연구

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서론: 모야모야병은 양측 내경동맥의 진행하는 협착증을 특징으로 하는 질환이다. 최근 들어 다양한 유전체 검사를 통해 예상되는 관련 유전자가 발견되기는 하였으나, 모야모야병의 정확한 발병원인은 아직 규명되지 않았다. 혈관내피전구세포 또한 모야모야병의 발병원인에 밀접히 관련이 있는 것으로 알려져 있다. 본 연구에서는 모야모야병에서 혈관내피전구세포의 미토콘드리아의 이상에 대해 알아보고자 한다.

방법: 모야모야병 환자(n=5)와 대조군인 건강한 자원자(n=5)로부터 말초 혈액을 체취하였다. 기존의 알려진 방법을 통해 혈관내피전구세포를 배양하여 이 세포의 미토콘드리아의 형태학적, 기능적 특이점에 대해 실험하였다. 형태학적 특징을 알아보기 위해 전자 현미경 및 MitoTracker Red를 이용하였다. 아울러 미토콘드리아 산소 소비율, 막전위, 세포내 칼슘 농도, 활성 산소농도 등을 알아보았다. 또한 혈관내피전구 세포의 혈관 생성능의 변화등을 알아보았다.

결과: 모야모야병을 가진 환자의 혈관내피전구세포의 미토콘드리아 모양은 그 모양이 절단되고 좀더 구형에 가까운 모습을 보였다. 아울러 미토콘드리아 내에는 액포가 형성되어 있었으며 crista 가 발달되지 않은 모습을 보였다.

또한 모야모야병 혈관내피전구세포의 미토콘드리아는 낮은 산소소비율과 함께 높은 세포내 칼슘 농도를 보였다. 또한 높은 활성산소 농도를 보였는데, 활성산소 소거제를 투약하면 미토콘드리아 형태적, 기능적 이상이 회복됨을 확인하였다. 흥미롭게도 활성산소 소거제를 처리하면, 모야모야병 혈관내피전구세포의 혈관 생성능이 회복됨도 발견하였다.

결론: 모야모야병의 혈관내피전구세포의 미토콘드리아에는 형태학적, 기능적 이상이 발견된다. 이러한 발견은 향후 모야모야병의 발병기전과 그 치료법에 대한 새로운 시각을 제시해줄 것으로 기대한다.

주요어: 뇌혈관 질환, 혈관내피전구 세포, 미토콘드리아, 모야모야병, 활성산소
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Abstract

Mitochondrial abnormalities are related to the dysfunction of circulating endothelial-colony-forming cells in moyamoya disease

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Introduction: Moyamoya disease (MMD) is a unique cerebrovascular disorder characterized by the progressive occlusion of the bilateral internal carotid artery. Although diverse genetic analyses of MMD have been performed, the etiology of MMD has not been fully clarified. Endothelial colony–forming cells (ECFCs), which were previously termed endothelial progenitor cells, play an important role in the pathogenesis of MMD. In this study, we performed morphological and functional studies of the mitochondria of ECFCs from MMD patients to present new insights into the pathogenesis of MMD.

Methods: The morphology of the ECFCs from the MMD patients and normal healthy volunteers was examined under both a transmission electron microscope and a confocal laser scanning microscope following staining with MitoTracker Red. The oxygen consumption rates (OCRs), mitochondrial membrane potentials (MMPs), intracellular Ca^{2+} concentrations, mitochondrial enzyme activities, and reactive oxygen species (ROS) levels were measured. The functional activity of the ECFCs was evaluated using a capillary tube formation assay.

Results: The ECFCs from the MMD patients displayed a disrupted mitochondrial morphology, including a shorter and more circular shape. The mitochondria of the ECFCs

from the MMD patients exhibited functional abnormalities, which were assessed as a decreased OCR and increased intracellular Ca^{2+} concentration. Moreover, the ECFCs from MMD patients showed increased ROS levels. Interestingly, treatment with a ROS scavenger not only rescued the mitochondrial abnormalities, but also restored the angiogenic activity of the ECFCs from the MMD patients.

Conclusions: The mitochondria of the ECFCs from the MMD patients exhibit morphological and functional abnormalities compared to normal ECFCs. This finding suggests that the mitochondrial abnormalities may have a possible role in the pathogenesis of MMD.

Key words : Cerebrovascular disease, Endothelial colony–forming cell, Endothelial progenitor cell, Mitochondria, Moyamoya, Reactive oxygen species

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Introduction

Moyamoya disease (MMD) is a unique cerebrovascular disorder of unknown cause characterized by the progressive occlusion of both the internal carotid artery and its major branches with the arterial collateral vessels at the base of the brain. As the definition of this disease implies, the pathogenesis of MMD is still unclear. Nonetheless, it is well established that this disease shows higher prevalence in East Asia, and 10-15 % of patients with MMD have a family history of this disease.[12] Moreover, it is well known that MMD occurs more frequently among women.[12] This information suggests that genetic factors participate in the etiology of MMD.

Recently, diverse genetic analyses of MMD have attempted to uncover the underlying pathogenic mechanism of MMD. For example, a number of genome-wide linkage studies identified some main loci that are linked to MMD, i.e., 3p24.2-p26, 6q25, 8q23, 12p12 and 17q25.[2] However, independent studies failed to identify the association between MMD and these loci, except for the 17q25 locus. Recent genetic studies have revealed that the RNF213 locus at 17q25 is an important susceptibility gene for MMD.[6] However, there is still a lack of data on the precise role of this gene, and there is even a report that this gene may not be solely responsible for MMD and other susceptibility genes for MMD may exist.[17]

Accordingly, new approaches and multi-modal studies of the pathogenesis of MMD are required. In previous reports, endothelial progenitor cells (EPCs) or vascular progenitor cells were shown to play an important role in physiological or pathological angiogenesis.[9] Abundant evidence suggests that circulating EPCs contribute to the development of diverse cardiovascular diseases. In particular, it was documented that dysfunction in or reduced numbers of circulating EPCs are related to the pathogenesis of stroke.[5] Interestingly, recent studies have shown an intimate correlation between the circulating EPCs and the development of MMD.[11,19] Moreover, it was also reported that the circulating EPCs in MMD

patients were dysfunctional.[11] However, the reason for the dysfunction of the circulating EPCs in MMD patients is not yet understood.

In this study, we investigated the cause of the dysfunction of circulating the endothelial colony–forming cells (ECFCs), which were previously termed EPCs, in MMD patients. We rephrase EPCs as ECFCs, because the outgrowth cells from adherent cultures of peripheral blood cells in vitro may have the different characteristics of pure EPCs. This study showed that the abnormalities in the mitochondria of the ECFCs were related to the dysfunction of the circulating ECFCs in MMD patients.

It is well known that MMD occurs more frequently among women.[1,12] This female predominance was significantly more noticeable in the familial group than in the sporadic group. In familial cases, the ratio of women to men significantly increases to 5.0.[18] Moreover, it was documented that the ratio of maternal transmission to paternal transmission was increased more than three-fold in familial MMD cases.[16] We designed this study according to these reports that MMD shows characteristic inheritance. This is the first study to analyze the mitochondria of ECFCs in MMD patients. Based on the results of our current study, we present new insights into the pathogenesis of MMD.

Materials and Methods

Study participants

For the study group, we obtained blood samples from the MMD patients during revascularization surgery after obtaining informed consent from the patients and their parents. All patients were confirmed to have MMD through the angiographic study. Blood samples of 5 MMD patients (2 men and 3 women) were included in this study and their median age was 13 years (range 2 to 36 years).

For the normal control group, 5 healthy volunteers (2 men and 3 women) were recruited, and their median age was 23 years old (range 20 to 27 years). They had no history of stroke, hypertension or smoking. Clinical features of the MMD patients and control group are summarized at Table.1. This study was approved by the Institutional Review Board of the Seoul National University Hospital (approval No. 1412149636). And all participating patients gave written informed consent. A blind observer who did not know the clinical information for each patient performed a series of *in vitro* expreiments to examine the unltrastructure of mitochondria and function of mitochondria (variety of assays).

Cell culture and phenotypic characterization of the ECFCs

All blood samples used for cultures were processed within 2 hours after collection. The peripheral blood was diluted 1:1 with phosphate-buffered saline (PBS), and the mononuclear cells were isolated from the buffy coat in Ficoll 1077 g/mL (Histopaque-1077; Sigma, St. Louis, MO) and washed in PBS 3 times. The cells were plated on culture dishes coated with collagen type I (BD BioCoat; BD Biosciences, Mountain View, CA) and grown in an endothelial cell growth medium (EGM-2; Clonetics, San Diego, CA) at a density of 2×10^6 cells per well. The cells were maintained at 37 °C and 5 % CO_2 in a humidified atmosphere.

To verify that the cells were ECFCs, we confirmed that the cells exhibited a typical

cobblestone morphology and immunofluorescence staining using antibodies against CD31, CD34, CD45, CD133, KDR, and vWF, as previously described in published studies.[13]

RNF213 gene sequencing

DNA samples were obtained from ECFCs using the DNA Mini kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After DNA extraction and quantification, the DNA samples were conducted to PCR amplification with the appropriate primer sets (sense 5′-CTGATGCGTCAGCTCCATAG-3′ and antisense 5′-TTCCTGCTTTGTGCAGTCAC-3′). Sequencing analysis of RNF213 c.14576G>A variant was conducted on the ABI 3730XL DNA sequencer, and data were analyzed by ABI sequencing Analysis software (Applied Biosystems, Norwalk, CT).

Electron microscope (EM) analysis

The normal and MMD ECFCs were fixed overnight in a mixture of cold 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and 2% paraformaldehyde in 0.1 M phosphate or cacodylate buffer (pH 7.2) and then embedded with epoxy resin. The epoxy resin-mixed samples were loaded into capsules and allowed to polymerize at 38°C for 12 h and 60°C for 48 h. Thin sections were sliced on an ultramicrotome (RMC MT-XL) and collected on a copper grid. Appropriate areas were cut into thin sectioning at 65 nm and stained with saturated 4% uranyl acetate and 4% lead citrate solutions, followed by examination under a transmission electron microscope (JEM-1400; JEOL, Tokyo, Japan) at 80 kV.

Quantitative morphological analysis of the mitochondria

The mitochondria were visualized after the cells were stained with MitoTracker Red

(Invitrogen, Carlsbad, CA). The images were captured under a confocal laser scanning microscope (FV10i-w; Olympus, Tokyo, Japan), and 20-50 cells from each group were analyzed with Image J software (National Institutes of Health, MD), as previously described.[3]

Measurements of the oxygen consumption rate (OCR)

A Seahorse Bioscience XF24–3 extracellular flux analyzer (Seahorse Bioscience, Billerica, MA) was used to measure the OCR in the medium immediately surrounding the ECFCs cultured in XF24 V7 cell culture microplates, according to the manufacturer's instructions. Briefly, the OCR measurement was performed after equilibration in assay medium (lacking supplemental CO_2). The Seahorse analyzer uses a cartridge with 24 optical fluorescent O_2 sensors that are embedded in a sterile disposable cartridge, one for each well. Prior to each rate measurement, the plungers mix the assay media in each well for 8 min to allow the partial oxygen pressure to reach equilibrium. To measure the rates, the plungers gently descend into the wells to form chambers. The O_2 concentration is periodically measured over 4 min, and the rates of oxygen consumption are obtained from the slopes of the changes in the concentration vs. time. After the rates are measured, the plungers ascend, and the wells are gently mixed to equilibrate the medium. The baseline rates are measured four times. One or more test chemicals are preloaded in the reagent delivery chambers of the sensor cartridge and then pneumatically injected into the wells to reach the desired final working concentration. After 2 min of mixing, the post-exposure OCR was measured four to six times. The averages of four baseline rates and two to six test rates were used for the data analyses.

Mitochondrial membrane potential (MMP) measurement

The MMP measurements were performed as previously described.[20] In depolarized cells, labeling of the mitochondria with potential-indicating probes like tetramethyl rhodamine

methyl ester (TMRM) disappears; therefore, the red fluorescence serves as an indicator of the MMP. The medium was replaced with phenol red-free medium containing 500 nM TMRM (100 μl/well; T-668; Invitrogen). The plates were incubated for 1 h at 37°C and washed three times with PBS (50 μl/well). The fluorescent signals were captured using a fluorescence microscope (Olympus), and > 100 cells from each group were analyzed.

Fluo-4 fluorescence imaging to measure the $[Ca^{2+}]_i$

To image the intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), the cells were loaded with the Ca^{2+}-sensitive dye, fluo-4-acetoxymethyl ester (Fluo-4 AM, 5 μM; F10471; Invitrogen) for 60 min at 37°C and then washed with PBS to remove the extracellular Fluo-4 AM. The fluorescent signals were captured using a fluorescence microscope. The fluorescence intensity reflected $[Ca^{2+}]_i$. The images for 100 cells from each group were analyzed using Image J software (National Institutes of Health).

MTT assay

To evaluate the activities of mitochondrial enzymes, MTT [(3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)] assays, which measure mitochondrial dehydrogenase activity, were performed as previously described.[4] Briefly, 2.5 mg/ml MTT (M2003; Sigma-Aldrich)in phenol red–free medium was added to the cells and incubated for 2 h at 37°C. Then, the MTT solution was aspirated, isopropanol was added to dissolve the formazan crystals, and the cells were incubated for 1h at 37°C. The absorbance was measured at 540 nm. At least three independent experiments were performed, and the data were expressed as a percentage of the normal ECFCs.

Measurement of reactive oxygen species (ROS) levels in the ECFCs

To detect the intracellular ROS levels, we used 2',7'-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR). Approzimately 1×10^6 cells were incubated with DCFH-DA (10 μM) for 30 min at 37 °C in the dark, and the cells were washed twice with PBS. Fluorescence was detected with a FACScan® flow cytometer (Becton Dickinson, Franklin Lakes, NJ) using CellQuest® software (Becton Dickinson).

Capillary tube formation assay

The functional activity of the ECFCs was evaluated using an *in vitro* capillary tube formation assay. The ECFCs (2×10^4 cells/well) were plated on a Matrigel (BD Biosciences)-coated 48-well plate and incubated for 18 hr. The number of tubes and branches attached to tubular structures that had formed from the ECFCs were counted in 4 randomly selected microscope fields (× 40 original magnification).

Data analysis

All data were expressed as the means ± standard error of the mean (S.E.M.) from at least three independent experiments for each data point. The Student's t-test was used for two-group comparisons, and an analysis of variance, followed by Fisher's LSD post hoc test was used to compare three or more groups using SigmaStat for Windows Version 3.10 (Systat Software, Inc., Point Richmond,CA).

Results

The ECFCs from MMD patients showed a disrupted mitochondrial morphology.

To investigate the mechanism of the impaired function of the MMD ECFCs, we focused on the mitochondria because previous studies showed that mitochondrial dysfunction leads to alterations in cellular function, and ultimately cell death. Using EM analysis, massive mitochondrial fission and a loss of cristae were observed in the MMD ECFCs, but not in the normal ECFCs (Fig. 1A). Consistent with the EM data, the mitochondria in the MMD ECFCs were shorter (represented by aspect ratio) and more circular (represented by form factor) than those in the normal ECFCs (Fig. 1B-1C). These data indicate that the MMD ECFCs displayed an altered mitochondrial morphology compared to the normal ECFCs.

The ECFCs from the MMD patients exhibited mitochondrial dysfunction and increased $[Ca2+]_i$.

We determined whether the MMD ECFCs exhibited mitochondrial dysfunction as well as an altered mitochondrial morphology. To investigate the alterations in mitochondrial function in the normal and MMD ECFCs, we first investigated cellular respiration in the normal and MMD ECFCs by measuring the OCR. To measure adenosine triphosphate (ATP) production, oligomycin was injected into culture wells prior to injection of the uncoupling agent carbonyl cyanide m-chlorophenyl hydrazone (CCCP). The MMD ECFCs consistently showed reduced basal OCRs, OCRs coupled to ATP generation, which is the oligomycin-sensitive OCR, and total oxidative capacity, which is the CCCP-sensitive OCR (Fig. 2A). The basal respiration rates, ATP levels and oxidative capacity of the MMD ECFCs were decreased by approximately 70%, 80% and 75% relative to the normal ECFCs, respectively (Fig. 2B). The

antimycin-insensitive OCR, which is interpreted as non-mitochondrial respiration, was slightly but non-significantly reduced in the MMD ECFCs (Fig. 2B).

Next, we showed that the MMP in the MMD ECFCs was significantly attenuated using TMRM assays (Fig. 2C). It is known that mitochondria are an important Ca^{2+} buffering organelle that sustains cellular Ca^{2+} hemostasis, and $[Ca^{2+}]_i$ accumulation induces cell death.[20] Because the MMD ECFCs exhibited mitochondrial dysfunction, we analyzed the $[Ca^{2+}]_i$ levels in ECFCs using a Fluo-4 assay. We found that the $[Ca^{2+}]_i$ levels in the MMD ECFCs were increased (Fig. 2C). Furthermore, the MTT assay was performed to investigate the activity of the mitochondrial enzymes in the ECFCs. We found that the MMD ECFCs exhibited significantly reduced mitochondrial reductase activity (Fig. 2D). Overall, these data indicate that the MMD ECFCs exhibited mitochondrial dysfunction compared to the normal ECFCs.

The MMD ECFCs exhibited increased ROS levels.

Because a depolarized MMP is known to induce ROS generation[22] and the intracellular ROS levels are inextricably linked with mitochondrial function, we measured the intracellular ROS levels in the ECFCs from the MMD patients and normal controls. The ROS levels were significantly increased in the MMD ECFCs compared to the normal ECFCs (Fig. 3A: 132% of normal control; $p=0.041$).

Furthermore, we treated the MMD ECFCs with the ROS scavenger N-acetylcysteine (NAC) The NAC treatment dose-dependently reduced the intracellular ROS production in the MMD ECFCs (Fig. 3B and 3C; $p=0.006$)

Treatment with the ROS scavenger rescued the mitochondrial abnormalities in the MMD ECFCs.

The MMD ECFCs were treated with NAC to determine whether the ROS scavenger can rescue the mitochondrial abnormalities observed in the MMD ECFCs. Surprisingly, the NAC-treated MMD ECFCs displayed healthy mitochondria in terms of their lengths and shapes (Fig. 4A). When the OCR was analyzed in the ECFCs after a 24 hr treatment with NAC, we found that the NAC treatment significantly rescued the reduced basal respiration rates, ATP levels and oxidative capacity in the MMD ECFCs (Fig. 4B). Using the TMRM assay, we showed that the MMP was also rescued in the NAC-treated MMD ECFCs (Fig. 4C). In addition, the Fluo-4 assay showed that the NAC-treated MMD ECFCs maintain normal $[Ca^{2+}]_i$ (Fig. 4C). These data indicated that the increased ROS levels induced harmful effects on the mitochondria in the MMD ECFCs, and treatment with a ROS scavenger can rescue the mitochondrial dysfunction in the MMD ECFCs.

Treatment with the ROS scavenger restored the impaired angiogenic activity in the MMD ECFCs.

Tube formation is an important function of ECFCs, and *in vitro* tube formation tests are the most frequently used assay for measuring angiogenic activity. We checked whether treatment with the ROS scavenger restored the impaired angiogenic activity in the MMD ECFCs. Interestingly, the NAC treatment (2 mM) significantly increased number of tubes formed and enhanced the angiogenic activity (Fig. 5).

Discussion

Our current study indicates that the mitochondria of the ECFCs from MMD patients exhibit morphological and functional abnormalities. The morphology of the mitochondria of the ECFCs from MMD patients were fragmented with vacuoles and disrupted cristae, whereas those of the normal ECFCs appeared to be elongated and filamentous. Functionally, the mitochondria of the ECFCs from MMD patients exhibited reduced basal respiration, ATP production and total oxidative capacity (maximal respiration), which indicates that the ECFCs in MMD patients are functionally defective. Furthermore, the mitochondria of the ECFCs from MMD patients were more depolarized and exhibited increased ROS and intracellular Ca^{2+} levels and reduced mitochondrial reductase activity. Notably, a scavenger that only decreased the ROS levels not only rescued the morphological and functional abnormalities of the mitochondria but also the angiogenic activity of the ECFCs. Accordingly, the mitochondria may have a significant role in the pathogenesis of MMD.

The mitochondria are not only involved in energy production but also in other cellular activities, such as signaling cascades, cellular proliferation, senescence, and cell death.[15] Thus, cellular dysfunction is highly associated with abnormalities in mitochondrial status and activity. Actually, mitochondrial dysfunction results in numerous diseases, including neurodegenerative disorders, metabolic diseases, and even cancers that are known as ‘mitochondrial diseases’ or ‘mitochondrial disorders’.[10]

Likewise, numerous studies demonstrated that mitochondrial damage and dysfunction also have an important role in various cardiovascular disorders, such as atherosclerosis, hypertension, stroke, heart failure and ischemic heart disease.[23] However, there has been no report about the correlation between mitochondria and MMD, until now. Our current study indicates that the impaired function of the ECFCs in MMD patients might result from mitochondrial abnormalities, suggesting the possibility that MMD might be mitochondria

related disease. However, to prove this hypothesis, mutations in the nuclear DNA that encode mitochondrial components or mutations in the mitochondrial DNA should be identified. These investigations are now under way. Additional studies of the diverse causes that contribute to the mitochondrial abnormalities of the ECFCs in MMD patients are required. These subsequent studies could clearly identify mitochondrial abnormalities in MMD as the primary phenomena rather than secondary result. This speculation that MMD might be a mitochondria-related disease could account for the specific epidemiological quality in MMD, because mitochondrial mutations exhibit maternal inheritance.

There is increasing evidence that circulating ECFCs, which were previously termed EPCs, have a very important role in endothelial homeostasis. For example, it was well documented that circulating ECFCs contribute to ongoing endothelial repair.[7] Moreover, the ECFCs dysfunction plays an important role in the pathogenesis in MMD. Although there are still controversial results and the exact role of ECFCs has not been conclusively defined, many previous studies demonstrated an intimate connection between ECFCs and MMD.[11,21]

In our previous studies, we not only demonstrated that there were fewer ECFCs in MMD patients, but they also showed impaired differentiation, dysfunction, and tendency to undergo senescence.[8,13] Our current study indicates that these impaired functions and the senescence of ECFCs in MMD patients might result from mitochondrial abnormalities. These abnormalities may be related to the delayed repair of the damaged vessel and to the development of vessel occlusions in MMD patients.

According to this study, mitochondrial dysfunction might be an important causative risk factor for the defects in ECFCs in MMD patients. This result implies the possibility that MMD might be a mitochondria-related disease. Furthermore, our study showed that ROS were closely related to the function of ECFCs. This finding provides a clue to pathological mechanism and therapeutic targets for MMD, such as ROS-sensitive transcription factors.

Recently, one study demonstrated that microRNA-424 is closely related to the cerebral ischemic-perfusion injury in the ischemic stroke by regulating endogenous ROS.[14] We assume that ROS plays an important role also in pathogenesis of MMD. We have an ongoing study about this issue.

There are several limitations in our current study. Firstly, the sample size in this study is relatively small. Our findings should be verified through a larger cohort. In addition, we could not recruit age-matched controls. However, it is ethically undesirable to obtain the blood from normal children volunteers, because children are vulnerable subjects in the medical research field. Lastly, due to the practical difficulty of acquiring the 'pathologic' tissue of MMD patients (the intracranial vessels such as moyamoya vessels or the stenotic carotid artery) ECFCs were utilized for the study.

Conclusions

Our current study revealed the morphological and functional abnormalities of the mitochondria of the ECFCs from MMD patients. This finding suggests the possibility that MMD might be a mitochondria-related disease. Additional studies on mitochondria could contribute to new insights into the pathogenesis of MMD.

Disclosure

The author report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

References

1. Baba T, Houkin K, Kuroda S: Novel epidemiological features of moyamoya disease. J Neurol Neurosurg Psychiatry 79:900-904, 2008

2. Bersano A, Guey S, Bedini G, Nava S, Herve D, Vajkoczy P, et al: Research Progresses in Understanding the Pathophysiology of Moyamoya Disease. Cerebrovasc Dis 41:105-118, 2016

3. Byun J, Son SM, Cha MY, Shong M, Hwang YJ, Kim Y, et al: CR6-interacting factor 1 is a key regulator in Abeta-induced mitochondrial disruption and pathogenesis of Alzheimer's disease. Cell Death Differ 22:959-973, 2015

4. Cha MY, Han SH, Son SM, Hong HS, Choi YJ, Byun J, et al: Mitochondria-specific accumulation of amyloid beta induces mitochondrial dysfunction leading to apoptotic cell death. PLoS One 7:e34929, 2012

5. Chu K, Jung KH, Lee ST, Park HK, Sinn DI, Kim JM, et al: Circulating endothelial progenitor cells as a new marker of endothelial dysfunction or repair in acute stroke. Stroke 39:1441-1447, 2008

6. Fujimura M, Sonobe S, Nishijima Y, Niizuma K, Sakata H, Kure S, et al: Genetics and Biomarkers of Moyamoya Disease: Significance of RNF213 as a Susceptibility Gene. Journal of Stroke 16:65-72, 2014

7. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, et al: Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med 348:593-600, 2003

8. Kang HS, Moon YJ, Kim YY, Park WY, Park AK, Wang KC, et al: Smooth-muscle progenitor cells isolated from patients with moyamoya disease: novel experimental cell model. J Neurosurg 120:415-425, 2014

9. Kang HS, Wang KC, Kim SK: Circulating Vascular Progenitor Cells in Moyamoya Disease. J Korean Neurosurg Soc 57:428-431, 2015

10. Khan NA, Govindaraj P, Meena AK, Thangaraj K: Mitochondrial disorders: Challenges in diagnosis & treatment. Indian Journal of Medical Research 141:13-26, 2015

11. Kim JH, Jung JH, Phi JH, Kang HS, Kim JE, Chae JH, et al: Decreased level and defective function of circulating endothelial progenitor cells in children with moyamoya disease. J Neurosci Res 88:510-518, 2010

12. Kuriyama S, Kusaka Y, Fujimura M, Wakai K, Tamakoshi A, Hashimoto S, et al: Prevalence and clinicoepidemiological features of moyamoya disease in Japan: findings from a nationwide epidemiological survey. Stroke 39:42-47, 2008

13. Lee JY, Moon YJ, Lee HO, Park AK, Choi SA, Wang KC, et al: Deregulation of Retinaldehyde Dehydrogenase 2 Leads to Defective Angiogenic Function of Endothelial Colony-Forming Cells in Pediatric Moyamoya Disease. Arterioscler Thromb Vasc Biol 35:1670-1677, 2015

14. Liu P, Zhao H, Wang R, Wang P, Tao Z, Gao L, et al: MicroRNA-424 protects against focal cerebral ischemia and reperfusion injury in mice by suppressing oxidative stress. Stroke 46:513-519, 2015

15. McBride HM, Neuspiel M, Wasiak S: Mitochondria: More than just a powerhouse. Current Biology 16:R551-R560, 2006

16. Mineharu Y, Takenaka K, Yamakawa H, Inoue K, Ikeda H, Kikuta KI, et al: Inheritance pattern of familial moyamoya disease: autosomal dominant mode and genomic imprinting. J Neurol Neurosurg Psychiatry 77:1025-1029, 2006

17. Moteki Y, Onda H, Kasuya H, Yoneyama T, Okada Y, Hirota K, et al: Systematic Validation of RNF213 Coding Variants in Japanese Patients With Moyamoya Disease. J Am Heart Assoc 4, 2015

18. Nanba R, Kuroda S, Tada M, Ishikawa T, Houkin K, Iwasaki Y: Clinical features of familial moyamoya disease. Childs Nerv Syst 22:258-262, 2006

19. Rafat N, Beck G, Pena-Tapia PG, Schmiedek P, Vajkoczy P: Increased levels of circulating endothelial progenitor cells in patients with Moyamoya disease. Stroke 40:432-438, 2009

20. Son SM, Byun J, Roh SE, Kim SJ, Mook-Jung I: Reduced IRE1alpha mediates apoptotic cell death by disrupting calcium homeostasis via the InsP3 receptor. Cell Death Dis 5:e1188, 2014

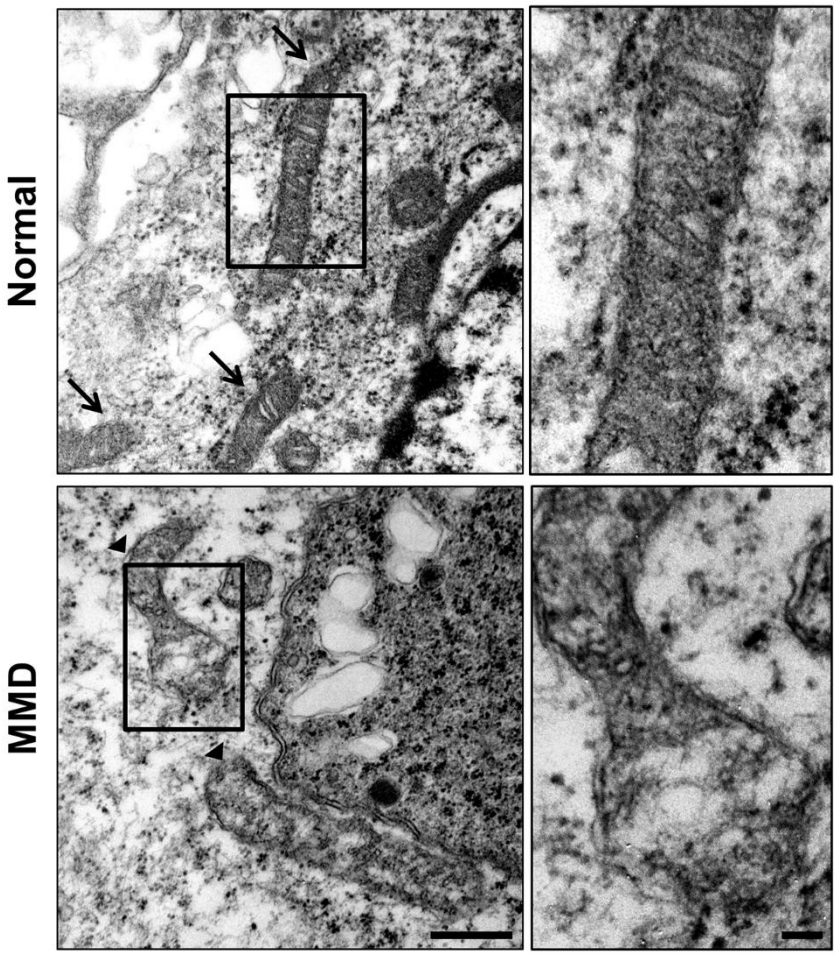
21. Sugiyama T, Kuroda S, Nakayama N, Tanaka S, Houkin K: Bone marrow-derived endothelial progenitor cells participate in the initiation of moyamoya disease. Neurol Med Chir (Tokyo) 51:767-773, 2011

22. Suski JM, Lebiedzinska M, Bonora M, Pinton P, Duszynski J, Wieckowski MR: Relation between mitochondrial membrane potential and ROS formation. Methods Mol Biol 810:183-205, 2012

23. Yu E, Mercer J, Bennett M: Mitochondria in vascular disease. Cardiovasc Res 95:173-182, 2012

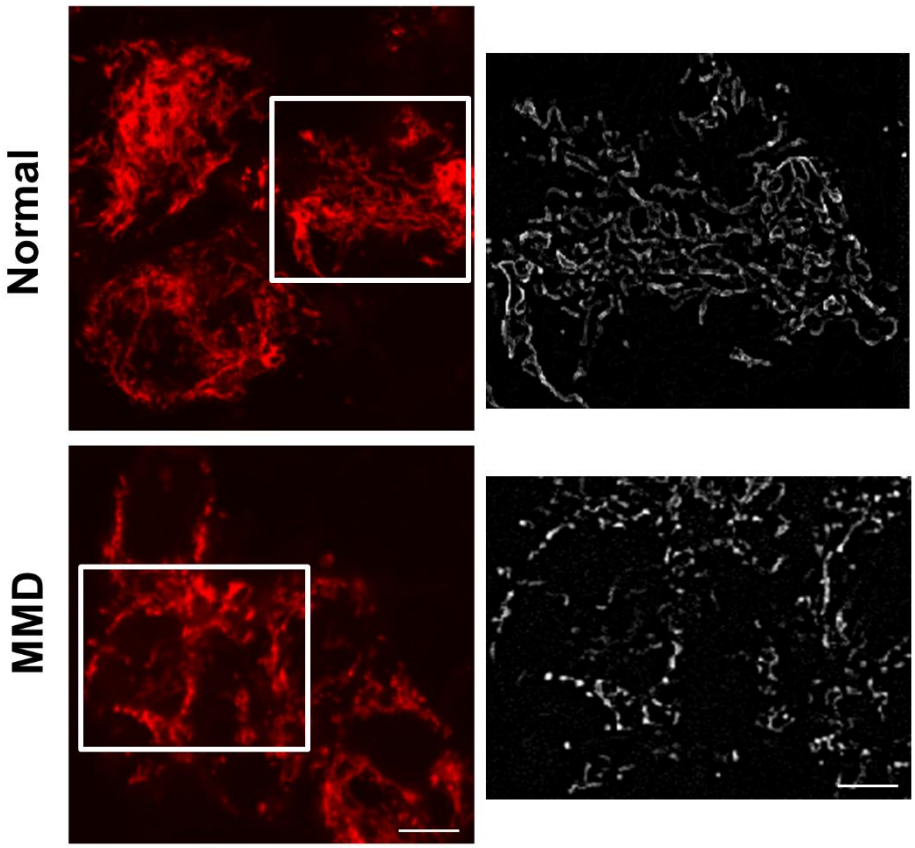
Figures

Figure 1. The endothelial colony–forming cells (ECFCs) from patients with moyamoya disease (MMD) show a disrupted mitochondrial morphology.



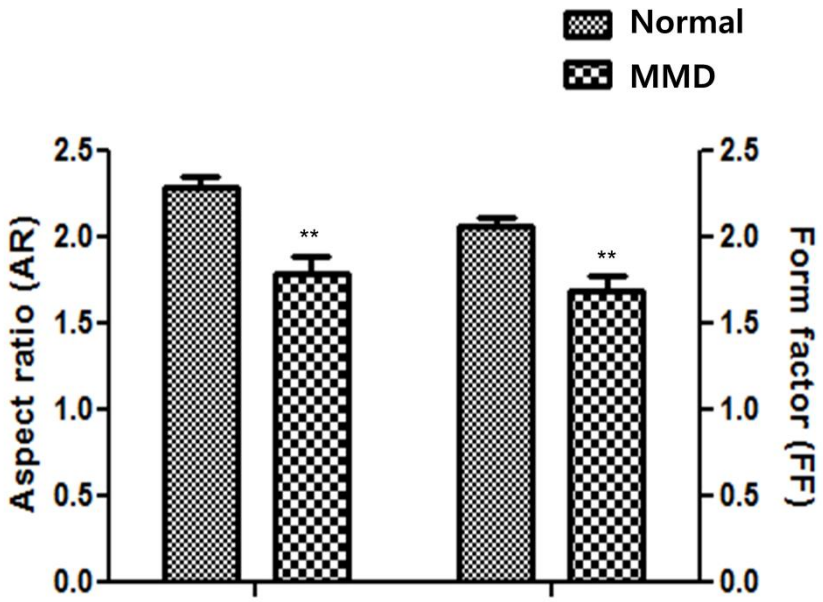
(A) Electron microscopy reveals a disrupted mitochondrial morphology in the MMD ECFCs. The arrows indicate the intact and healthy mitochondria, whereas the arrowheads indicate the disrupted mitochondria. The scale bars represent 0.5 μm and 0.1 μm.

Figure 1. The endothelial colony–forming cells (ECFCs) from patients with moyamoya disease (MMD) show a disrupted mitochondrial morphology.



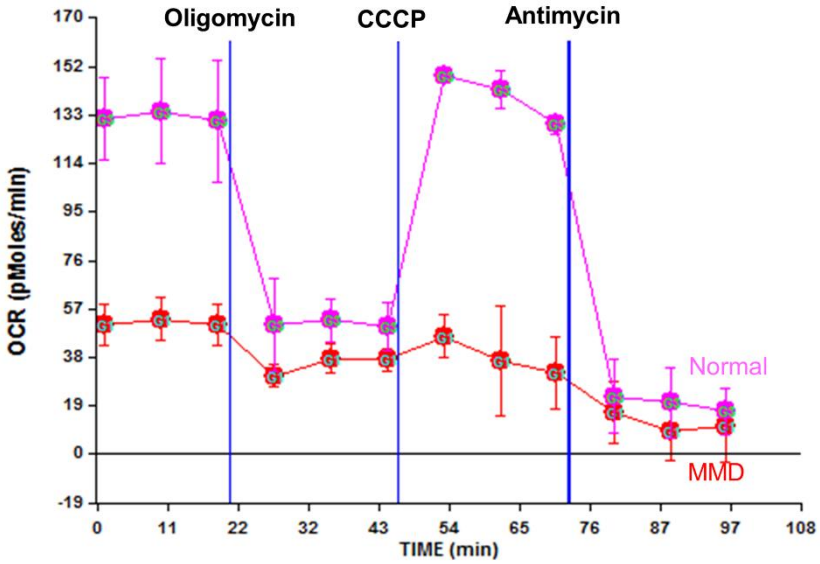
(B) Changes in the mitochondrial morphology in the MMD ECFCs are shown by MitoTracker staining. In the right panels, the enlarged images are converted to an 8-bit format to analyze mitochondrial morphology using the Image J program. The scale bars represent 5 μm and 2 μm.

Figure 1. The endothelial colony–forming cells (ECFCs) from patients with moyamoya disease (MMD) show a disrupted mitochondrial morphology.



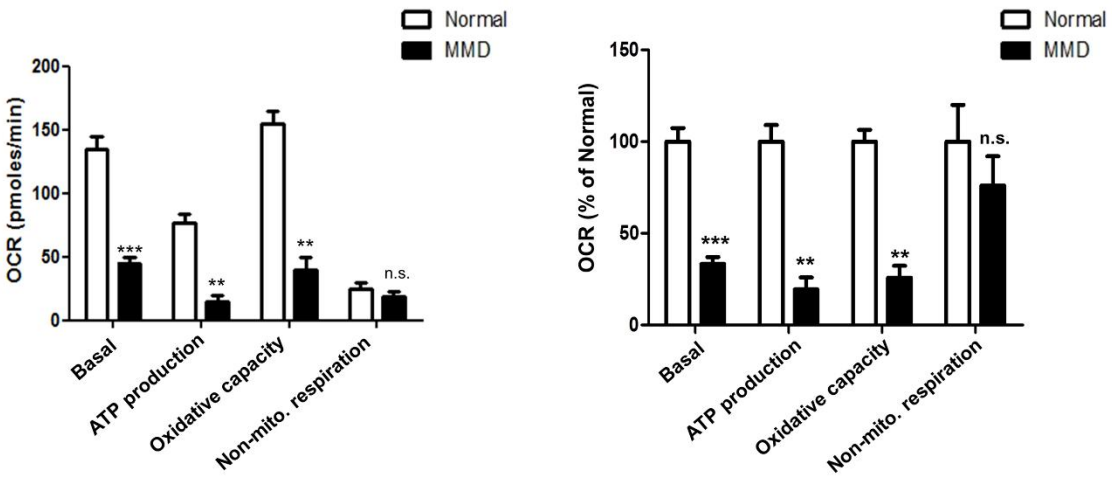
(C) Quantitative analysis of the changes in mitochondrial morphology. The mitochondria in the MMD ECFCs are shorter (represented by the aspect ratio) and have a more circular shape (represented by the form factor) compared to the normal ECFCs. ** $p < 0.01$ vs. the normal ECFCs.

Figure 2. The endothelial colony–forming cells (ECFCs) from patients with moyamoya disease (MMD) display mitochondrial dysfunction and increased $[Ca^{2+}]_i$.



(A) The representative data from a single Seahorse experiment that was representative of three similar experiments depict the oxygen consumption rate (OCR) in normal (pink) and MMD (red) ECFCs. Each OCR data point represents a mean ± S.E.M. The concentrations of oligomycin (complex V inhibitor), carbonylcyanide-3-chlorophenylhydra-zone (CCCP; uncoupler of mitochondrial respiration and ATP synthesis), and antimycin (complex III inhibitor) are 2 μM, 5 μM, and 1 μM, respectively. The MMD ECFCs display reduced OCR levels.

Figure 2. The endothelial colony–forming cells (ECFCs) from patients with moyamoya disease (MMD) display mitochondrial dysfunction and increased $[Ca^{2+}]_i$.



(B) Total OCR as well as the fractions of OCR that are changed following the administration of oligomycin, CCCP, and antimycin. The data are derived from the experiment presented in Figure 2A. ** $p < 0.01$, *** $p < 0.001$ vs. the normal ECFCs. The abbreviation n.s. indicates not significant.

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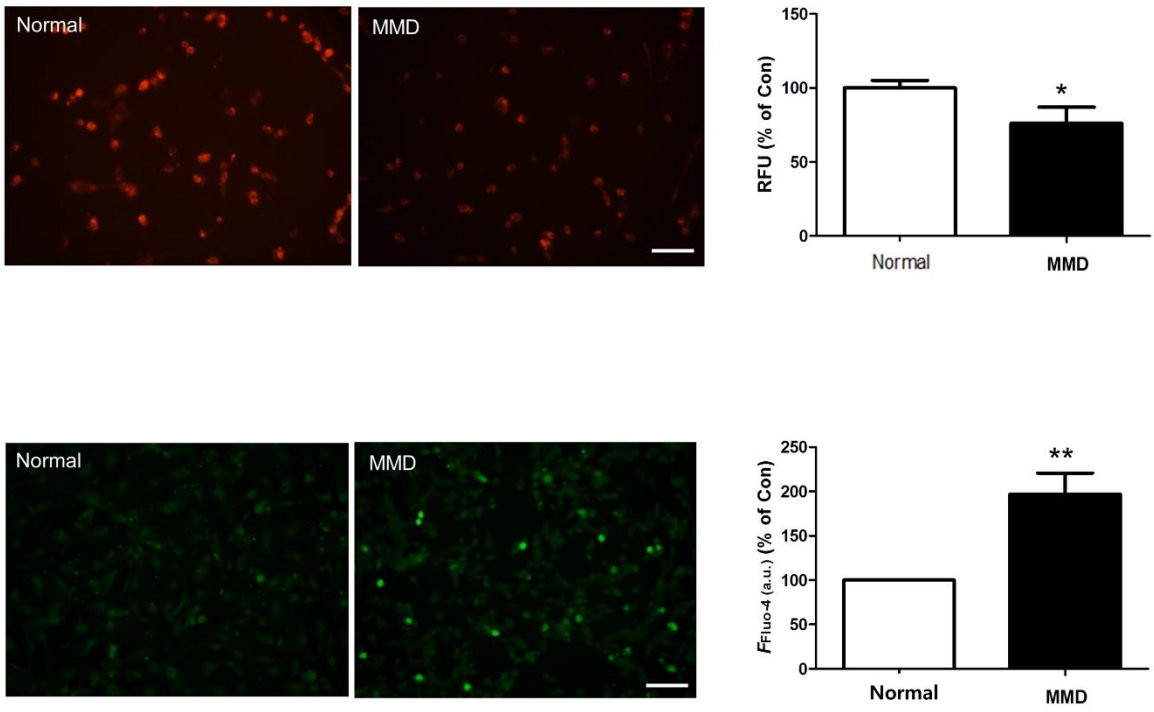
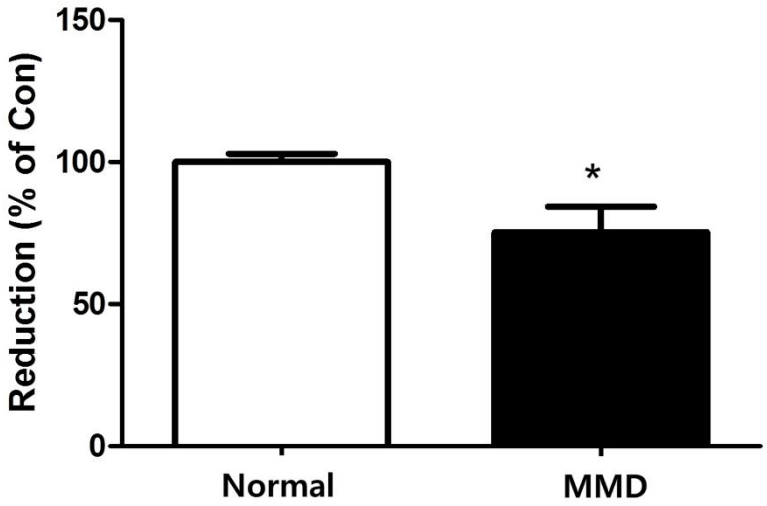


(C) The mitochondrial membrane potential is measured using the tetramethyl rhodamine methyl ester (TMRM) assay (top). The data are presented as the means ± S.E.M. * $p< 0.05$ vs. the normal ECFCs.

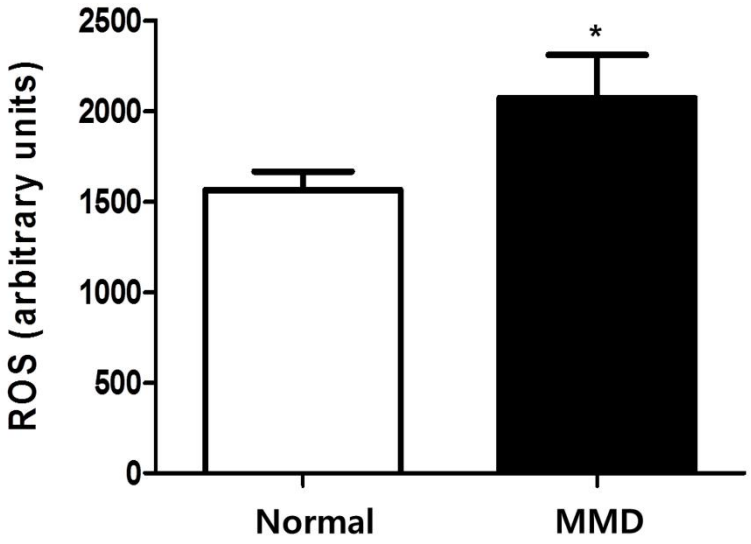
Intracellular Ca^{2+} ($[Ca^{2+}]_i$) is measured using the Fluo-4 assay (bottom). Representative images are shown. The data are presented as the means ± S.E.M. * $p< 0.05$, ** $p< 0.01$ vs. the normal ECFCs. The scale bar represents 50 μm.

Figure 2. The endothelial colony–forming cells (ECFCs) from patients with moyamoya disease (MMD) display mitochondrial dysfunction and increased $[Ca^{2+}]_i$.



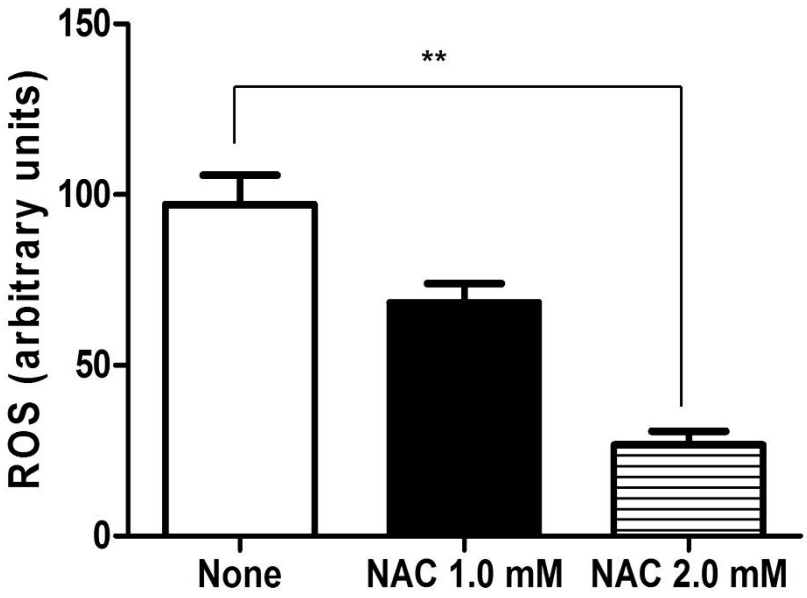
(D) Mitochondrial dehydrogenase activity is determined by the MTT assay. * $p< 0.05$ vs. the normal ECFCs.

Figure 3. The endothelial colony–forming cells (ECFCs) from patients with moyamoya disease (MMD) show increased reactive oxygen species (ROS) levels.



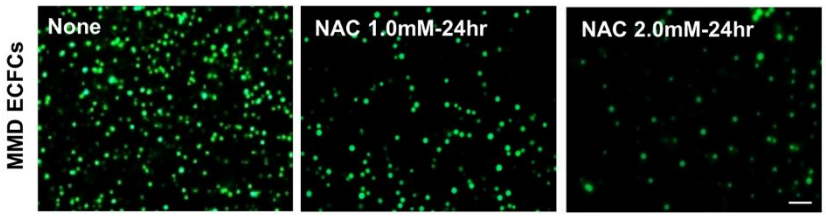
(A) The ROS levels in ECFCs are measured using the oxidation-dependent fluorescent dye 6-carboxy-2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). ROS generation is significantly increased in the MMD ECFCs. * $p < 0.05$ vs. the normal ECFCs.

Figure 3. The endothelial colony–forming cells (ECFCs) from patients with moyamoya disease (MMD) show increased reactive oxygen species (ROS) levels.



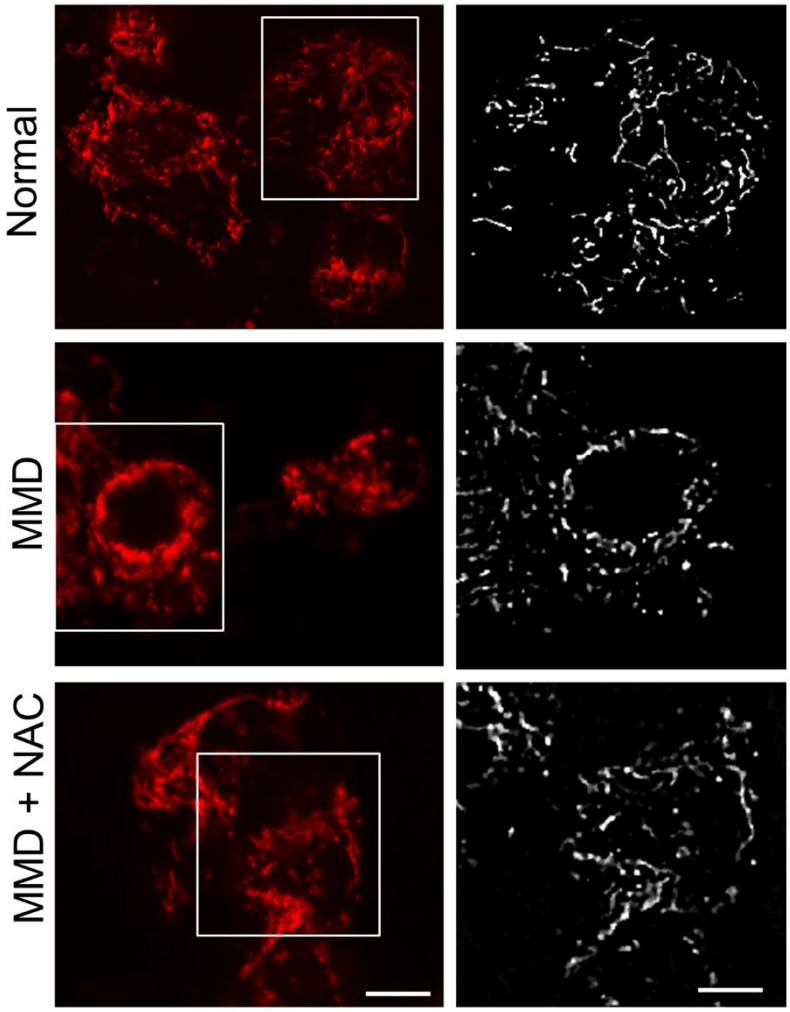
(B) The N-acetylcysteine (NAC) treatment dose-dependently reduces the ROS levels in the MMD ECFCs. ** $p < 0.01$ vs. the normal ECFCs.

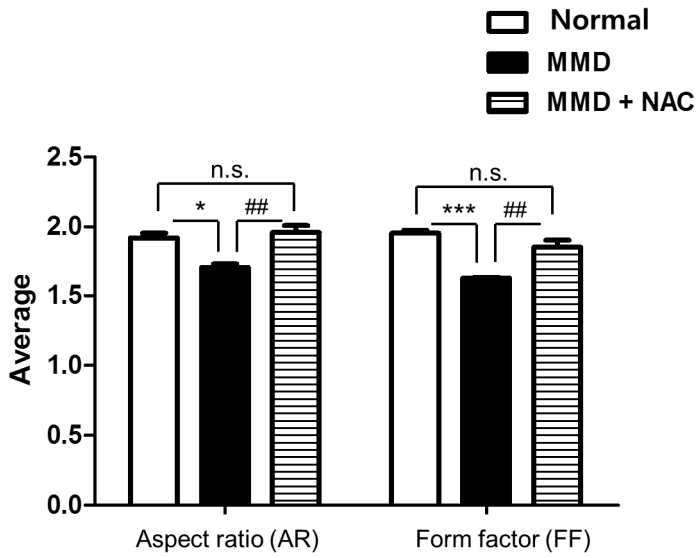
Figure 3. The endothelial colony–forming cells (ECFCs) from patients with moyamoya disease (MMD) show increased reactive oxygen species (ROS) levels.



(C) Representative image of DCFH-DA

Figure 4. Treatment with the reactive oxygen species (ROS) scavenger rescues mitochondrial dysfunction in endothelial colony–forming cells (ECFCs) from patients with moyamoya disease (MMD).

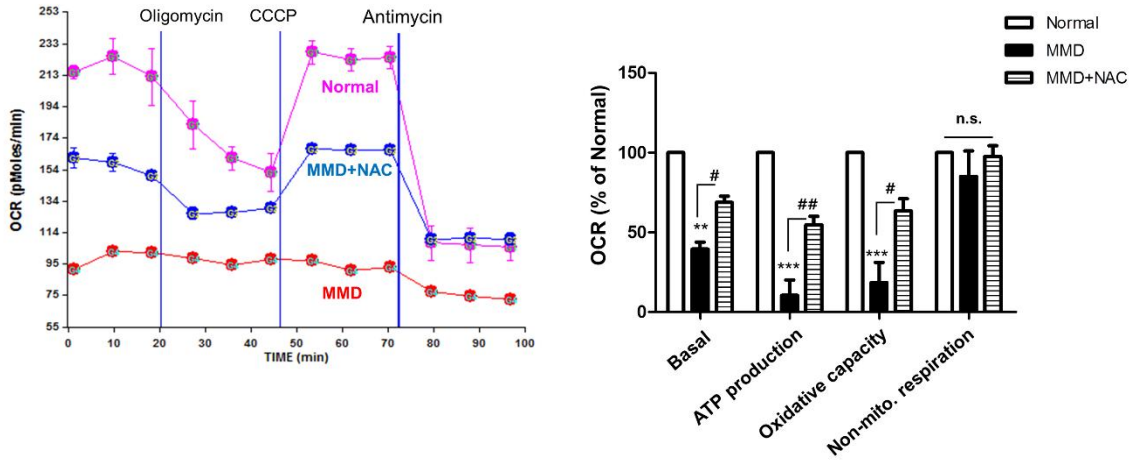




(A) Treatment with the ROS scavenger rescues the mitochondrial morphology in the MMD ECFCs. In the right panels, the enlarged images are converted to an 8-bit format to analyze mitochondrial morphology using the Image J program. The scale bars represent 5 μm and 2 μm.

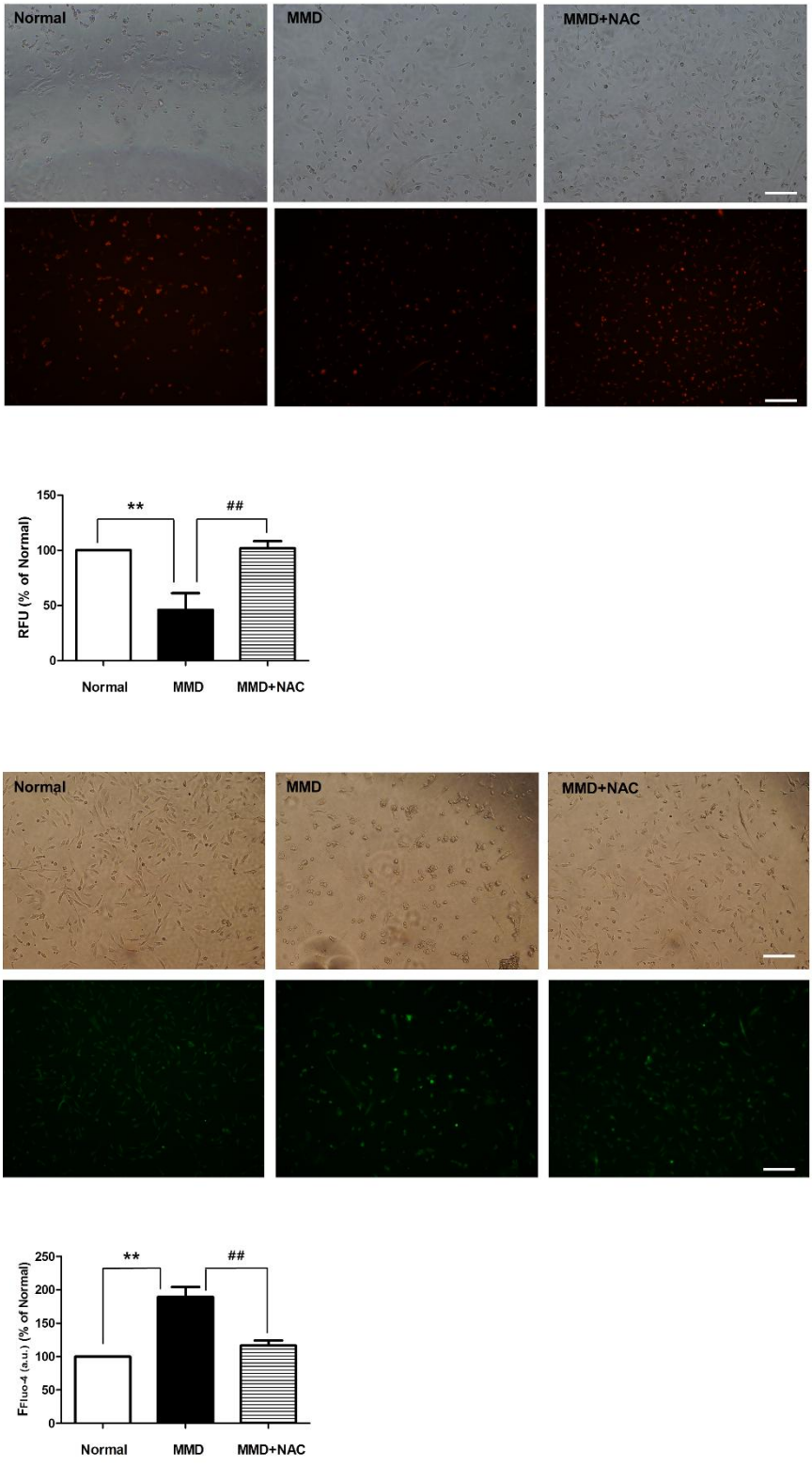
Quantitative analysis of the mitochondrial morphology after the ROS scavenger treatment (bottom). * $p < 0.05$, *** $p < 0.001$ vs. the normal ECFCs; ## $p < 0.01$ vs. the MMD ECFCs (n=30 each).

Figure 4. Treatment with the reactive oxygen species (ROS) scavenger rescues mitochondrial dysfunction in endothelial colony–forming cells (ECFCs) from patients with moyamoya disease (MMD).



(B) The representative data from a single Seahorse experiment that was representative of three independent experiments depict the oxygen consumption rate (OCR) in normal (pink), MMD (red) and N-acetylcysteine (NAC)-treated MMD (Blue) ECFCs. Each OCR data point represents a mean ± S.E.M. The concentrations of oligomycin, carbonylcyanide-3-chlorophenylhydra-zone (CCCP), and antimycin were 2 μM, 5 μM, and 1 μM, respectively. ** $p < 0.01$, *** $p < 0.001$ vs. the normal ECFCs; # $p < 0.05$, ## $p < 0.01$ vs. the MMD ECFCs. The abbreviation n.s. indicates not significant.

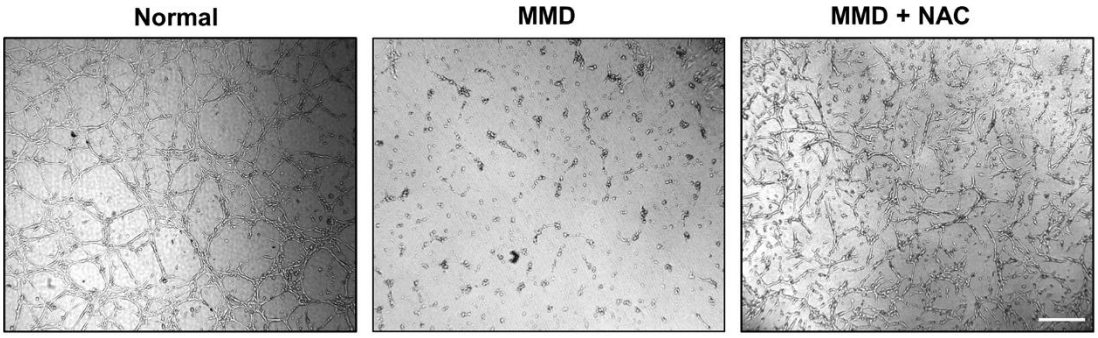
Figure 4. Treatment with the reactive oxygen species (ROS) scavenger rescues mitochondrial dysfunction in endothelial colony–forming cells (ECFCs) from patients with moyamoya disease (MMD).



(C) The mitochondrial membrane potential is measured with the tetramethyl rhodamine methyl ester (TMRM) assay (top). The data are presented as the means ± S.E.M. ** $p< 0.05$ vs. the normal ECFCs; ## $p < 0.01$ vs. the MMD ECFCs.

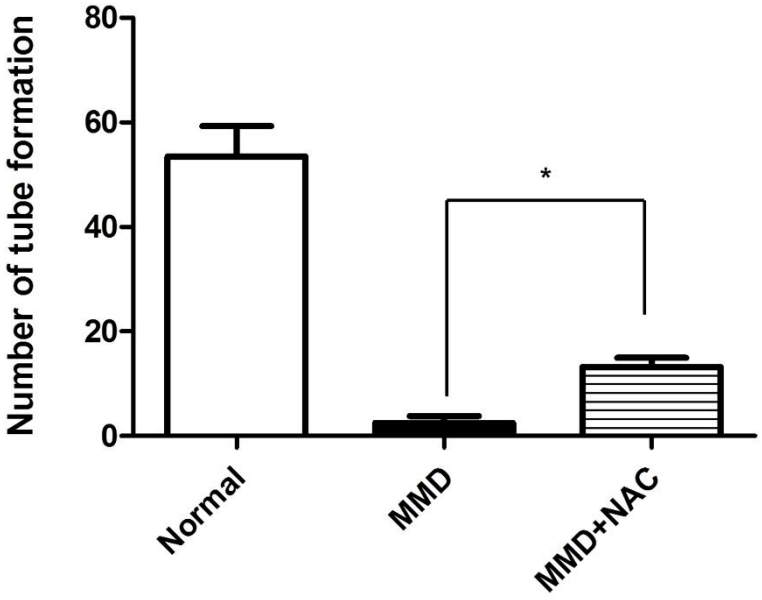
Intracellular Ca^{2+} ($[Ca^{2+}]_i$) is measured using the Fluo-4 assay (bottom). Representative images are shown. The data are presented as the means ± S.E.M. ** $p< 0.01$ vs. the normal ECFCs; ## $p < 0.01$ vs. the MMD ECFCs. The scale bar represents 50 μm.

Figure 5. Treatment with the reactive oxygen species (ROS) scavenger restores the impaired angiogenic activity in the endothelial colony–forming cells (ECFCs) from patients with moyamoya disease (MMD).



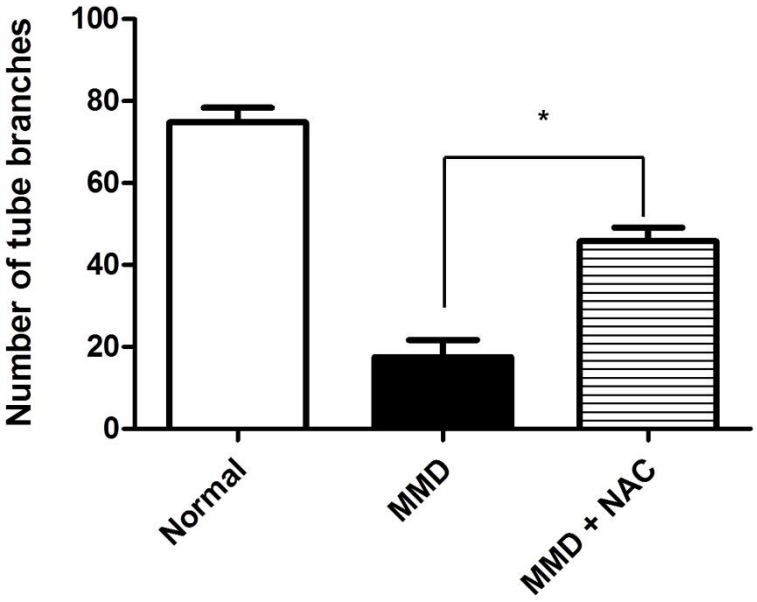
(A) Tube formation assay using ECFCs cultivated on Matrigel.

Figure 5. Treatment with the reactive oxygen species (ROS) scavenger restores the impaired angiogenic activity in the endothelial colony–forming cells (ECFCs) from patients with moyamoya disease (MMD).



(B) The number of tubes formed by the N-acetylcysteine (NAC)-treated MMD ECFCs is significantly increased. *$p < 0.01$ vs. the MMD ECFCs.

Figure 5. Treatment with the reactive oxygen species (ROS) scavenger restores the impaired angiogenic activity in the endothelial colony–forming cells (ECFCs) from patients with moyamoya disease (MMD).



(C) The number of tube branches is also significantly increased in the NAC-treated MMD ECFCs. * $p < 0.01$ vs. the MMD ECFCs.

Table

Table 1. Clinical features of the moyamoya disease (MMD) patients and healthy normal subjects.

Case No.	Sex	Age (yrs)	RNF213 c.14576G>A Variant[a]	Symptoms	MRI findings	Angiographic findings	Suzuki grade	Associated conditions[b]	Family history of stroke
MMD 1	F	36	G/A	Headache, TIA	Ischemic lesions in the both WM	Bilateral, no PCA involvement	Stage IV/IV	None	No
MMD 2	M	8	G/A	TIA	No infarct	Bilateral, no PCA involvement	Stage III/III	None	No
MMD 3	F	4	G/A	Headache, TIA	Multiple old lacunar infarcts	Bilateral, no PCA involvement	Stage III/III	None	No
MMD 4	F	17	A/A	TIA	No infarct	Bilateral, no PCA involvement	Stage III/II	None	No
MMD 5	M	1.5	A/A	TIA	No infarct	Bilateral, no PCA involvement	Stage II/II	None	No
Control 1	F	27	G/G	None	-	-	-	None	No
Control 2	M	23	G/G	None	-	-	-	None	No
Control 3	F	23	G/G	None	-	-	-	None	No
Control 4	M	20	G/A	None	-	-	-	None	No
Control 5	F	24	G/G	None	-	-	-	None	No

HA, headache; MRI, magnetic resonance images; PCA, posterior cerebral artery; TIA, transient ischemic attack; WM, white matter

[a] G/G: wild type (genotype GG), G/A: heterozygote (genotype GA), A/A: homozygote (genotype AA)

[b] Associated conditions include neurofibromatosis type 1, Down's syndrome, previous cranial irradiation, diabetes mellitus, hypertension, high cholesterol level, excessive alcohol intake, carotid or other artery disease and heart disease.