Deregulation of Retinaldehyde Dehydrogenase 2 Leads to Defective Angiogenic Function of Endothelial Colony–Forming Cells in Pediatric Moyamoya Disease

Ji Yeoun Lee,* Youn Joo Moon,* Hae-Ock Lee,* Ae-Kyung Park, Seung-Ah Choi, Kyu-Chang Wang, Jung Woo Han, Je Gun Joung, Hyun Seung Kang, Jeong Eun Kim, Ji Hoon Phi, Woong-Yang Park, Seung-Ki Kim

Objective—Moyamoya disease (MMD) is a common cause of childhood stroke, in which the abnormal function of the endothelial colony–forming cell (ECFC) plays a key role in the pathogenesis of the disease. This study was designed to identify genes involved in MMD pathogenesis using gene expression profiling and to understand the defective function of MMD ECFCs.

Approach and Results—We compared gene expression profiles of ECFCs isolated from patients with MMD and normal controls. Among the differentially expressed genes, we selected a gene with the most downregulated expression, retinaldehyde dehydrogenase 2 *(RALDH2).* The activity of RALDH2 in MMD ECFCs was assessed by in vitro tube formation assay and in vivo Matrigel plug assay in the presence of all-trans retinoic acid. The transcriptional control of RALDH2 was tested using ChIP assays on acetyl-histone H3. Through the gene expression profiling of 7 MMD ECFCs, we were able to identify 537 differentially expressed genes. Notably, the expression of RALDH2 was markedly suppressed in MMD ECFCs. MMD ECFCs inefficiently formed capillary tubes in vitro and capillaries in vivo, a defect restored by all-trans retinoic acid treatment. Knockdown of *RALDH2* mRNA in normal ECFCs also induced decreased activity of capillary formation in vitro. The decreased level of *RALDH2* mRNA in MMD ECFCs was attributed to defective acetyl-histone H3 binding to the promoter region.

Conclusions—From these results, we conclude that the expression of RALDH2 was epigenetically suppressed in ECFCs from patients with MMD, which may play a key role in their functional impairment. **(*Arterioscler Thromb Vasc Biol*. 2015;35:00-00. DOI: 10.1161/ATVBAHA.115.305363.)**

Key Words: arteries ■ moyamoya disease ■ RALDH2 protein

Moyamoya disease (MMD) is a cerebrovascular disorder characterized by idiopathic, progressive occlusion of the major bilateral intracranial arteries.[1,2] Previous research on the pathogenesis of MMD found that cytokines involved in intimal hyperplasia were increased in MMD.[3] Angiogenic cytokines that may explain the steno-occlusion of major intracranial arteries and the development of small collaterals (moyamoya vessels) have been noted.[4] The possible contribution of infection and autoimmune phenomena has also been elucidated.[5,6] Recent genome-wide association studies have suggested RNF213 as the susceptibility gene for the disease.[7] However, no single theory has been able to explain the pathogenesis of MMD to date.

We have previously reported a significant overexpression of cellular retinoic–binding protein-I, a mediator of the biological activity of retinoic acid (RA), in the cerebrospinal fluid of patients with MMD compared with matched controls.[8] RA is known to be a critical regulator of vascular smooth muscle cell differentiation and growth, and associated with vasculoproliferative diseases.[9] These findings implicate alterations in RA metabolism or RA signaling during development in the pathogenesis of MMD. To test this hypothesis, we need in vitro and in vivo experimental models recapitulating the functional defects in patients with MMD.

Endothelial colony–forming cells (ECFCs), previously termed endothelial progenitor cells, have been gaining

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From the Department of Anatomy (J.Y.L.), Division of Pediatric Neurosurgery, Seoul National University Children's Hospital (J.Y.L., Y.J.M., S.-A.C., K.-C.W., J.W.H., J.H.P., S.-K.K.), and Department of Neurosurgery (H.S.K., J.E.K.), Seoul National University College of Medicine, Seoul, Korea; Samsung Genome Institute, Samsung Medical Center, Seoul, Korea (H.-O.L., J.G.J., W.-Y.P.); College of Pharmacy, Sunchon National University, Jeonnam, Korea (A.-K.P.); and Department of Molecular Cell Biology, Sungkyunkwan University School of Medicine, Suwon, Korea (W.-Y.P.).

*These authors contributed equally to this article.

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Correspondence to Woong-Yang Park, MD, PhD, Samsung Genome Institute, Samsung Medical Center, 50 Irwondong, Gangnamgu, Seoul 135-710, Korea. E-mail woongyang.park@samsung.com; or Seung-Ki Kim, MD, PhD, Division of Pediatric Neurosurgery, Seoul National University Children's Hospital, 101 Daehakro, Jongno-gu, Seoul, 110-744, Korea. E-mail nsthomas@snu.ac.kr

attention as a critical player in the pathogenesis of MMD.[10–13] Decreased numbers and defective functioning of ECFCs have been observed in pediatric patients with MMD.[10] In addition, molecular reconstitution experiments using ECFCs isolated from patients with MMD enabled mechanistic studies and opened a new era in the field of MMD research.[14]

In this study, we compared the mRNA expression of ECFCs from patients with MMD and normal controls and identified 537 differentially expressed genes. Of these, retinaldehyde dehydrogenase 2 (RALDH2), an enzyme involved in the synthesis of RA, was found to be significantly underexpressed in MMD ECFCs. We then provided an evidence of epigenetic deregulation of RALDH2 gene promoter. Furthermore, we found that all-trans RA (ATRA) rescued defective tube formation in MMD ECFCs or RALDH2-knockdown normal ECFCs. Altogether, these results suggest the critical involvement of RA in the pathogenesis of MMD.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

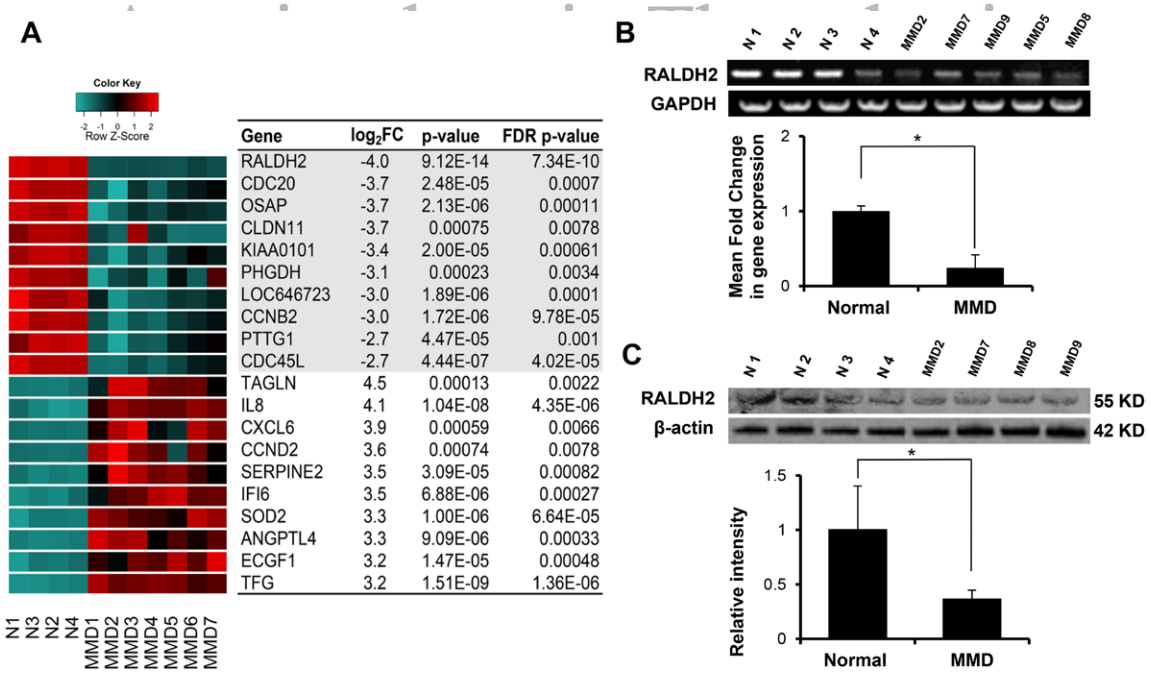
Results

ECFC Culture and Characterization

We isolated late ECFCs from 9 patients with MMD and 4 normal controls (Table I in the online-only Data Supplement). Colonies of spindle shape and positive DiI-Ac-low-density lipoprotein uptake appeared 7 days after seeding and sequential morphological changes were observed (Figure IA and IC in the online-only Data Supplement). The typical endothelial cobblestone morphology of late ECFCs from patients with MMD were not different from that of normal ECFCs in culture. We also checked the expression level of cell surface markers of late ECFCs, such as CD34, KDR, CD133, CD31, and CD45 by FACS to find similar patterns in both the groups (Figure IB in the online-only Data Supplement). Further validation of the differentiation status of isolated ECFCs was performed by immunostaining against CD31 and von Willebrand factor (Figure ID in the online-only Data Supplement). On the basis of the expression pattern of all the known surface markers, MMD ECFCs were indistinguishable from normal ECFCs.

Gene Expression Profiling of Late ECFC Isolated From Patients With MMD

To understand the functional defects of MMD ECFCs,[10] we analyzed gene expression profiles of MMD and normal ECFCs. A total of 537 differentially expressed genes were identified by applying our selection criteria, such as an absolute fold-change (≥ 2.0) and *P* value (<0.01). Gene enrichment analyses of gene ontology (biological processes) and pathways enabled us to recognize that the biological processes involving immune response and chemotaxis were significantly enhanced in MMD ECFCs. However, biological processes related to cell cycle and DNA repair were suppressed in MMD ECFCs. In aspects of metabolic and signaling pathways, the genes related to the chemokine signaling pathway, extracellular matrix–receptor interaction, and cell adhesion molecules were activated in MMD ECFCs, whereas the genes for DNA



Gene	log_2FC	p-value	FDR p-value
RALDH2	-4.0	9.12E-14	7.34E-10
CDC20	-3.7	2.48E-05	0.0007
OSAP	-3.7	2.13E-06	0.00011
CLDN11	-3.7	0.00075	0.0078
KIAA0101	-3.4	2.00E-05	0.00061
PHGDH	-3.1	0.00023	0.0034
LOC646723	-3.0	1.89E-06	0.0001
CCNB2	-3.0	1.72E-06	9.78E-05
PTTG1	-2.7	4.47E-05	0.001
CDC45L	-2.7	4.44E-07	4.02E-05
TAGLN	4.5	0.00013	0.0022
IL8	4.1	1.04E-08	4.35E-06
CXCL6	3.9	0.00059	0.0066
CCND2	3.6	0.00074	0.0078
SERPINE2	3.5	3.09E-05	0.00082
IFI6	3.5	6.88E-06	0.00027
SOD2	3.3	1.00E-06	6.64E-05
ANGPTL4	3.3	9.09E-06	0.00033
ECGF1	3.2	1.47E-05	0.00048
TFG	3.2	1.51E-09	1.36E-06

Figure 1. Differentially expressed genes in endothelial colony–forming cells (ECFCs) from patients with moyamoya disease (MMD). **A**, Expression profiles and changes of top 20 upregulated or downregulated genes. Notice retinaldehyde dehydrogenase 2 (*RALDH2*) is the most downregulated gene in MMD ECFCs compared with normal ECFCs. **B**, The mRNA expression of *RALDH2* is significantly decreased in MMD ECFCs (4.2-fold; **P*<0.05 by rank test). **C**, Representative immunoblot of RALDH2 protein expression showing a corresponding decrease in protein levels of RALDH2 in MMD ECFCs. Graph shows mean relative intensity of RALDH2 protein expression in normal and MMD ECFCs (2.8-fold reduction in MMD ECFCs; **P*<0.05 by rank test). MMD1 indicates MMD sample no. 1; MMD2, MMD sample no. 2; N1, normal sample no. 1; and N2, normal sample no. 2.

replication, cell cycle, and mismatch repair were downregulated. The pattern of those enriched biological processes or pathways reflected the defective function of MMD ECFCs. Among differentially expressed genes, RALDH2, which is an enzyme mediating the reduction of retinal to RA during the biosynthetic process, was consistently downregulated in MMD ECFCs (Figure 1A).

Altered RA Biosynthesis and RA Signaling in MMD ECFC

A 4.2-fold reduction in the expression of RALDH2 in MMD ECFCs compared with normal ECFCs were confirmed by reverse transcription polymerase chain reaction ($P<0.001$; Figure 1B) and corroborated at the protein level (Figure 1C). Because RALDH2 catalyzes the reduction of retinal to RA, we speculated that RA is required for the normal biological function of ECFCs. Therefore, first we checked whether RA stimulates in vitro tube formation of ECFCs, indicative of vessel formation in vivo. As shown in Figure 2A, the addition of ATRA stimulated the tube formation of MMD ECFCs in a dose-dependent manner. By comparison, tube formation of normal ECFCs was not affected by ATRA treatment. For MMD ECFCs, ATRA treatment significantly increased both the number of tube branches and number of formed tubes (Figure 2B). To rule out the possibility that the observed enhancement in tube formation was because of increased cell proliferation, we evaluated cell viability, cell proliferation, and cell cycle composition. The results showed no difference in all 3 parameters with or without ATRA treatment (Figure 2C and 2D). In addition, there was no change in the migration of MMD ECFCs by ATRA treatment (Figure 2E). These results suggest that the stimulatory effect of RA in the tube formation is not a result of altered cell proliferation or migration.

To investigate the effect of downregulation of RALDH2 in MMD ECFCs, we introduced siRNA against RALDH2 to normal ECFCs and measured tube formation activity. As expected, after knockdown of RALDH2 in normal ECFCs (Figure II in the online-only Data Supplement), the in vitro capacity for capillary formation was significantly decreased. The morphology of the tubes was markedly disrupted (Figure 3A), and the number of tube branches and number of formed tubes were all drastically decreased (Figure 3B). The cell viability, proliferation, and cell cycle of ECFCs from normal controls were not altered in siRNA-treated cells (Figure 3C). Importantly, the effect of RALDH2 knockdown in normal ECFCs was

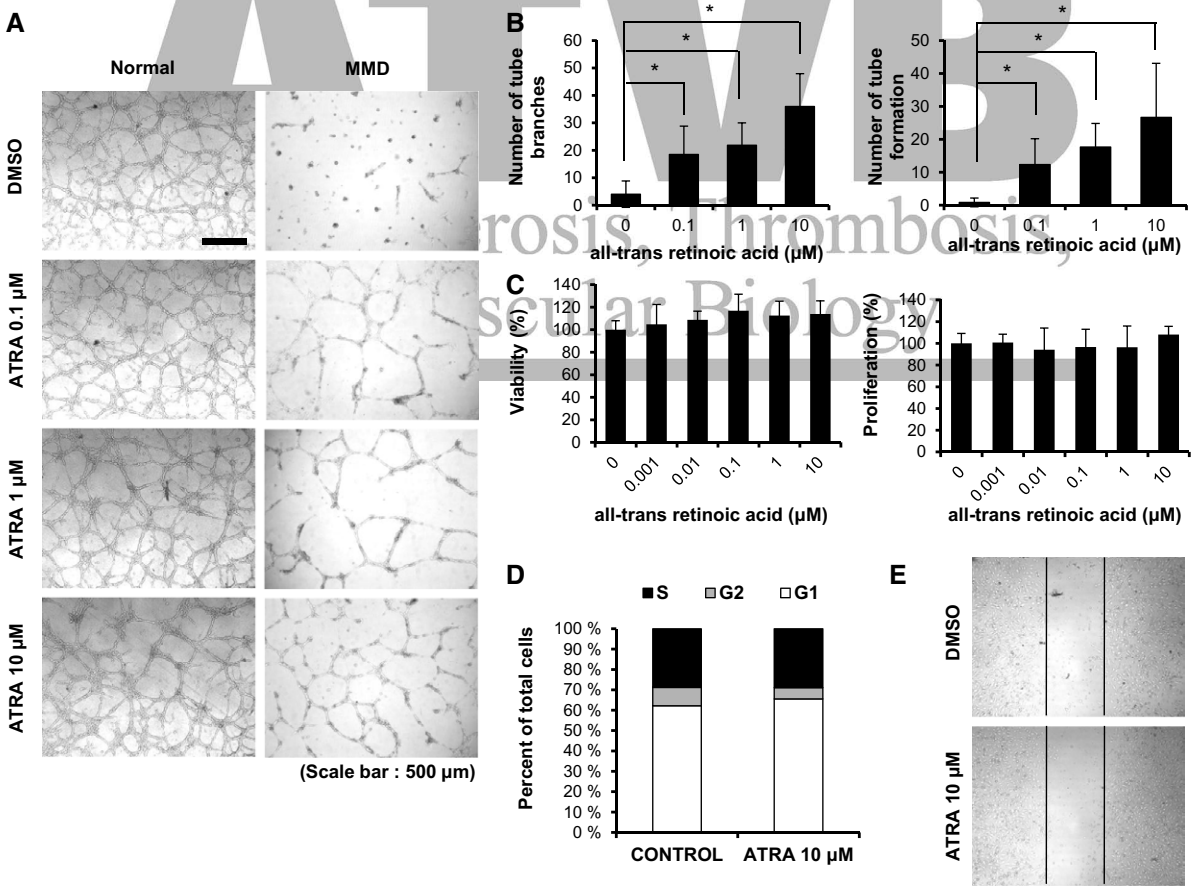


Figure 2. All-trans retinoid acid (ATRA) treatment rescues angiogenic function in moyamoya disease (MMD) endothelial colony–forming cells (ECFCs). **A**, Tube formation assay by cultivated normal and MMD ECFCs on Matrigel. The ability of MMD ECFCs to form tubes in vitro was dramatically enhanced after treatment with ATRA. **B**, These results are confirmed by the number of tube branches as well as the number of formed tubes compared with control (*$P<0.05$ by rank test). **C** and **D**, There was no difference in cell viability, proliferation, and cell cycle profile before and after ATRA treatment. **E**, Migration assay showed no effect of ATRA treatment. DMSO indicates dimethyl sulfoxide; G1, gap 1 cell cycle phase; G2, gap 2 cell cycle phase; and S, synthesis.

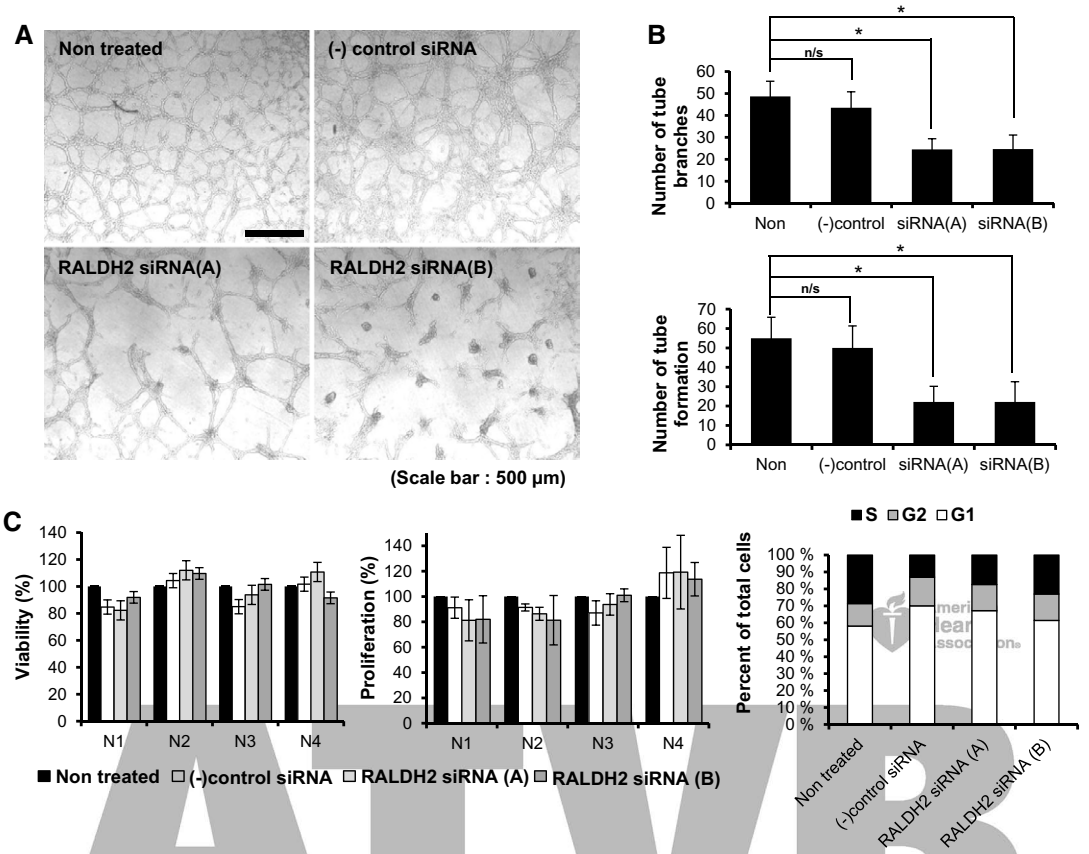


Figure 3. Retinaldehyde dehydrogenase 2 (RALDH2) knockdown inhibits tube formation in normal endothelial colony–forming cells (ECFCs). **A**, The in vitro tube formation capabilities of normal ECFCs were inhibited after RALDH2 siRNA transfection. **B**, The number of tube branches and the number of formed tubes were also decreased after RALDH2 knockdown in normal ECFCs (*P<0.05 by rank test). **C**, The viability, proliferation, and cell cycle profile were not affected by transfection with RALDH2 siRNA. G1 indicates gap 1 cell cycle phase; G2, gap 2 cell cycle phase; and S, synthesis.

significantly reversed by ATRA treatment. As shown in Figure 4, normal ECFCs with RALDH2 knockdown partially recovered their ability to form capillary tubes, suggesting that the effect of RALDH2 knockdown was exerted through modulation of RA. Tube formation after knockdown of RALDH2 with or without ATRA was repeated using human umbilical

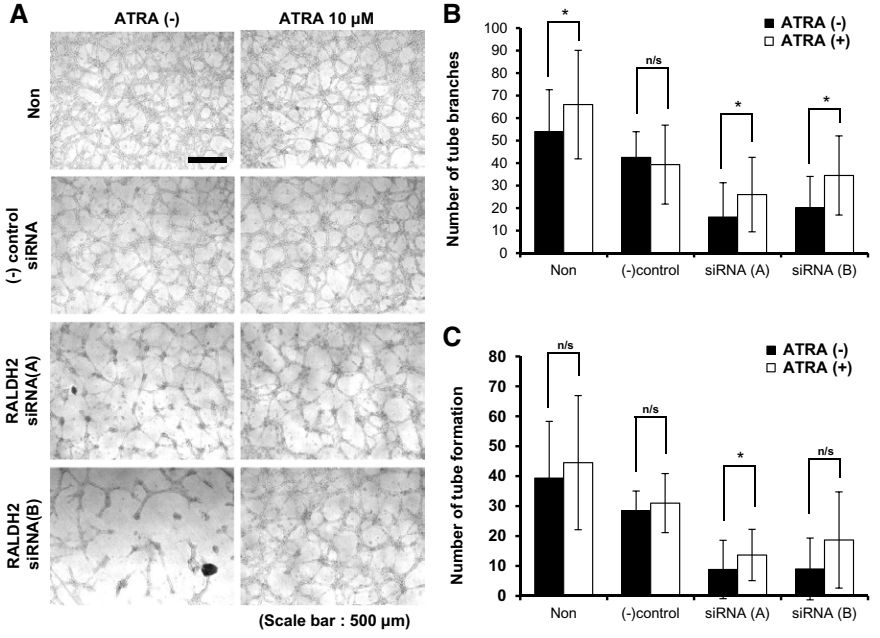


Figure 4. All-trans retinoid acid (ATRA) treatment restores tube formation capability of retinaldehyde dehydrogenase 2 (RALDH2) knocked down normal endothelial colony–forming cells (ECFCs). **A**, The inhibited tube formation ability of normal ECFCs after RALDH2 knockdown was partially restored after treatment with ATRA. **B**, These results were confirmed by the number of branches as well as the number of formed tubes compared with before and after ATRA treatment (*P<0.05 by rank test).

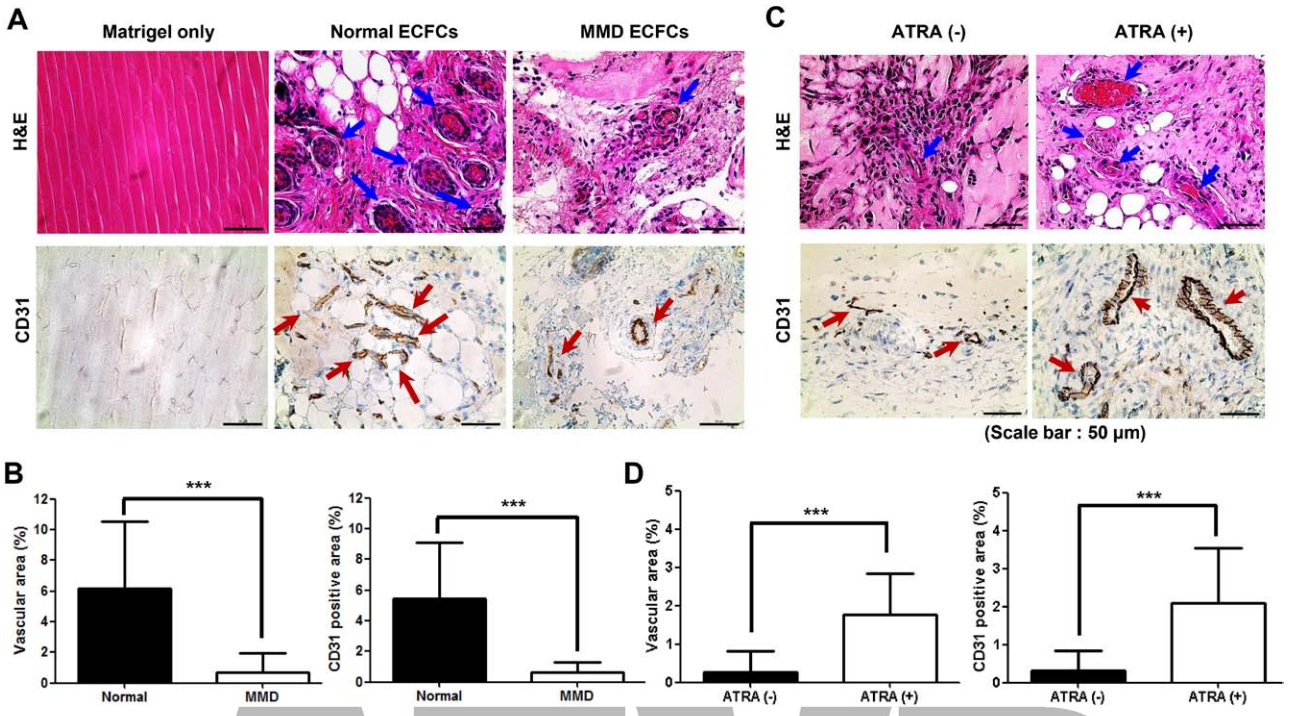


Figure 5. All-trans retinoid acid (ATRA) increases angiogenesis by moyamoya disease (MMD) endothelial colony–forming cells (ECFCs) in vivo. **A** and **C**, Representative micrographs of hematoxylin and eosin–stained (H&E) sections of Matrigel plugs and CD31 immunohistochemical staining show the vascular structures with lumens and red blood cell area (blue arrows), and the vascular density (red arrows), respectively. **B** and **D**, The stained sections were quantified and expressed as the percentage of vascular area or CD31 positive area. The quantification results show the vascular area and density of CD31 positive cells were significantly reduced in MMD ECFCs (normal vs MMD: 6.14±4.43 vs 0.63±0.72; ***P<0.001 in vascular area; 5.46±4.01 vs 0.54±1.02, ***P<0.001 in CD31 positive cells by rank test). The angiogenesis by MMD ECFCs was significantly increased after ATRA treatment (vascular area: ATRA [–] vs ATRA [+], 0.26±0.55 vs 1.78±1.06; ***P<0.001; CD31 positive cells: ATRA [–] vs ATRA [+]: 0.32±0.51 vs 2.01±1.45; ***P<0.001).

vein endothelial cells, and similar trend was observed (Figure III in the online-only Data Supplement).

Second, we evaluated the effect of ATRA on in vivo angiogenic potential capillary formation using the Matrigel plug assay. In agreement with the defective in vitro tube formation, MMD ECFCs were significantly inferior to normal ECFCs in angiogenic potential (normal versus MMD: 6.14±4.43 versus 0.63±0.72, P<0.001 in vascular area; 5.46±4.01 versus 0.54±1.02, P<0.001 in CD31 positive cells; Figure 5A and 5B). More importantly, when the degree of angiogenesis was compared between MMD ECFC with or without ATRA treatment, ATRA significantly increased the typical capillary-like structure formation in MMD ECFCs (ATRA [–] versus ATRA [+]: 0.26±0.55 versus 1.78±1.06; P<0.001; Figure 5C and 5D), as well as blood vessel formation (ATRA [–] versus ATRA [+]: 0.32±0.51 versus 2.01±1.45 in MMD; P<0.001; Figure 5C and 5D).

To test whether ATRA-modulated soluble factors involved in the vessel formation for MMD ECFCs, levels of cytokines, matrix metalloproteinases (MMPs), and angiogenic factors were assessed by a multiplex assay. Indeed, the results showed significant decrease in the production of transforming growth factor-β1, MMP-9, and vascular cell adhesion molecule-1 in the supernatant from cultures of MMD ECFCs after ATRA treatment (Table). MMP-9 is noteworthy as the observed decrement was >10-fold (1308.4±518.7 pg/mL before ATRA treatment, 115.3±45.0 pg/mL after ATRA treatment; P=0.025).

Epigenetic Deregulation of RALDH2

To elucidate the mechanisms for the reduced *RALDH2* gene expression in the MMD ECFCs, we first investigated the

Table. Mutiplex Assay for Matrix Metalloproteinases, Cytokines, and Angiogenic Factors

Cytokines	Moyamoya disease (DMSO) Mean±SM, pg/mL	Moyamoya Disease (RA) Mean±SM, pg/mL	*P* Value
TGF-β1	183.2±8.1	133.4±3.0	0.0001*
MMP-9	1308.4±518.7	115.3±45.0	0.0253*
VCAM-1	4608.2±607.9	3409.9±73.1	0.0424*
bFGF	74.1±1.7	72.6±1.7	0.2647
ICAM-1	714.6±85.0	666.7±87.4	0.3498
E-selectin	85.6±40.4	72.9±34.7	0.4072
MMP-3	812.4±269.9	728.1±246.0	0.4102
HGF	7.0±1.5	7.5±2.4	0.434
VEGF	99.6±37.4	105.3±35.7	0.4572
MCP-1/CCL2	1367.4±530.7	1420.1±566.3	0.4779
IL-β1	2.6±0.1	2.6±0.1	0.3871

bFGF indicates basic fibroblast growth factor, DMSO, dimethyl sulfoxide; HGF, hepatocyte growth factor, ICAM-1, intercellular adhesion molecule-1, IL-β1, interleukin-β1, MCP-1, monocyte chemoattractant protein-1, MMP, matrix metalloproteinase, RA, retinoic acid; TGF-β1, transforming growth factor-β1, VCAM-1, vascular cell adhesion molecule-1, and VEGF, vascular endothelial growth factor.

*Significantly different from control, P<0.05 by Student t test.

polymorphic or somatic variations in promoter sequences >5-kb upstream to the transcription initiation site, but nothing was found (data not shown). Next, we checked the DNA methylation of CpG islands in the RALDH2 promoter region. There was a 1467-bp CpG island in the promoter region, where most CpG sites were unmethylated in bisulfate sequencing (data not shown). Finally, we tested the chromatin regulation of RALDH2, which had not been reported previously. In the encyclopedia of DNA elements (ENCODE) database, a 5-kb active promoter region was predicted for the *RALDH2* gene based on the ChIP-seq data, including an H3K27Ac histone mark (http://genome.ucsc.edu/ENCODE). Because the H3K27Ac histone mark is strongly associated with active transcription,[15,16] we checked the association of the RALDH2 promoter and acetyl-histone H3 by ChIP (Figure 6A and 6B). Indeed, the RALDH2 promoter was associated with acetyl-H3 in normal ECFCs, ascertained by 2 primer sets (RALDH2 promoter region1 and RALDH2 promoter region2). In contrast, MMD ECFCs lost the acetyl-H3 association at the RALDH2 promoter region. The first intron of RALDH2 located outside the H3K27Ac histone mark served as a negative control, whereas GAPDH gene promoter showed a comparable level of acetyl-H3 association in both normal and MMD ECFCs, suggesting that the deregulated acetyl-H3 association occurred at a selected region. Our results suggest that the decreased RALDH2 production in MMD ECFCs can be attributed to the altered histone mark and promoter deregulation.

Discussion

Through gene expression profiling, we generated a list of differentially expressed genes in MMD ECFCs against normal controls. We found that RALDH2, which can control the biosynthesis of RA, was markedly downregulated in MMD ECFCs. Because RA can induce the tube formation of endothelial cells, the altered function of RALDH2 can lead to the defective angiogenic function of MMD ECFCs. The underlying mechanism of the downregulation of RALDH2 was because of the reduction in acetyl-histone H3-promoter binding. In a recent report, ECFCs from patients with MMD had a significantly reduced ability to form tubes compared with ECFCs from normal controls.[10] In this study, we demonstrated that the impaired function of MMD ECFCs could be restored by ATRA treatment. A shortage of RA in MMD ECFCs may be proposed as a pathogenic mechanism of MMD. This hypothesis is further supported by the impairment of tube formation after a knockdown of RALDH2 and its subsequent rescue after ATRA treatment in normal ECFCs. Concordant results were shown in the in vivo Matrigel plug assay. On the basis of these findings, we suggest that RA deficiency led by epigenetic suppression of RALDH2 expression is a key factor in causing functional defects in the ECFCs of patients with MMD.

RA has been implicated as a regulator of vasculogenesis[17] related to embryological formation and the remodeling of vasculature,[18,19] as well as of vasculoproliferative diseases, such as atherosclerosis.[9,20,21] RA controls the proliferation, migration, and differentiation of vascular smooth muscle cells, which are the key steps in the response to vascular injury and atherosclerosis.[22] By modulating the expression of inflammatory cytokines and endothelial adhesion molecules, RA also seems to regulate the formation of atheromas.[23] In line with this study, a recent report showed that RA, specifically ATRA, induced in vitro tube formation of human umbilical vein endothelial cells in a dose-dependent manner.[24] The authors suggested that RA has a stimulatory effect on angiogenesis in terms of capillary-like tube formation.

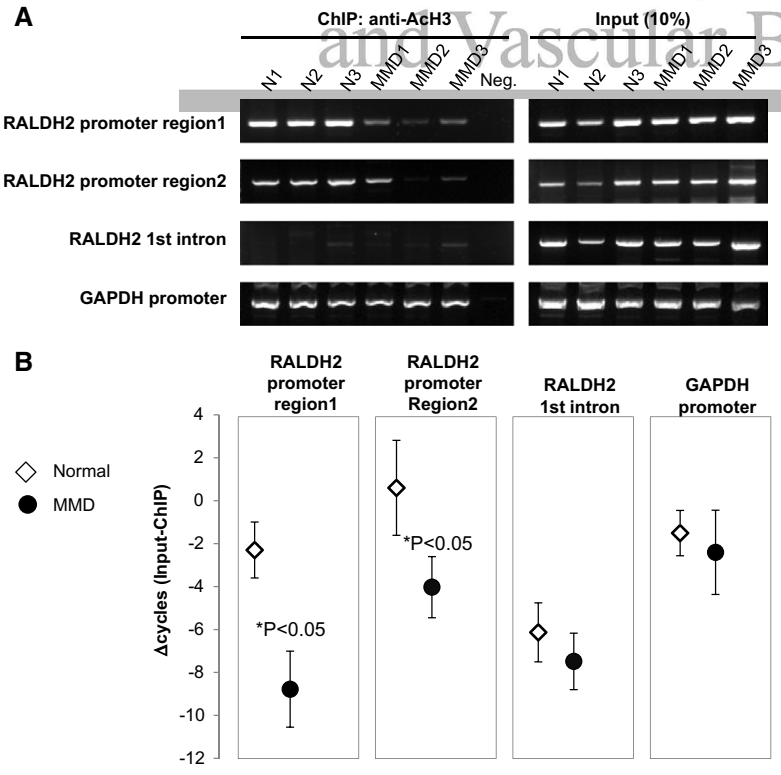


Figure 6. Low expression of retinaldehyde dehydrogenase 2 (RALDH2) is ascribed to defective acetyl-histone H3 association at the promoter region. **A**, Chromatin immunoprecipitation (ChIP) was performed using antiacetyl-histone H3 antibodies on normal (N) or moyamoya disease (MMD) endothelial colony–forming cells. The association of RALDH2 promoter and acetyl-H3 was assessed by polymerase chain reaction (PCR) with 2 primer sets targeting the active promoter region. A primer pair targeting the first intron was used as a negative control, and another pair targeting GAPDH promoter was used as a positive control. **B**, The association of acetyl-H3 with RALDH2 promoter was quantified by quantitative PCR on the ChIP DNA. The *y* axis represents the differences in the Ct values in input subtracted by ChIP sample. The differences in the normal and MMD samples reached to the statistical significance for the RALDH2 promoter region. Data were obtained from 2 independent experiments. *P* values were given by rank test. (N1: normal sample no.1, N2: normal sample no. 2, MMD1: MMD sample no. 1, MMD2: MMD sample no. 2).

Embryological studies of RA deficiency in animal models, established by targeted deletion of *RALDH2*, are helpful in elucidating the potential associations of RALDH2, RA, and vasculogenesis. Disrupted formation of extraembryonic vessels,[25] and defects in heart looping morphogenesis[19] have been reported. Further study of *Raldh2*$^{-/-}$ embryos revealed malformation of the capillary plexus and the uncontrolled growth of endothelial cells. Maternally administered RA restored disrupted cell cycle control and vascular patterning.[18]

Various matrixins, cytokines, and angiogenic factors were compared from cultured media of MMD ECFCs with and without RA treatment. The most notable finding was the significantly decreased level of MMP-9, by >10-fold, after RA treatment. This finding is in line with previous reports showing increased expression of MMP-9 in serum[26] and plasma[3] in patients with MMD. The increased expression of MMP-9 may be related to the intimal hyperplasia found in MMD pathology, because an MMP-9 knockout animal model showed decreased hyperplasia of the internal carotid artery intima.[27] Furthermore, there is similar evidence that RA acts directly to inhibit MMP-9 in cancer cells.[28,29] In sum, although a direct causal relationship has not been proven, the demonstrated decrease in MMP-9 in cultured media of MMD ECFCs after the addition of ATRA may yield clues to how RA may be involved in the dysfunction of ECFC in patients with MMD.

Previous studies have also implied an association between RA signaling and MMD. Increased protein levels of cellular retinoic–binding protein-I were found in the cerebrospinal fluid of patients with MMD in comparison with control subjects.[8] In addition, an association with a single nuclear polymorphism was found in the promoter of transforming growth factor-β1 and the first exon of PDGF in an single nucleotide polymorphism analysis between the genes of normal controls and patients with MMD.[30] Transforming growth factor-β1 and PDGF are genes controlled by RA signaling.

In summary, through gene expression profiling and in vitro functional validation in ECFCs, we found the critical step of MMD pathogenesis related to altered RA biosynthesis. Epigenetic suppression of RALDH2 expression critically contributes to the defective functioning of MMD ECFCs, which can be rescued by supplying RA in vitro and in vivo. Further analysis focusing on the epigenetic mechanism–regulating RALDH2 expression needs to be conducted.

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Disclosures

None.

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Significance

Moyamoya disease (MMD) is a cerebrovascular occlusive disease of the bilateral internal carotid arteries that causes an abnormal vascular network at the base of the brain. The role of endothelial colony–forming cells in cerebrovascular disease has been emphasized, and various studies have shown the defective function of endothelial colony–forming cells in patients with MMD. We found that retinaldehyde dehydrogenase 2 is downregulated in MMD endothelial colony–forming cells and retinoic acid treatment improved the in vitro and in vivo capillary formation function of MMD endothelial colony–forming cells. Decreased binding of acetyl-H3 to retinaldehyde dehydrogenase 2 promoter caused downregulation of *RALDH2* mRNA. This is the first study to report a key molecule and the causal gene expression in retinoic acid pathway contributing to the possible pathogenesis of MMD.