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. In the results, 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;35:1670-1677. 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 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 flowcytometry to find similar patterns in both 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 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 late ECFCs, such as CD34, KDR, CD133, CD31, and CD45 by flowcytometry to find similar patterns in both 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.

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]. FDR indicates false discovery rate; MMD1, MMD sample no. 1; MMD2, MMD sample no. 2; N1, normal sample no. 1; and N2, normal sample no. 2.
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 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 vein

**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.
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 ($P<0.001$, 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 human nuclei and CD31-double positive vascular area in MMD ECFCs ($P<0.001$, Figure 5A and 5C).

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-$\beta_1$, 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 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

**Table. Multiplex Assay for Matrix Metalloproteinases, Cytokines, and Angiogenic Factors**

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bFGF indicates basic fibroblast growth factor; DMSO, dimethyl sulfoxide; HGF, hepatocyte growth factor; ICAM-1, intercellular adhesion molecule-1; IL-$\beta_1$, interleukin-$\beta_1$; MCP-1, monocyte chemoattractant protein-1; MMP, matrix metalloproteinase; RA, retinoic acid; TGF-$\beta_1$, transforming growth factor-$\beta_1$; VCAM-1, vascular cell adhesion molecule-1; and VEGF, vascular endothelial growth factor.

*Significantly different from control, $P<0.05$ by Student $t$ test.
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 in MMD ECFCs 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 vasculogenesis17 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.

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 morphogenesis19 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

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 antibody 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).

A

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<th>ChIP: anti-AcH3</th>
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B

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ΔP<0.05
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 and plasma 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. Furthermore, there is similar evidence that RA acts directly to inhibit MMP-9 in cancer cells. 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. 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. 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.

References

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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.
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Supplement Material

Supplemental Table. Demographics of moyamoya disease patients and normal controls

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Moyamoya disease patients

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Normal Controls

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*TIA: transient ischemic attack*
Supplemental Figure I. Phenotypic characterization of endothelial colony forming cells (ECFCs).

(A) ECFCs show temporal morphological changes and colonies display a cobblestone morphology. (B) FACS analysis of CD34, KDR, CD133, CD31 and CD45. (C) Dil-labeled Ac-LDL uptake by ECFCs. (D) Immunofluorescence staining of ECFCs labeled with antibodies against CD31 and vWF.
Supplemental Figure II. Retinaldehyde dehydrogenase 2 (RALDH2) knockdown by siRNA transfection in normal endothelial colony forming cells (ECFCs).

(A) Visualization of efficient transfection of siRNA into normal ECFCs as green cells. (B) RT-PCR shows effective knockdown of RALDH2 by using two types of siRNA. (C) Similar results from real-time quantitative PCR, demonstrates that the expression of the RALDH2 mRNA is decreased after transfection with siRNA. (D) Gene knockdown was confirmed at the protein level. (N1: normal sample #1, N2: normal sample #2, N3: normal sample #3, N4: normal sample #4)
Supplemental Figure III. Effective knockdown retinaldehyde dehydrogenase 2 (RALDH2) and the effect of all-trans retinoic acid (ATRA) treatment on tube formation in human umbilical vein endothelial cell (HUVEC).

(A) Normal tube formation capabilities of HUVECs in vitro are inhibited after RALDH2 siRNA was transfected. The inhibited tube formation ability of HUVECs after RALDH2 knock down is partially restored after treatment with ATRA. (B) RT-PCR shows effective knockdown of RALDH2 by using 2 types of siRNA in HUVECs. Real-time quantitative PCR also confirms that the expression of RALDH2 mRNA is decreased after transfection with siRNA. (C) The viability and proliferation are not affected by transfection with RALDH2 siRNA.
Materials and Methods

Study subjects
Blood samples from moyamoya disease (MMD) patients and healthy controls were obtained with informed consent under institutional review board approval (SNUH IRB 1404-006-567). All MMD patients had confirmed diagnoses based on cerebral angiography. For ethical reasons, recruitment of age-matched controls was not possible; therefore, young adults with no history of stroke, hypertension, or smoking were chosen.

For mRNA expression microarray experiments, 7 samples each from the MMD patients (1 male/6 females, mean age: 18 years) and normal controls (2 males/2 females, mean age: 23 years) were used. For the in vitro experiments, samples from 6 MMD patients (3 males/3 females, mean age: 11 years) and 4 normal controls (2 males/2 females, mean age: 23 years) were used. For the in vivo experiments, samples from 3 MMD patients (1 male/2 females, mean age: 11 years) and 3 normal controls (2 males/1 female, mean age: 23 years) were used (Supplementary Table).

Isolation and characterization of endothelial colony forming cells (ECFCs)
The procedures for buffy coat preparation and mononuclear cell (MNC) culture towards endothelial colony forming cell (ECFC) lineage have been described previously.1 All blood samples (40 mL) were processed within 2 hours after collection. The MNCs were plated on culture dishes coated with collagen type I (BD BioCoat; BD Biosciences, Mountain View, CA) in an endothelial cell growth medium (EGM-2; Clonetics, San Diego, CA) with 10 % fetal bovine serum. Human umbilical vein endothelial cells (HUVEC: Catalog number CRL-1730; ATCC, Manassas, VA) were grown in EGM-2 medium. All cell cultures were maintained at 37 °C with 5 % CO₂ in a humidified atmosphere. All experiments were conducted before the 6th cell passage.

ECFCs were characterized by fluorescence-activated cell sorting (FACS) analysis and immunofluorescence staining using antibodies against CD34, KDR, CD133, CD31, CD45 and vWF.2-5 For flow cytometry analysis, 1 X 10⁶ cells were incubated for staining with 10 μL each of phycoerythrin (PE) conjugated anti-human CD34 (BD Biosciences: Catalog # 555822), KDR (R&D: Catalog # FAB357P), CD133 (Miltenyi biotec: Catalog # 130-080-801), CD31 (BD Biosciences: Catalog # 560983) and CD45 (BD Biosciences: Catalog # 560975) antibodies. The data were analyzed using a FACScan flow cytometer (Becton Dickinson) and CellQuest software (Becton Dickinson). Immunofluorescence staining was performed using anti-CD31 (Santa Cruz Biotechnology: Catalog # sc-1506) and anti-vWF (DAKO: Catalog # M0616) antibodies.

Gene expression analysis
Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and purified using RNeasy mini kits (Qiagen, Valencia, CA). The purity and concentration of the RNA were determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) and Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). The RNA was amplified and hybridized to Affymetrix Human Gene 1.0 ST arrays as previously described.6 The scanned raw expression values were background-corrected, normalized, and summarized using the robust multi-array average approach.7 The resulting log2-transformed data were used for further analyses. Using Significance analysis of microarray (SAM)8, a thousand permutations were performed in order to calculate the expected scores and False Discovery Rate (FDR). Statistical criteria for identifying the DEGs were defined by an absolute fold-change (≥ 2.0) and p-value (< 0.01). For the adjustment of multiple comparisons, the Benjamini-Hochberg
false discovery rate (BH FDR)-adjusted p-values were calculated. The DAVID bioinformatics resource (http://david.abcc.ncifcrf.gov) was used to detect over-represented gene ontology, biological process (GO_BP) categories, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways among the differentially up- or down-regulated genes.

We used AccuPower PCR premix (Bioneer, Daejeon, Korea) with gene specific primer sets (RALDH2: sense 5'-AGGCCCTCCTCGTCAAG, antisense 5'-TGCCCAGAGTGACATCAC and GAPDH: sense 5'-CAGCCACCTTTGTCAAGCTCA, antisense 5'-AGGGTTCTACATGGCAACTG) for RT-PCR. The PCR products were resolved on a 1.5% agarose gel stained with SYBR® Safe DNA gel stain (Invitrogen) and were visualized using a UV transilluminator. Real-time quantitative RT-PCR (qRT-PCR) was also performed to measure the quantity of the RALDH2 transcript on a 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) with SYBR® green master mix (Applied Biosystems).

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference. Relative gene expressions were determined from the Ct values obtained using the 2^-ΔΔCt method.9

The protein levels of RALDH2 were analyzed by western blot using an anti-human RALDH2 (Santa Cruz Biotechnology: Catalog # sc-22593) antibody. The chemiluminescence was analyzed using Fluorchem HD2 (Alpha Innotech/Hns Bio, San Leandro, CA).

For siRNA transfection, normal ECFCs were transfected with two RALDH2 siRNAs of different sequences (RALDH2 (A) : sense 5'-CUCAGACUUUGGACUCGUA, antisense 5'-UACGAGUCCAAGGUCUGAG and RALDH2 (B) : sense 5'-GACAUGAACCAUGGAGU, antisense 5'-ACUCCAUGGGUCAUGGC) and a nonspecific control siRNA (Bioneer) using an aRNAiMAX transfection kit (Invitrogen).

**all-trans retinoic acid (ATRA) preparation and treatment**

ATRA (Sigma, Deisenhofen, Germany) was dissolved in dimethyl sulfoxide (DMSO) and administered to the ECFCs at the indicated concentrations in cell culture medium. As control, the same amount of DMSO was given in each experiment.

**Capillary tube-formation assay**

Briefly, the cells (2 x 10^4 cells/well) were plated in a Matrigel (BD Biosciences)-coated 48-well plate and incubated for 18 hours with or without ATRA. The experiments were performed in triplicate. The number of tubes and branches were counted, and tube length was measured at 4 random microscopic fields by two independent observers (x 40 original magnification).

**Cell viability, proliferation and cell cycle analysis**

Cell viability was measured with Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s instructions. Briefly, the cells were seeded in 96-well plates (1 x 10^4 cells/well). After incubation for 18 hours, the viability of the cells was measured using a microplate reader and expressed as the mean concentration of 5 wells. The experiments were performed in triplicate.

Cell proliferation assays were performed using the BrdU-ELISA (Colorimetric Cell Proliferation ELISA; Roche applied science, Mannheim, Germany) according to the manufacturer’s instructions. The cells (1 x 10^4 cells/well) were seeded into a well of a 96-well plate and cultured in 100 μl of culture medium. After 18 hours of incubation, the cells were labeled with BrdU for 12 hours and their absorbance was measured. The mean concentration in each set of 5 wells was expressed. The experiments were performed in triplicate.

The cell cycle profile was analyzed by propidium iodide (PI) staining and flow cytometry. Cells (1 x 10^6 cells) were cultured for 18 hours after treatment with ATRA or transfection with RALDH2 siRNA, respectively. The cells were harvested and fixed in 70 % cold ethanol. The
fixed cells were incubated with 50 μg/ml PI solution, 0.1 mg/ml RNase A and 0.05 % triton X-100 for 40 minutes at 37 °C. Flow cytometric analysis was performed using a FACScan flow cytometer (Becton Dickinson), and the percentages of cells in different phases of the cell cycle were calculated from 3 independent experiments.

**Migration assay**

Migration of ECFCs was detected using a monolayer scratched wound assay. Cells (5 × 10⁵ cells/well in 6 well plate, n = 3) were seeded into 6-well plates and cell monolayers scraped. The remaining cells were gently washed with PBS and incubated with or without treatment of ATRA. After 24 hours, the migration area was measured using a computer-assisted microscope. The experiments were performed in triplicate.

**In vivo Matrigel angiogenesis assay**

The Matrigel plug assay was performed using 6-7-week-old BALB/c-nude mice (Orient Bio Inc, Sung-nam, Korea) as described previously. All procedures of animal experiments were approved by the Institutional Animal Care and Use Committees (IACUC) of the Seoul National University Hospital (IACUC number: 12-0383-C2A3). Anesthesia was induced with 4% isofluorane and maintained in 1–3% oxygen. The mice were divided randomly into 4 groups (n = 8) and Matrigel plugs were prepared under the experimental conditions; Matrigel (200 μl, BD Biosciences, San Jose, CA) containing a mixture of normal ECFCs (1 × 10⁶) ECFCs in the presence or absence of 10 μM of ATRA, or mixture of MMD ECFCs (1 × 10⁶) in the presence or absence of 10 μM of ATRA. Growth factors were not added. The Matrigel mixtures were subcutaneously injected. On day 7, the mice were humanely sacrificed and the Matrigel plugs were excised from the skin.

To identify the vascularization of human endothelial cells, double immunofluorescence staining was performed. The tissue slides were deparaffinized, rehydrated, incubated in antigen retrieval solution (Invitrogen), permeabilized with 0.2% Triton-X100 in PBS for 30 minutes, and blocked for 1 hour at RT with 1% BSA and 10% normal goat serum (Jackson Immunoresearch Laboratories) in PBS. Sections were immunostained with anti-human nuclei (1:200, Millipore, Temecula, CA) and anti-human CD31 (1:400, Millipore, Temecula, CA, USA) overnight at 4°C, followed by anti- goat IgG conjugated to Alexa-488 and Alexa-594 (1:600, Invitrogen) for 1 hour. After washing, tissue sections were mounted with an anti-fading solution containing 4’,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). The sections were analyzed with a confocal microscope (Zeiss). The percentage of tumor vessel area was calculated as the number of image pixels that stained human nuclei and CD31 double-positive per high-power field. Quantifications were performed from Matrigel plugs obtained from eight independent experiments. Values were obtained from three random sections of each plug by two independent observers.

It should be noted that despite the ‘in vivo’ condition of this Matrigel plugs assay, it does not recapitulate the actual environment setting of MMD. Also, because ‘inhibition of growth factors and cytokines by RA’ is one of our main hypotheses on the pathogenetic role of ECFC in MMD, growth factors were deliberately excluded.

**Enzyme-linked immunosorbent assay (ELISA)**

A commercially available multiplexed sandwich ELISA assay (VersaMAP Development System; R&D Systems, Minneapolis, MN) was used to measure the concentration of basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), matrix metalloproteinase-3 (MMP-3), MMP-9, monocyte chemoattractant
protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin, and interleukin-1 (IL-1). Conditioned media samples from MMD ECFCs treated with or without ATRA were read by the LX200 instrument (Millipore, Billerica, MA) in triplicate.

**Chromatin immunoprecipitation (ChIP) Assay**

Cultured ECFCs were treated with 1% formaldehyde to harvest cell lysates for sonication using Bioruptor (Diagenode, Liege, Belgium). The supernatants were diluted ten-fold in ChIP dilution buffer and 10 % samples were set aside as inputs. The supernatants were pre-cleared with Salmon Sperm DNA/Protein Agarose and incubated with anti-acetyl H3 antibodies. Acetyl-H3 bound DNA fragments were isolated using a NucleoSpin column (Macherey-Nagel, Duren, Germany). The association of the RALDH2 promoter to acetyl H3 was evaluated by quantitative PCR on Mx3005P (Agilent Technologies) using specific primers: RALDH2 promoter region1 (sense 5’-GGAGAGCGATTCTCTTGGT, antisense 5’-AGTTACTGCCTTGCCCTGCT); RALDH2 promoter region2 (sense 5’-TGTGGCATTGCTGGGTTC, antisense 5’-CTGCTGGGATGTGCTACAGA); RALDH2 1st intron (sense 5’-CCCTCAAGACCCACACTCACC, antisense 5’-TACCTGCGCTGAGAGGTCC); GAPDH promoter (sense 5’-CCCAACTTTCCCGCCTCTC, antisense 5’-CAGCGGC CCTGGTCACTG).

**Statistical analysis**

Data were presented as mean ± SD. For the microarray data analysis, non-parametric SAM (Significance analysis of microarrays) test was used. For the other experimental data, Wilcoxon rank sum test was used to aid visualization of the descriptive data, unless indicated otherwise. P < 0.05 was considered as statistically significant.

**REFERENCE**

8. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing