MicroRNA-34a regulates the longevity-associated protein SIRT1 in coronary artery disease: effect of statins on SIRT1 and microRNA-34a expression

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ABSTRACT
Endothelial senescence is thought to play a role in CAD (coronary artery disease). miR-34a (microRNA-34a) and other SIRT1 (silent information regulator 1)-related miRs have recently been found to target SIRT1 leading to endothelial senescence. In the present study, we investigated whether SIRT1-related miRs, including miR-9, miR-34a, miR-132, miR-181a, miR-195, miR-199a, miR-199b and miR-204, and SIRT1 were expressed in EPCs (endothelial progenitor cells) obtained from patients with CAD, and whether statins (atorvastatin or rosuvastatin) affected these levels. To determine the effects of miR-34a on SIRT1, cultured EPCs transfected with miR-34a were analysed for total SIRT1 protein levels. EPCs were obtained from 70 patients with CAD and 48 subjects without CAD. Patients with CAD were randomized to 8 months of treatment with atorvastatin or rosuvastatin. EPCs were obtained from peripheral blood at baseline and after 8 months of statin therapy. Levels of miRs and SIRT1 in EPCs were measured by real-time RT–PCR (reverse transcription–PCR) and FACS. Functional approaches to miR-34a have shown that transfection of miR-34a into EPCs resulted in regulation of SIRT1 expression. Levels of miR-34a were higher in the CAD group than in the non-CAD group, whereas levels of SIRT1 protein were lower in the CAD group than in the non-CAD group. There were no significant differences in other miRs (miR-9, miR-132, miR-181a, miR-195, miR-199a, miR-199b and miR-204) between the two groups. Levels of miR-34a were mildly negatively correlated with SIRT1 protein levels. A randomized clinical study has shown that the atorvastatin group had markedly decreased miR-34a levels and increased SIRT1 levels, whereas the rosuvastatin group showed no change in these levels. Levels of other miRs remained unchanged in the atorvastatin and rosuvastatin groups. In conclusion the results of the present study suggest that miR-34a may regulate SIRT1 expression in EPCs and that atorvastatin up-regulates SIRT1 expression via inhibition of miR-34a, possibly contributing to the beneficial effects of atorvastatin on endothelial function in CAD.

Key words: atherosclerosis, atorvastatin, endothelial progenitor cell, randomized prospective study, rosuvastatin.
Abbreviations: acLDL, acetylated low-density lipoprotein; BMI, body mass index; CAD, coronary artery disease; Cₜ, threshold cycle value; DiI, 1′-dioctadecyl-3,3′,3′,3′-tetramethylindocarbocyanine; EBM-2, endothelial basal medium-2; EPC, endothelial progenitor cell; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HbA₁c, glycated haemoglobin; HDL; high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; KDR, kinase insert domain-containing receptor; LDL, low-density lipoprotein; mAb, monoclonal antibody; MFI, mean fluorescence intensity; miR, microRNA; PBMC, peripheral blood mononuclear cell; PerCP, peridinin–chlorophyll–protein complex; SIRT1, silent information regulator 1; TAG, triacylglycerol; t-BHP, t-butyl hydroperoxide; UEA-1, Ulex europeaus agglutinin I.
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INTRODUCTION

The integrity and functional activity of the endothelial monolayer is essential for protection against the initiation of atherosclerosis [1]. In particular, senescence of endothelial cells may play an important role in the pathogenesis of atherosclerotic disorders including CAD (coronary artery disease) [2–4].

It has been reported that circulating EPCs (endothelial progenitor cells) may have a key role in the maintenance of endothelial integrity and the replacement of apoptotic or damaged endothelial cells [5,6]. Our previous studies have also demonstrated that a decreased EPC count due to telomere shortening may be involved in the pathogenesis of CAD [7,8].

SIRT1 (silent information regulator 1), a member of the class of proteins referred to as sirtuins, is a mammalian homologue of yeast Sir2 and belongs to the Sir2 family [9]. SIRT1 has been identified as an NAD+-dependent deacetylase that is responsible for maintenance of chromatin silencing and genome stability [10]. SIRT1 has recently been reported to be a novel modulator of vascular endothelial cell homeostasis, and has been shown to exert anti-atherosclerotic effects against endothelial dysfunction by preventing stress-induced senescence in vitro models [11,12]. It has been reported that miRs (microRNAs) are the most abundant family of small non-coding RNAs and these regulate mRNA translation of target genes through the RNA interference pathway [13,14]. miR-34a has recently been reported to bind directly to SIRT1 mRNA and regulate cell apoptosis by repressing SIRT1 in a cultured cell model, suggesting that miR-34a may be a negative regulator of SIRT1 [15,16]. It has also been reported that some miRs, such as miR-9, miR-132, miR-181a, miR-195, miR-199a, miR-199b and miR-204, target SIRT1 [17–20].

In clinical studies, statins (3-hydroxy-3-methylglutaryl-CoA reductase inhibitors) have been demonstrated to have anti-atherosclerotic effects such as increasing the bioavailability of NO and improving endothelium-mediated vasodilation in patients with cardiovascular risk factors or CAD [21,22]. A recent study has reported that treatment with statins such as atorvastatin, pravastatin and pitavastatin, inhibit endothelial senescence and enhance SIRT1 in HUVECs (human umbilical vein endothelial cells) stimulated with H2O2 [23]. However, it remains unknown whether statins have these anti-senescent properties in EPCs obtained from patients with CAD.

The aim of the present study was to determine whether miR-34a and other SIRT1-related miRs are expressed with SIRT1 in EPCs obtained from patients with CAD compared with controls. In addition, to evaluate the effects of statins (atorvastatin or rosuvastatin) on these miRs and SIRT1 levels, we carried out a prospective randomized single-blinded study (atorvastatin compared with rosuvastatin).

MATERIALS AND METHODS

Study population

A total of 70 patients with stable CAD were admitted to our hospital for PCI (percutaneous coronary intervention) and stent implantation. None of these patients had previous exposure to statin therapy. CAD was diagnosed on the basis of the presence of: (i) a history of typical chest pain on effort, (ii) documented exercise-induced myocardial ischaemia, (iii) angiographically proven significant coronary stenosis and (iv) absence of ACS (acute coronary syndromes) for 6 months before blood sampling. Patients were excluded from the study if they had clinical signs of acute infection, severe renal failure (serum creatine levels >3 mg/dl) or rheumatoid disease, or if they were suspected of having a malignant or primary wasting disorder.

Peripheral blood samples were obtained from 48 subjects with no evidence of CAD by history or physical examination and these were designated as the non-CAD group.

Approval for the study protocol was obtained from the ethics committee of the Iwate Medical University School of Medicine (H17-73), and written informed consent was obtained from all subjects.

Study design

The present study was designed as a prospective randomized single-blinded (the researchers were blinded to patients’ treatment groups) study. Patients with CAD were randomized to receive atorvastatin (10 mg/day, n = 35) or rosuvastatin (2.5 mg/day, n = 35) for a period of 8 months.

Blood sampling

Fasting peripheral blood was collected from patients with CAD in the morning after an overnight fast for baseline data and again after 8 months of statin treatment. Fasting peripheral blood was also collected from controls in the morning after an overnight fast.

Quantification of circulating EPCs and cell culture enrichment of EPCs

PBMCs (peripheral blood mononuclear cells) were isolated from heparinized blood samples obtained from all subjects by Ficoll–Paque density-gradient centrifugation. PBMCs were incubated with PerCP (peridinin–chlorophyll–protein complex)-conjugated anti-(human CD34) mAb (monoclonal antibody) (Becton Dickinson) and FITC-conjugated anti-(human KDR (kinase insert domain-containing receptor)) mAb (R&D Systems). The numbers of CD34- and KDR-double positive cells among
10^6 cells were counted using a FACScan analyser (Becton Dickinson).

PBMCs (8 x 10^6 cells) were plated on fibronectin-coated culture dishes (Sigma–Aldrich) and maintained in EBM-2 (endothelial basal medium–2) supplemented with EGM-2–MV-SingleQuots (Clonetics) containing 5% fetal bovine serum, 50 ng/ml human VEGF (vascular endothelial growth factor), 50 ng/ml human IGF-1 (insulin-like growth factor 1) and 50 ng/ml human EGF (epidermal growth factor). To exclude contamination with mature circulating endothelial cells, we carefully removed the culture supernatant 8 h after initial seeding and placed it into new fibronectin-coated culture dishes. Adherent cells were passaged once at day 4, and the endothelial phenotype was confirmed by FITC-conjugated UEA-1 (Ulex europeaus agglutinin I; Sigma–Aldrich) and the uptake of DiI (1′-dioctadecyl-3,3′,3′-tetramethylindocarbocyanine)-labelled acLDL (acylated low-density lipoprotein; Invitrogen). Two independent investigators evaluated the number of EPCs per mm^2 by counting UEA-1- and DiI-acLDL-double-positive cells in 15 randomly selected high-power fields using an inverted fluorescence microscope.

Real-time PCR for SIRT1-related miRs and SIRT1 mRNA

Total RNA including the small RNA fraction was extracted from isolated EPCs using a mirVanaTM Paris miR isolation kit (Ambion), according to the manufacturer’s instructions.

Extracted RNA from isolated EPCs and human total RNA as control (Applied Biosystems) were initially reverse transcribed using a High Capacity cDNA Archive kit (Applied Biosystems) and then amplified in a 10 μl PCR and primer set for amplification of human miR-34a (Assay ID: 000426), miR-9 (Assay ID: 000583), miR-132 (Assay ID: 000457), miR-181a (Assay ID: 000480), miR-195 (Assay ID: 000494), miR-199a (Assay ID: 000498), miR-199b (Assay ID: 000500), miR-204 (Assay ID: 000508) and U6 (Assay ID: 001093) using TaqMan® miR assays, according to the manufacturer’s recommended protocol. Levels of SIRT1 (Assay ID: Hs01009006_m1) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Assay ID: Hs99999905_m1) mRNA were amplified using TaqMan® Gene Expression assays (Applied Biosystems). The amplification steps consisted of denaturation at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s and then annealing at 60 °C for 1 min. All reactions were carried out on the 7500 real-time PCR system (Applied Biosystems) using the TaqMan® Universal PCR master Mix and Assays on demand (Applied Biosystems). Relative quantification was carried out using the ΔΔCt (threshold cycle value) method for recurrent compared with primary, with U6 or GAPDH as an endogenous control and fold changes were calculated for each gene [25]. Replicates with a Ct > 40 were excluded. The assay was run in triplicate for each case to allow for assessment of technical variability. To account for PCR amplification of contaminating genomic DNA, a control without RT was included. To improve the accuracy of real-time RT–PCR (reverse transcription–PCR) for quantification, amplifications were performed in triplicate for each RNA sample.

Flow cytometric analysis

To analyse the amount of intracellular SIRT1 and cell-surface CD34 in EPCs, flow cytometric analysis was carried out with a FACScan flow cytometer and CellQuest software (Becton Dickinson). EPCs were incubated with PerCP-conjugated CD34 antibody (Becton Dickinson). EPCs were washed and permeabilized with FACS Permeabilizing Solution (Becton Dickinson) for 10 min. Intracellular SIRT1 was then stained with anti-(human SIRT1) antibody (Epitomics) that was labelled using Zenon rabbit IgG labelling kits (Molecular Probes) for 30 min. Isotype-matched irrelevant control IgG (FITC-conjugated mouse anti-human mAb) was used as a control (Becton Dickinson). Levels of total SIRT1 protein in CD34-positive cells were measured by a FACScan flow cytometer (Becton Dickinson) and are shown as MFI (mean fluorescence intensity).

Transfection with miR-34a mimic and inhibitor

Cultured EPCs from five patients with CAD before statin therapy (random sampling) were used for an in vitro model transfected with miR-34a mimic and inhibitor. For miR-34a mimic and precursor transfections, EPCs were transfected with pre-miR™ miR-34a precursor molecules (20 ng/ml; Ambion) and anti-miRTM miR-34a inhibitors (20 ng/ml; Ambion) using the NeoFx transfection agent (Ambion). Those cells were used for experiments 24 h after transfection and were exposed at each passage to 0.1 μM t-BHP (t-butyl hydroperoxide; Sigma–Aldrich) for an additional 48 h. Levels of miR-34a and SIRT1 were measured by the above methods.

Cell culture with oxidant treatments

Atorvastatin (Pfizer) and rosuvastatin (AstraZeneca) were dissolved in DMSO (final concentration, 0.05 %). Atorvastatin and rosuvastatin were applied at 5-fold diluted concentrations (atorvastatin, 0.02–0.5 μM; rosuvastatin, 0.4–10 μM). To induce oxidative stress,
Table 1  Baseline and clinical characteristics of study population

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CAD group</th>
<th>Atorvastatin (n = 35)</th>
<th>Rosuvastatin (n = 35)</th>
<th>Non-CAD group (n = 48)</th>
</tr>
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<tbody>
<tr>
<td>Age (years)</td>
<td>67.9 ± 9.1</td>
<td>66.6 ± 10.4</td>
<td>69.2 ± 7.47</td>
<td>65.1 ± 8.7</td>
</tr>
<tr>
<td>Male (n)</td>
<td>56 (80 %)</td>
<td>27 (77 %)</td>
<td>29 (82 %)</td>
<td>37 (77 %)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.6 ± 3.2</td>
<td>24.0 ± 2.78</td>
<td>25.2 ± 3.63</td>
<td>25.5 ± 2.7</td>
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<tr>
<td>Hypertension (n)</td>
<td>24 (68 %)</td>
<td>26 (74 %)</td>
<td>18 (48 %)</td>
<td>7 (15 %)</td>
</tr>
<tr>
<td>Diabetes mellitus (n)</td>
<td>9 (26 %)</td>
<td>11 (31 %)</td>
<td>7 (15 %)</td>
<td></td>
</tr>
<tr>
<td>Previous angina (n)</td>
<td>9 (25 %)</td>
<td>10 (28 %)</td>
<td>2 (4 %)</td>
<td></td>
</tr>
<tr>
<td>Smoking (n)</td>
<td>6 (20 %)</td>
<td>12 (34 %)</td>
<td>5 (10 %)</td>
<td></td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>126.6 ± 15.8</td>
<td>124.2 ± 15.5</td>
<td>132.0 ± 13.5</td>
<td></td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>69.9 ± 9.73</td>
<td>69.4 ± 9.12</td>
<td>70.5 ± 10.4</td>
<td>78.3 ± 11.6</td>
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<td>HbA1c (%)</td>
<td>5.92 ± 1.15</td>
<td>6.00 ± 1.29</td>
<td>5.77 ± 0.95</td>
<td>5.27 ± 0.40</td>
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<td>HDL-C (mg/dl)</td>
<td>52.5 ± 15.6</td>
<td>51.0 ± 13.8</td>
<td>54.0 ± 17.3</td>
<td>64.0 ± 19.3</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>105.3 ± 30.2</td>
<td>107.7 ± 33.2</td>
<td>102.9 ± 27.1</td>
<td>93.1 ± 26.8</td>
</tr>
<tr>
<td>hsCRP (mg/dl)</td>
<td>0.40 ± 0.24</td>
<td>0.39 ± 0.26</td>
<td>0.41 ± 0.23</td>
<td>0.11 ± 0.12</td>
</tr>
</tbody>
</table>

cultured EPCs from five patients with CAD before statin therapy were grown in EBM-2 lacking ascorbic acid and each statin or vehicle and exposed at each passage to 0.1 μM t-BHP (Sigma–Aldrich) or PBS. After 8 days of culture, the percentage changes in SIRT1 and miR-34a in stimulated EPCs were compared with those in unstimulated EPCs.

Laboratory data

Laboratory data were measured by standard biochemistry methods in our hospital laboratory. All analyses were performed in the same run from samples that were frozen and stored at −80°C immediately after centrifugation. Fasting serum HDL (high-density lipoprotein)-cholesterol and TAG (triacylglycerol) were measured by routine enzymatic methods. Levels of LDL (low-density lipoprotein)-cholesterol were measured using a direct Friedewald’s formula [26]. hsCRP (high sensitivity C-reactive protein) was quantified by a latex-enhanced immunonephelometric assay (CardioPhase hsCRP; Dade Behring). HbA1c (glycated haemoglobin) was determined by HPLC.

Statistical analysis

All values are presented as means ± S.D. A Kolmogorov–Smirnov analysis was performed to assess data distribution. An unpaired Student’s t test was performed for normally distributed data, and a non-parametric Mann–Whitney test was performed where this was not appropriate. Statistical analysis of categorical variables was also carried out using χ² analysis and Fisher exact analysis. After 8 months of treatment with statins, comparisons between the two groups (atorvastatin group and rosuvastatin group) were analysed by two-way repeated measures ANOVA for normally distributed variables and by the Kruskal–Wallis test for non-normally distributed variables. When applicable, significant differences were further analysed with Dunnett post-hoc tests. Pearson’s correlation coefficients were used to examine the relationship between miR-34a and SIRT1 protein levels. A P < 0.05 was considered statistically significant.

RESULTS

Baseline and clinical characteristics

Baseline characteristics of the study populations are shown in Table 1. There were no significant differences in age, the percentage of males and BMI (body mass index) between the CAD and non-CAD groups. There were significant differences in other parameters between the two groups (P < 0.05).

Clinical characteristics in atorvastatin and rosuvastatin groups

As shown in Table 1, there were no significant differences in baseline characteristics between the atorvastatin and rosuvastatin groups.

Lipid profiles after 8 months of statin therapy

All medications in the atorvastatin and rosuvastatin groups remained unchanged during the 8 months of statin therapy. As shown in Table 2, there was no significant difference in baseline lipid levels between the atorvastatin and rosuvastatin groups. Both groups showed reduced LDL-cholesterol and TAG levels, but
Lipidaemic profile in CAD patients treated with statin therapy

Comparison of circulating EPC counts and levels of SIRT1 mRNA and SIRT1 MFI between CAD and non-CAD groups

There was a negative correlation between baseline EPC count and SIRT1 protein levels in all subjects ($r = -0.59$, $P < 0.01$).

Levels of miR-34a were higher in the CAD group than the non-CAD group ($2.6 ± 1.57$ compared with $1.43 ± 0.83$; $P < 0.01$) (Figure 2A). There were no significant differences in levels of miR-9, miR-132, miR-181a, miR-199a, miR-199b and miR-204 between the CAD and the non-CAD groups ($miR-9$, $0.49 ± 0.12$ compared with $0.51 ± 0.21$; $miR-132$, $1.10 ± 0.32$ compared with $1.09 ± 0.17$; $miR-181a$, $1.30 ± 0.35$ compared with $1.17 ± 0.25$; $miR-195$, $0.90 ± 0.29$ compared with $0.82 ± 0.36$; $miR-199a$, $0.38 ± 0.19$ compared with $0.37 ± 0.21$; $miR-199b$, $0.13 ± 0.08$ compared with $0.13 ± 0.07$; $miR-204$, $0.11 ± 0.07$ compared with $0.10 ± 0.06$) (Figure 2A). There was no difference in average Cts of U6 between the CAD and non-CAD groups ($21.4 ± 2.9$ compared with $20.9 ± 2.6$). Levels of miR-34a were negatively correlated with SIRT1 MFI in all subjects ($r = -0.51$, $P < 0.01$, Figure 2B). There were no significant correlations between other miRs and SIRT1 MFI in any of the subjects ($miR-9$ compared with SIRT1: $r = 0.10$, $P = 0.61$; $miR-132$ compared with SIRT1: $r = 0.14$, $P = 0.45$; $miR-181a$ compared with SIRT1, $r = -0.06$, $P = 0.77$; $miR-195$ compared with SIRT1: $r = -0.35$, $P = 0.06$; $miR-199a$ compared with SIRT1; $r = -0.29$, $P = 0.12$; $miR-199b$ compared with SIRT1: $r = 0.06$, $P = 0.77$; $miR-204$ compared with SIRT1; $r = -0.10$, $P = 0.59$).

Table 2  Lipidaemic profile in CAD patients treated with statin therapy

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Atorvastatin group (n = 35)</th>
<th>Rosuvastatin group (n = 35)</th>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>8 months</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dl)</td>
<td>107.7 ± 33.2</td>
<td>82.3 ± 19.3*</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl)</td>
<td>51.0 ± 13.8</td>
<td>53.3 ± 9.6</td>
</tr>
<tr>
<td>TAG (mg/dl)</td>
<td>153.7 ± 88.3</td>
<td>108.3 ± 45.0*</td>
</tr>
</tbody>
</table>

HDL-cholesterol levels in CAD patients were unchanged after 8 months of therapy. Decreases in LDL-cholesterol levels after 8 months of therapy did not differ significantly between the two groups (fold change in LDL-cholesterol, 0.75 ± 0.24 compared with 0.71 ± 0.26 in the atorvastatin group and rosuvastatin group respectively; $P$ value was not significant).

Numbers of circulating EPCs in the CAD and non-CAD groups

The number of circulating EPCs was lower in the CAD group than in the non-CAD group ($46.7$ cells/$10^6$ events compared with $43.9$ cells/$10^6$ events respectively; $P < 0.05$) (Figure 1A). There was no significant difference in the number of baseline circulating EPCs between the atorvastatin and rosuvastatin groups ($43.9 ± 22.0$ cells/$10^6$ events; $P$ value was not significant).

Levels of SIRT1 and miR-34a in the CAD and non-CAD groups

There was no difference in average Cts of GAPDH between the CAD and non-CAD groups ($20.3 ± 3.1$ compared with $20.4 ± 3.0$; $P$ value was not significant). Levels of SIRT1 mRNA ($2.34 ± 1.04$ compared with $3.79 ± 1.44$ respectively, $P < 0.01$) and SIRT1 protein ($16.9 ± 2.8$ compared with $21.9 ± 4.1$; $P < 0.01$) were lower in the CAD group than in the non-CAD group (Figures 1B and 1C). There was a positive correlation between baseline EPC count and SIRT1 protein levels in all subjects ($r = 0.59$, $P < 0.01$).

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and circulating EPC count in all subjects \( (r = -0.55, P < 0.01) \).

There were no significant differences in levels of SIRT1 and miRs between the atorvastatin and rosuvastatin groups \( (SIRT1 \text{ mRNA}, 2.16 \pm 1.11 \text{ compared with } 2.52 \pm 0.95; \text{SIRT1 MFI}, 16.7 \pm 2.6 \text{ compared with } 17.1 \pm 3.0; \text{miR-9}, 0.51 \pm 0.11 \text{ compared with } 0.48 \pm 0.13; \text{miR-34a}, 2.91 \pm 1.42 \text{ compared with } 2.81 \pm 1.93; \text{miR-132}, 1.11 \pm 0.27 \text{ compared with } 1.10 \pm 0.37; \text{miR-181a}, 1.26 \pm 0.32 \text{ compared with } 1.29 \pm 0.35; \text{miR-195}, 0.90 \pm 0.26 \text{ compared with } 0.90 \pm 0.33; \text{miR-199a}, 0.38 \pm 0.19 \text{ compared with } 0.40 \pm 0.23; \text{miR-199b}, 0.15 \pm 0.07 \text{ compared with } 0.12 \pm 0.09; \text{miR-204}, 0.10 \pm 0.08 \text{ compared with } 0.11 \pm 0.07) \).

**EPCs transfected with miR-34a mimic and inhibitor**

Cultured EPCs were transfected with miR-34a mimic and inhibitor followed by challenge with t-BHP after 48 h. Levels of miR-34a expression were higher in miR-34a mimic transfected cells than the mock transfected cells (Figure 3A). On the other hand, miR-34a levels were lower in miR-34a inhibitor transfected EPCs than the mock-transfected EPCs (Figure 3A). EPCs transfected with miR-34a mimic showed a reduction in levels of SIRT1 MFI compared with mock-transfected cells (Figure 3B). EPCs transfected with miR-34a inhibitor showed an increase in levels of SIRT1 MFI compared with mock-transfected cells (Figure 3B). Levels of SIRT1 mRNA did not change in EPCs transfected with miR-34a inhibitor and mimic compared with mock-transfected cells (Figure 3C).

**In vitro study with statin treatments**

Levels of miR-34a were lower in cultured EPCs treated with atorvastatin and rosuvastatin than in those with vehicle (Figure 4A). On the other hand, levels of SIRT1 (both mRNA and protein) were higher in cultured EPCs treated with atorvastatin and rosuvastatin than in those with vehicle (Figure 4B). Fold changes in SIRT1 and miR-34a were higher in cultured EPCs treated with atorvastatin compared with those treated with rosuvastatin (Figure 4). Fold changes in SIRT1 and miR-34a treated with atorvastatin and rosuvastatin was dose-dependent with a maximal inhibitory effect.
Expression of SIRT1 and miR-34a in CAD

Figure 4  Effect of atorvastatin and rosuvastatin on miR-34a and SIRT1 in cultured EPCs stimulated with oxidative stress
Levels of miR-34a decreased in EPCs treated with atorvastatin and rosuvastatin compared with vehicle (A), whereas levels of SIRT1 increased in those treated with atorvastatin and rosuvastatin compared with vehicle (B and C). Fold changes in miR-34a and SIRT1 were higher in EPC treated with atorvastatin than in those treated with rosuvastatin. ∗P < 0.05 compared with vehicle. †P < 0.05 compared with the maximum dose.

achieved at 0.5 and 10 μM (atorvastatin and rosuvastatin respectively).

Effect of statin therapy on EPC count in the CAD group
As shown in Figure 5(B), treatment with atorvastatin markedly increased the number of circulating EPCs (43.9 ± 22.0 compared with 81.8 ± 19.2 cells/10⁶ events; P < 0.01), whereas treatment with rosuvastatin had no impact on the number of circulating EPCs (43.6 ± 22.7 compared with 43.8 ± 20.3 cells/10⁶ events; P value was not significant).

Effect of statin therapy on levels of SIRT1 and miR-34a in the CAD group
After 8 months of statin therapy, levels of miR-34a decreased in the atorvastatin group but remained unchanged in the rosuvastatin group (Figure 5C). In addition, levels of other miRs remained unchanged in both groups (Table 3). Levels of SIRT1 mRNA and total SIRT1 protein increased in the atorvastatin group (Figures 6A, 6C and 6D) but remained unchanged in the rosuvastatin group (Figures 6B–6D).

DISCUSSION
The main findings of the present study are: (i) endothelial cells transfected with miR-34a resulted in a negative regulation of SIRT1 expression; (ii) SIRT1 levels were lower in EPCs obtained from the CAD group than in the non-CAD group; (iii) levels of miR-34a were higher in the CAD group than the non-CAD group, and were negatively correlated with SIRT1 protein levels; (iv) a randomized clinical study showed the atorvastatin group to have markedly decreased miR-34a and increased SIRT1 levels, whereas the rosuvastatin group showed no change in these levels; and (v) treatment with atorvastatin up-regulated SIRT1 levels via repression of miR-34a compared with treatment with rosuvastatin in our in vitro study.

Endothelial cell injury is regarded as the classical stimulus for the development of atherosclerotic lesions [27]. It has been reported that endothelial injury in the absence of sufficient circulating EPC may affect the progression of CVD (cardiovascular disease) [28]. Work on an in vitro model has reported that knockdown of SIRT1 mitigated the antioxidant and anti-inflammatory vascular effects against oxidative stress in cultured coronary vascular endothelial cells [29]. Work on an experimental model with diabetic atherosclerosis has reported that SIRT1 mRNA levels in abdominal aorta obtained from diabetic rats were significantly lower.

| Table 3  Effect of atorvastatin and rosuvastatin on miRs in patients with CAD |
| Values are means ± S.D. of the fold changes in miRs. No significant difference was observed compared with baseline for any of the miRs. |

<table>
<thead>
<tr>
<th>miR</th>
<th>Baseline (n = 35)</th>
<th>8 months</th>
<th>Baseline (n = 35)</th>
<th>8 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-9</td>
<td>0.51 ± 0.11</td>
<td>0.51 ± 0.09</td>
<td>0.48 ± 0.13</td>
<td>0.49 ± 0.11</td>
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<tr>
<td>miR-132</td>
<td>1.11 ± 0.27</td>
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<td>1.10 ± 0.37</td>
<td>1.11 ± 0.20</td>
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<tr>
<td>miR-181a</td>
<td>1.26 ± 0.32</td>
<td>1.28 ± 0.35</td>
<td>1.29 ± 0.35</td>
<td>1.29 ± 0.27</td>
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<tr>
<td>miR-195</td>
<td>0.90 ± 0.26</td>
<td>0.92 ± 0.24</td>
<td>0.90 ± 0.33</td>
<td>0.91 ± 0.18</td>
</tr>
<tr>
<td>miR-199a</td>
<td>0.38 ± 0.19</td>
<td>0.41 ± 0.21</td>
<td>0.40 ± 0.23</td>
<td>0.40 ± 0.12</td>
</tr>
<tr>
<td>miR-199b</td>
<td>0.15 ± 0.07</td>
<td>0.16 ± 0.10</td>
<td>0.12 ± 0.09</td>
<td>0.14 ± 0.07</td>
</tr>
<tr>
<td>miR-204</td>
<td>0.10 ± 0.08</td>
<td>0.12 ± 0.06</td>
<td>0.11 ± 0.07</td>
<td>0.11 ± 0.05</td>
</tr>
</tbody>
</table>
Figure 5  Effect of atorvastatin (n = 35) and rosuvastatin (n = 35) on the number of EPCs and levels of miR-34a in patients with CAD

Representative examples are shown in (A). Treatment with atorvastatin significantly increased the number of CD34/KDR-positive cells compared with rosuvastatin (B). Treatment with atorvastatin decreased levels of miR-34a compared with rosuvastatin (C).

Figure 6  Effect of atorvastatin (n = 35) and rosuvastatin (n = 35) on levels of SIRT1 in patients with CAD

Representative examples are shown in (A) and (B). Treatment with atorvastatin significantly increased both SIRT1 mRNA (C) and SIRT1 protein levels (D) compared with rosuvastatin.

than levels from non-diabetic rats [30]. The present study has shown that both circulating EPC count and SIRT1 levels were lower in the CAD group compared with the non-CAD group. In addition, there was a weak positive correlation between SIRT1 levels and EPC count. SIRT1 likely plays a critical role in endothelial homoeostasis by regulating eNOS (endothelial NO synthase) [31]. A recent report has shown a causal relationship between EPC senescence and down-regulation of SIRT1 [32]. From these observations, down-regulation of SIRT1 may be involved in EPC senescence and progression of coronary atherosclerosis in patients with CAD. However, the mechanism of progression of coronary atherosclerosis by which SIRT1 repression is related to EPC senescence in patients with CAD has remained unknown.

Accumulating evidence suggests that some miRs, such as miR-34a, let7-f, miR-27b and miR-130a, are
involved in the process of angiogenesis, modulating new vessel formation through their up-regulation or down-regulation [33–36]. In particular, miR-34a overexpression induces senescence in normal EPCs via repression of SIRT1 and may thereby contribute to their impaired angiogenic function [33]. It has also been reported that miR-9, miR-132, miR-181a, miR-195, miR-199a, miR-199b and miR-204 targeted SIRT1 [17–20]. Importantly, the miR-34 family and particularly miR-34a have been shown to mediate some of the functional consequences of p53 activation, including apoptosis, cell-cycle arrest and senescence in different cell lines and primary cells [37,38]. In agreement with this observation, the present study showed an increase in miR-34a in EPCs obtained from the CAD group compared with the non-CAD group. Levels of miR-34a were negatively correlated with SIRT1 protein levels and circulating EPC counts. In addition, our gain-of-function and loss-of-function approaches to miR-34a showed that transfection of miR-34a into EPCs resulted in the down-regulation of SIRT1 expression. On the other hand, there were no differences in levels of other miRs, including miR-9, miR-132, miR-181a, miR-195, miR-199a, miR-199b and miR-204, between the CAD and the non-CAD groups. Levels of these miRs were not correlated with SIRT1 protein levels. These observations suggest that this impairment of SIRT1 regulation via up-regulation of miR-34a may be related to EPC senescence and may thus play an important role in the progression of coronary atherosclerosis. A recent report demonstrated dysregulation of angiogenesis-related miR, such as miR-126, miR-130a, miR-221, miR-222 and miR-92a in EPCs from patients with CAD [39]. Further study will be needed to clarify the mechanism by which other miR dysregulate SIRT1 expression in EPCs from patients with CAD.

Another important finding of the present study was that treatment with atorvastatin reduced miR-34a levels and increased SIRT1 levels and circulating EPC count in patients with CAD, whereas treatment with rosvastatin had no impact on either miR-34a, SIRT1 levels or EPC count. Although the reason underlying the difference between these two statins (atorvastatin and rosvastatin) remains uncertain, it may be at least in part explained by a difference in lipophilicity. Atorvastatin is more lipophilic than rosuvastatin and would therefore be more likely to permeate through cell membranes [40]. Atorvastatin exerts potent anti-atherosclerotic effects, which are mediated not only by their lipid-lowering effect but also by improvement of vascular endothelial damage [40]. Our previous and other reports have already demonstrated that lipid lowering therapy with atorvastatin increased EPC numbers in patients with CAD [41,42]. Although our randomized study showed that treatment with rosvastatin did not change circulating EPC counts, a recent study reported that circulating EPCs were significantly increased after rosvastatin treatment in patients with systolic heart failure [43]. This discrepancy may due to differences in rosvastatin dose (2.5 mg/day compared with 10 mg/day). Indeed, our in vitro study has shown treatment with rosvastatin affected both miR-34a and SIRT1 levels in a dose-dependent manner. In addition, rosvastatin had less effect on miR-34a and SIRT1 levels than atorvastatin. A SIRT1-heterozygous knockout mouse model has shown a senescence phenotype in aorta with streptozotocin treatment which was not reinstated by a strongly lipophilic statin (pitavastatin), suggesting that the maintenance of SIRT1 expression via statin treatment may be an important factor in the development of stress tolerance [12].

**Study limitations**

A limitation of the present study is the small number of CAD patients receiving each type of statin therapy. The EPC study examined cultured EPC samples, but not isolated EPCs because the numbers of isolated EPCs were low. Most of the results in the present study may therefore be influenced by an in vitro effect after culturing of EPCs. The present study used relatively low doses of statins (atorvastatin, 10 mg/day; rosvastatin, 2.5 mg/day) compared with other reports from Western countries, because these are the most commonly prescribed statin doses in Japan (maximum approved doses are: atorvastatin, 20 mg daily; rosvastatin, 10 mg daily). However, there was no difference between the atorvastatin and rosvastatin groups in LDL-cholesterol levels (<100 mg/dl) after 8 months of therapy, which suggests that atorvastatin (10 mg/day) and rosvastatin (2.5 mg/day) have the same lipid-lowering effect. AHA/ACC (American Heart Association/American College of Cardiology) guidelines for secondary prevention in patients with CAD state that the goal of treatment is a LDL-cholesterol level less than 100 mg/dl [44].

**Conclusions**

The present study has suggested that impairment of SIRT1 regulation via up-regulation of miR-34a may contribute to EPC senescence and thereby play an important role in the progression of coronary atherosclerosis. In addition, statins, and in particular atorvastatin, up-regulate SIRT1 expression via inhibition of miR-34a in CAD, possibly contributing to the beneficial effects of statins on endothelial function in this disorder.

**AUTHOR CONTRIBUTION**

The study was designed and conducted by Tsuyoshi Tabuchi, Tomonori Itoh and Mamoru Satoh. Data analysis was performed by Tsuyoshi Tabuchi and...
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Expression of SIRT1 and miR-34a in CAD


Received 2 November 2011/26 January 2012; accepted 27 February 2012
Published as Immediate Publication 27 February 2012. doi:10.1042/CS20110563