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Adiponectin promotes migration activities of endothelial progenitor cells via Cdc42/Rac1

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ABSTRACT

Adiponectin has anti-atherosclerotic effects through its direct actions on vascular cells. The present study investigates the molecular mechanisms of adiponectin in the migration of endothelial progenitor cells (EPCs) which play an important role in neovascularization and re-endothelization. The phosphorylation of Akt and the activations of Cdc42 and Rac1 were significantly increased by adiponectin. Adiponectin increased the migration activity of EPCs, which was completely inhibited by a PI3-kinase inhibitor. siRNA of Cdc42 or Rac1 completely inhibited the adiponectin-induced migration, but siRNA of Akt had no effects, indicating that adiponectin promotes the migration activities of EPCs mainly through PI3-kinase/Cdc42/Rac1.

Structured summary:
MINT-7217629: *PAK1* (uniprotkb:Q13153) *physically interacts* (MI:0914) with *CDC42* (uniprotkb:P60953) by *pull down* (MI:0096)
MINT-7217644: *PAK1* (uniprotkb:Q13153) *physically interacts* (MI:0914) with *Rac1* (uniprotkb:P63000) by *pull down* (MI:0096)

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1. Introduction

Adiponectin is an adipocyte-specific secretory protein that has been implicated as a mediator of systemic insulin sensitivity with the liver and muscle as target organs [1–3]. The plasma adiponectin levels, which are decreased in obese patients, animal models of obesity, coronary artery disease and type 2 diabetes [4–6], correlate inversely with insulin resistance [7,8]. Adiponectin has also been recently revealed to have anti-atherogenic and anti-inflammatory properties. Overexpression of adiponectin reduces atherosclerotic lesions in mouse models, whereas adiponectin-deficient mice exhibit excessive intimal responses to vascular injury and diet-induced insulin resistance [9,10]. Moreover, adiponectin was found to be capable of stimulating angiogenesis [11]. Adiponectin promoted the formation of capillary-like structures from human umbilical vein endothelial cells (HUVECs) in vitro, functioned as a chemoattractant for HUVECs in migration and stimulated blood vessel growth in mouse [12].

On the other hand, endothelial progenitor cells (EPCs), which were first identified in adult peripheral blood mononuclear cells (MNCs) in 1997 [13], play an important role in postnatal neovascularization. EPCs contribute to the process of vasculogenesis, which comprises the adhesion of EPCs to the sites of vascularization and their subsequent infiltration and partial digestion of the target tissue resulting in the growth of a new blood vessel [14]. A number of experimental and clinical studies have revealed that transplantation of EPCs is an effective treatment for ischemic heart disease and arteriosclerosis obliterans because of the resulting neovascularization [15,16]. We revealed that transplantation of EPCs ameliorated diabetic neuropathy by increasing the tissue blood flow [17]. Many successful outcomes of experimental and clinical studies suggest that EPCs have a stronger potential for neovascularization than mature endothelial cells.

The mobilization and differentiation of EPCs were shown to be important in the process of adult neovascularization [18]. EPCs, cooperating with local endothelial cells, play a role in the formation

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of new blood vessels. Recent studies have provided evidence that the number and migratory activity of circulating EPCs inversely correlate with risk factors for coronary artery disease, suggesting that EPCs could serve as a surrogate biological marker for vascular function and cumulative cardiovascular risk [19]. Furthermore, decreased numbers and functional deficits of EPCs were observed in types 1 and 2 diabetic patients [20,21], and the recruitment of EPCs into the aorta is decreased in diabetic animals. These results suggest that such deficits in the number and function of EPCs may contribute to the macrovascular complications in diabetic patients.

Although adiponectin is reported to increase the numbers and migration of EPCs [22,23], the precise mechanisms are still obscure. In this study, we have identified that adiponectin induces the migration of EPCs via the PI3-kinase/Cdc42/Rac1 pathway. Our findings reveal not only the role of adiponectin in neovasculogenesis and vascular repair, but also the therapeutic potency of adiponectin.

2. Materials and methods

2.1. Human umbilical cord blood

Human umbilical cord blood (50–120 ml) was obtained from each donor after childbirth. Written informed consent was obtained from all mothers before labor and delivery. Protocols for sampling human umbilical cord blood were approved by the Institutional Review Board.

2.2. Purification and cell culture of EPCs

EPCs were isolated from human cord blood using a Histopaque-density centrifugation method previously described [17]. Non-adherent cells were discarded after 48 h incubation. For the identification of EPCs, FACS analyses of freshly isolated cells were performed after 7 days in culture. Cells were fixed with 1% paraformaldehyde and labeled with phycoerythrin-conjugated CD31 (clone WN59; BD Biosciences, San Jose, CA); CD34 (clone 8G12; BD Biosciences, San Jose, CA); KDR (clone 89106; TECUNE Corporation, Minneapolis, MN) and Tie-2 (clone 83715; R&D Systems, Minneapolis, MN). Isotype-identical antibodies served as controls.

2.3. Identification of adiponectin receptor

The adiponectin receptor was identified by Western blot analyses using anti-human AdipoR1 antibody and anti-mouse AdipoR2 antibody (Alexis Biochemicals, San Diego, CA).

2.4. RNA interference

The siRNA-mediated knockdown of Akt, Cdc42 and Rac1 was performed using previously described methods [24]. The targeted sequences that effectively mediated the silencing of the expression of Akt, Cdc42 and Rac1 were prepared by Qiagen (Hilden, Germany). EPCs were transfected with the siRNAs or a 21-nucleotide irrelevant RNA (Qiagen) as a control, by using RNAifect (Qiagen) according to the manufacturer's protocol.

2.5. Western blot analyses

Confluent-grown EPCs were stimulated with 5 μg/ml full-length adiponectin (Biovender; Candler, NC) for the indicated periods. A sample (20 μg) of lysate protein was subjected to SDS–PAGE and detected by the first antibodies of anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-ERK (Thr202/Tyr204) and anti-ERK (Cell Signaling Technology Inc., Beverly, MA).

2.6. Cdc42/Rac1 activity assay

To investigate whether adiponectin activates Cdc42 and Rac1, we used a Cdc42/Rac1 activation assay kit (from Cytoskeleton) according to the instructions provided by the manufacturer. This is a direct pull-down experiment performed by measuring the binding capability of GTP-bound Cdc42 or Rac1 to GSTPAK1 PBD fusion protein immobilized onto glutathione agarose beads. The amounts of active Cdc42 and Rac1 were measured by Western blot analyses.

2.7. Migration assay

To assess the migration of EPCs, we performed a modified Boyden chamber migration assay. The chambers were placed in 24-well dishes filled with M199 containing 0.1% BSA with adiponectin (5–10 μg/ml) or VEGF (10–20 ng/ml) added to the lower chamber, and the chambers were incubated for 12 h. After incubation, cells were labeled with Hoechist 33342. The adiponectin-stimulated migratory capacity was then quantified by counting the migrated EPCs on the lower surface of the filter using fluorescence microscopy.

2.8. Immunofluorescent staining of EPCs

Cultured cells were fixed with 4% paraformaldehyde in 0.1 M PBS, permeabilized with 0.05%-triton X in PBS and stained with Alexa Fluor 488-conjugated phalloidin (Invitrogen Co., Carlsbad, CA). Fluorescence was examined using a fluorescent microscope (BX51, Olympus, Tokyo, Japan).

2.9. Statistical analysis

All the group values were expressed as means ± S.E. Statistical analyses were assessed by one-way ANOVA. The level of significance was set at $P < 0.05$.

3. Results

3.1. Identification of EPCs

EPCs, isolated from the culture of cord blood-derived mononuclear cells, expanded from the attached cells (Fig. 1A). A linear formation was occasionally observed during the expansion of EPCs (Fig. 1B). More than 90% of the isolated cells were identified by DiI-acetylated LDL, which is a marker of vascular endothelial cells (Fig. 1C). On the other hand, HUVECs never showed the linear formation (data not shown). Flow cytometric analyses revealed positive staining with CD34 (81.3%), CD31 (89.4%), KDR (68.2%) and Tie-2 (78.8%) (Fig. 1D).

3.2. Identification of adiponectin receptor

To examine whether EPCs have adiponectin receptors, we evaluated the expressions of adiponectin receptors, AdipoR1 and AdipoR2 in EPCs using Western blot analyses. AdipoR1 was identified as a single band at 40 kDa (Fig. 2A). We also detected a single band stained with anti-mouse AdipoR2 (Fig. 2B), which had 93% homology with the human AdipoR1 sequences.

3.3. Adiponectin stimulates the phosphorylation of Akt, but not ERK

Adiponectin significantly stimulated the phosphorylation of Akt in a time-dependent manner (Fig. 3A). The phosphorylation of Akt was rapidly increased at 10 min after adiponectin stimulation. On

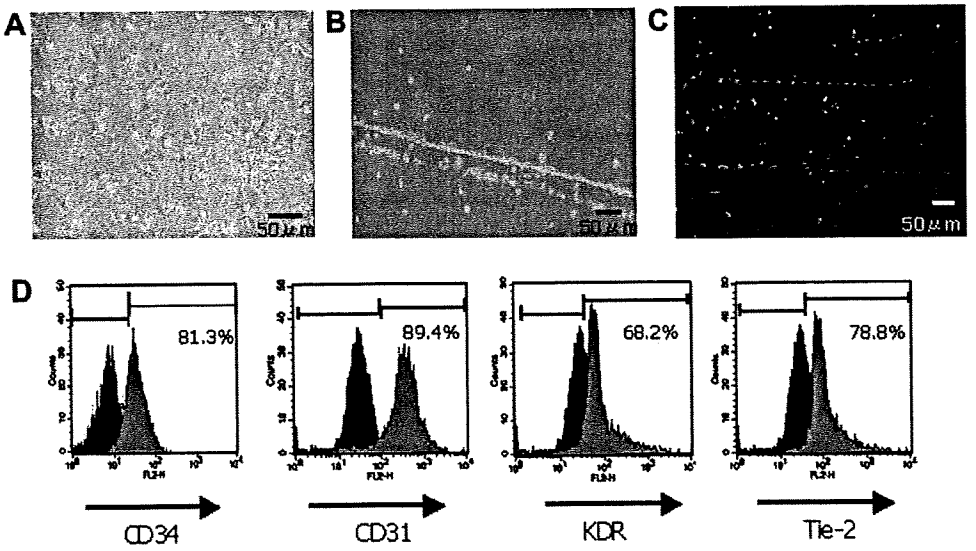

Fig. 1. Culture and identification of EPCs from cord blood. (A) EPCs, isolated from the culture of cord blood MNCs, expanded from attached cells. (B) A linear formation is occasionally observed during the expansion of EPCs. (C) The uptake of DiI-acetylated LDL. (D) Flow cytometric analysis revealed positive staining with CD34, CD31, KDR and Tie-2.

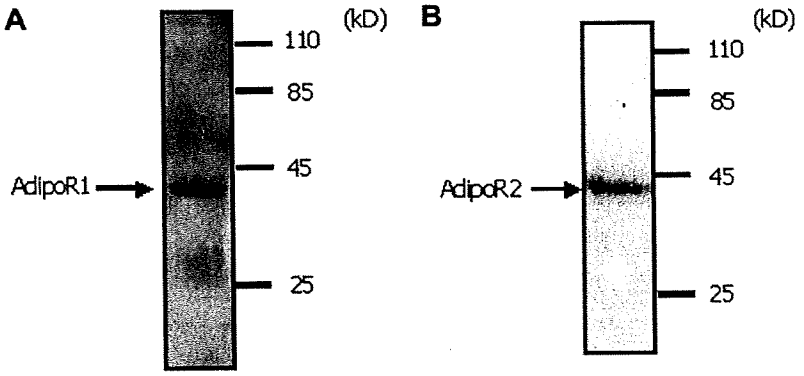

Fig. 2. Expression of adiponectin receptors, AdipoR1 (A) and AdipoR2 (B), in EPCs. Adiponectin receptors were identified by Western blot analyses using anti-human AdipoR1 antibody and anti-mouse AdipoR2 antibody.

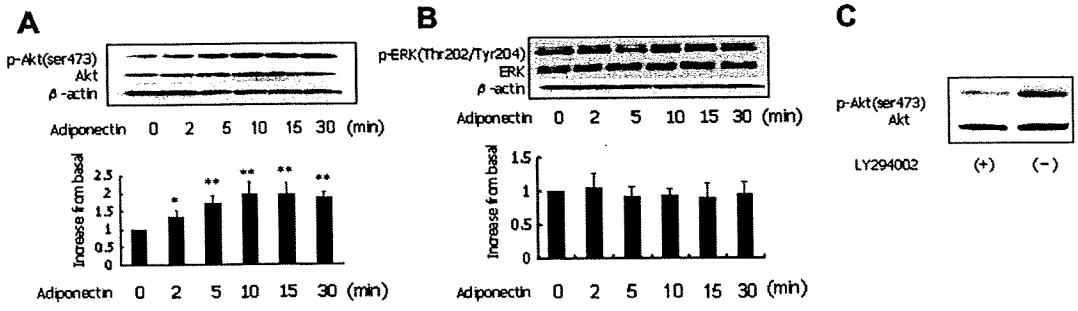

Fig. 3. The phosphorylations of Akt and ERK stimulated by adiponectin. Adiponectin stimulation (5 μg/ml) was conducted for the indicated time. (A) Adiponectin increased the phosphorylation of Akt. (B) The phosphorylation of ERK did not change by adiponectin. Results are shown as the means ± S.E. ($n = 3$) ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. 0 min). One out of three experiments with similar results is shown. (C) The phosphorylation of Akt was inhibited by LY294002.

the other hand, adiponectin did not increase the phosphorylation of ERK (Fig. 3B). To assess whether adiponectin-stimulated Akt phosphorylation via PI3-kinase (PI3K), we measured the effect of LY294002, a PI3K inhibitor, on the phosphorylation of Akt. The phosphorylation of Akt was completely inhibited by LY294002 (Fig. 3C).

3.4. Adiponectin stimulates the activities of Cdc42 and Rac1

As shown in Fig. 4, both Cdc42 and Rac1 were activated by adiponectin stimulation. Maximum Cdc42 and Rac1 activation occurred at 2 min after adiponectin stimulation (Fig.4A and B). Next, we measured the effect of LY294002 on the activation of Cdc42 and

Rac1. Both Cdc42 and Rac1 were inhibited by LY294002, indicating that adiponectin-stimulated Cdc42 and Rac1 via PI3K (Fig. 4C).

3.5. Adiponectin promotes migration activity of EPCs via PI3K/Cdc42/Rac1

The number of migrated cells attracted by adiponectin was about seven times greater than that under the serum-free condition (Fig. 5A). The migratory activities of EPCs were increased in a dose-dependent manner by adiponectin as well as by VEGF. The adiponectin-induced enhancement of migratory activities was completely inhibited by LY294002 (91.7% suppression) (Fig. 5B). On the other hand, the MEK inhibitor, PD98059 did not inhibit the adiponectin-induced EPC migration, suggesting that an ERK-dependent pathway is not involved in the migratory effect of adiponectin in EPCs.

To elucidate the signaling pathway of adiponectin-stimulated migration, targeted knockdown using siRNA was performed. Western blot analyses showed that transfection with the Cdc42, Rac1 and Akt siRNAs effectively reduced the expression levels by over 90% (Fig. 6A). As shown in Fig. 6B, the adiponectin-induced migratory activities were completely inhibited by siRNA of Cdc42 and Rac1 (85.7% suppression by Cdc42-siRNA and 88.5% suppression by Rac1-siRNA). However, siRNA of Akt had no effect on the adiponectin-stimulated migration (1.4% suppression). These observations suggest that adiponectin stimulates Akt, Cdc42 and Rac1 via PI3K, but the migratory effects of adiponectin on EPCs mainly act through PI3K/Cdc42/Rac1 (Fig. 7).

3.6. Adiponectin promotes actin organization of EPCs via Rac1

The actin structure was visualized by staining EPCs with Alexa Fluor 488-conjugated phalloidin, which probes filamentous actin. EPCs without stimulation did not form clear actin organization (Fig. 8A). Adiponectin induced actin organization and lamellipodia formation at the cortex of EPCs (Fig. 8B). Rac1-siRNA blocked adiponectin-induced actin organization and lamellipodia formation in EPCs (Fig. 8C). On the other hand, Akt-siRNA did not effect adiponectin-induced actin polymerization and lamellipodia formation in EPCs (Fig. 8D).

4. Discussion

EPCs contribute to the process of vasculogenesis, which comprises the adhesion of EPCs to the sites of vascularization and their subsequent infiltration and partial digestion of the target tissue, resulting in the growth of a new blood vessel. In the present study, we have reported, for the first time, that adiponectin at its physiological plasma concentration could promote EPC migration through PI3K/Cdc42/Rac1.

Many successful outcomes of experimental and clinical studies suggest that EPCs have a stronger potential for neovascularization than ECs. We reported that transplantation of EPCs ameliorated diabetic neuropathy by increasing the tissue blood flow [17]. Enhanced cell migration is one of the underlying mechanisms in angiogenesis of EPCs. Therefore, inducing EPCs migration is considered to be a potentially effective strategy for angiogenesis. On the other hand, decreased numbers and functional deficits of EPCs in diabetic patients have been reported. In both types 1 and 2 diabetic patients, the numbers of EPCs were significantly decreased compared with normal subjects, and this was inversely correlated with HbA1c [20,21]. Tube formation of EPCs was also decreased in types 1 and 2 diabetic patients. In vitro experiments revealed that high glucose decreased the proliferation of EPCs [25]. These results suggest that the impaired function of EPCs may contribute to the diabetic macroangiopathy.

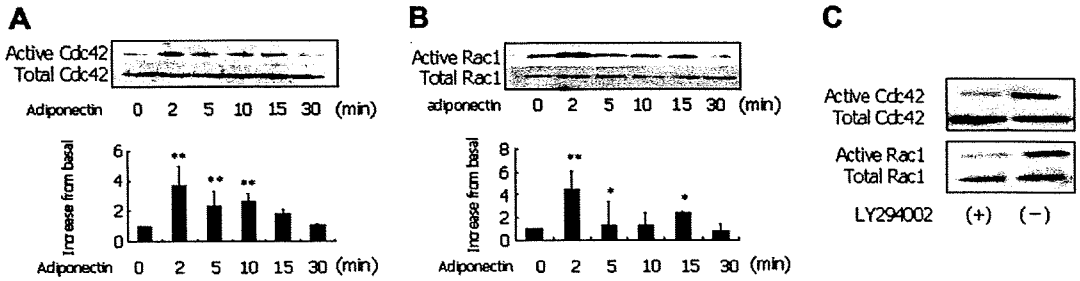

Fig. 4. The activations of Cdc42 and Rac1 stimulated by adiponectin. Adiponectin stimulation (5 μg/ml) was conducted for the indicated time. The activations of Cdc42 and Rac1 were detected by pull-down assay. (A) Adiponectin stimulated the activation of Cdc42. (B) Adiponectin stimulated the activation of Rac1. Results are shown as the means ± S.E. ($n = 3$) ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. 0 min). One out of three experiments with similar results is shown. (C) Both Cdc42 and Rac1 were inhibited by LY294002.

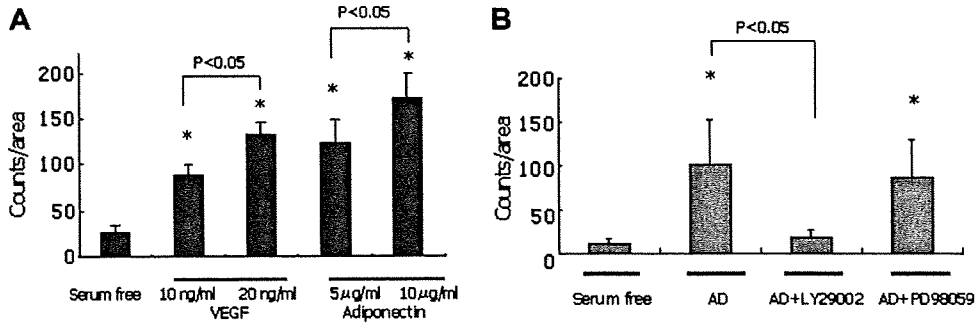

Fig. 5. Migration of EPCs stimulated by adiponectin. (A) Modified Boyden chamber assay was performed with adiponectin or VEGF as chemoattractant. (B) Adiponectin-induced enhancement of migration activities was inhibited completely by LY294002, but not by PD98059 ($^{*}P < 0.05$, vs. serum free control).

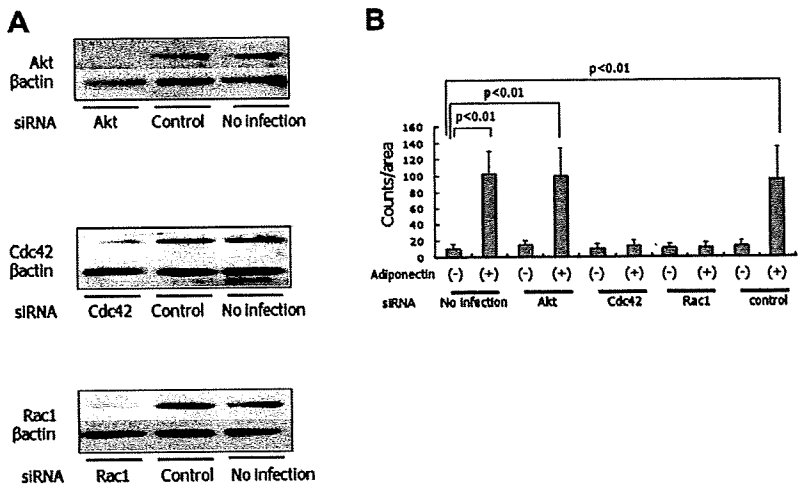

Fig. 6. The effects of Akt, Cdc42 and Rac1 on adiponectin-induced migration of EPCs. (A) The siRNA-mediated knockdown of Akt, Cdc42 and Rac1. (B) Adiponectin-induced migration activities were completely inhibited by siRNA of Cdc42 and Rac1 (85.7% suppression by Cdc42-siRNA and 88.5% suppression by Rac1-siRNA). However, the migration activity was not inhibited by a siRNA of Akt (1.4% suppression). Results are shown as the means ± S.E. ($n = 3$).

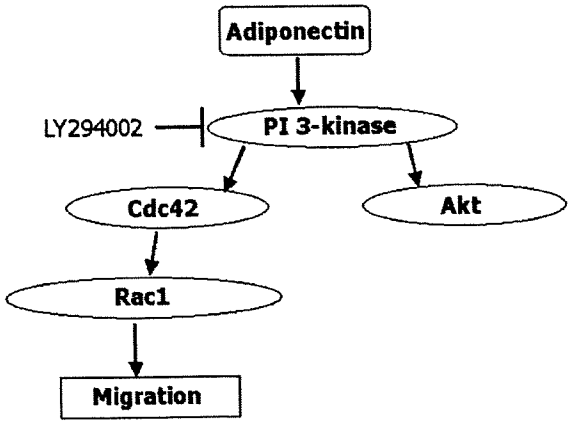

Fig. 7. Proposed scheme for adiponectin-stimulated signaling in the migration of EPCs.

Adiponectin was found to be capable of stimulating angiogenesis. Adiponectin also potently increased both the EPC numbers and migration capability [22,23]. Another report revealed that adiponectin knockout mice had a primary state of endothelial dysfunction with increased leukocyte–endothelium adhesiveness [26]. The present study confirmed the promotion of the migration of EPCs by adiponectin. PI3K, which is upstream of Akt and Cdc42, is one of the most important regulatory proteins involved in controlling several key functions of the cell, such as cell growth, aging, and transformation [27,28]. It was demonstrated in this study that adiponectin caused a dose-dependent migration of EPCs. This adiponectin-mediated migration was markedly suppressed by the administration of a PI3K inhibitor, LY294002. These results indicate that the effect of adiponectin on the EPC function was mediated in a PI3K-dependent manner.

Akt is an important regulator of various cellular processes including glucose metabolism and cell survival. Akt has been reported to directly activate eNOS, suggesting that Akt may regulate the increased production of nitric oxide in response to adiponectin stimulation [29,30]. Adiponectin has recently been revealed to stimulate the PI3K/Akt signaling pathway to enhance EC proliferation and migration [12]. However, in this study, we found that the activation of Akt was not essential for the adiponectin-induced migration of EPCs. Conflicting reports exist as to the role of Akt in cell migration. Several studies have demonstrated that Akt plays a positive role in migration, whereas other studies have reported conflicting results [31–33]. The discrepancy in the results, obtained in response to different stimulators, could be due to the activation of different signaling pathways in the different cell types.

The Rho family GTPases play critical roles in various situations such as cell growth, metastasis, morphogenesis, organogenesis and pathogenesis [34]. Cdc42 and Rac1 were reported to act as downstream effectors of PI3K in several growth factor-stimulated pathways [35,36]. We have discovered that Cdc42 and Rac1 were essential downstream proteins in EPC migration. Migrating cells cause a special actin organization, called "lamellipodium", at the leading edge of migration [37]. We have shown that adiponectin stimulates the lamellipodium formation at the cortex of EPC, which is suppressed by the inhibition of Rac1. These data suggest that the pro-angiogenic effects of the adiponectin-stimulated PI3K activity are due in large part to an activation of Cdc42/Rac1.

On the other hand, adiponectin did not affect the phosphorylation of ERK1/2. We also observed that the MEK inhibitor, PD98059, did not inhibit the adiponectin-induced EPC migration suggesting that none of those kinase-dependent pathways is involved in the migratory effect of adiponectin. Our data are consistent with a previous report that adiponectin affected no phosphorylation of ERK induced by TNF-α in HAEC.

Adiponectin in the circulation forms a complex of a trimer (high molecular weight form). We used the full-length adiponectin comprised of both the high and low molecular weight forms. We found that EPCs have both adiponectin receptor types 1 and 2 (AdipoR1 and R2). These results provide important insights into the molecular mechanisms underlying the promotion of EPC migration by adiponectin. However, we were not able to elucidate whether it was adiponectin receptor R1 or R2 that contributes to the EPC migration induced by adiponectin. Further study is being conducted in our laboratory to answer this question.

In conclusion, we revealed that adiponectin at the physiological plasma concentration can promote the migration of EPCs, mainly through PI3K/Cdc42/Rac1. Our present study provides important insights into the molecular mechanisms underlying EPC migration. Taken together, these results suggest that exogenous supplementation of adiponectin could be useful for therapeutic angiogenesis in patients who suffer from vascular complications such as ischemic heart disease and diabetes. This study may provide a better

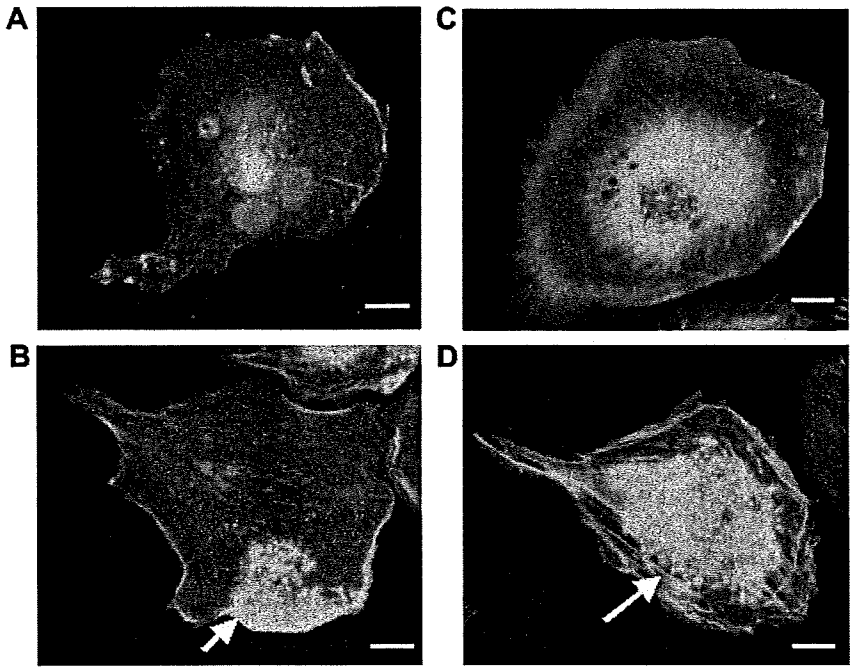

Fig. 8. The actin structure was visualized with the staining of the cells with Alexa Fluor 488-conjugated phalloidin. (A) The filamentous actin in EPC without stimulation. (B) The actin organization and lamellipodium formation at the cortex of EPC under adiponectin stimulation. (C) Rac1-siRNA blocked adiponectin-induced actin organization and lamellipodia formation in EPC. (D) Akt-siRNA did not effect the adiponectin-induced actin organization and lamellipodia formation in EPCs (scale-bar = 20 μm).

understanding of the biological functions of adiponectin and its potential clinical applications.

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Confirmation of Multiple Risk Loci and Genetic Impacts by a Genome-Wide Association Study of Type 2 Diabetes in the Japanese Population

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OBJECTIVE—To identify novel type 2 diabetes gene variants and confirm previously identified ones, a three-staged genome-wide association study was performed in the Japanese population.

RESEARCH DESIGN AND METHODS—In the stage 1 scan, we genotyped 519 case and 503 control subjects with 482,625 single nucleotide polymorphism (SNP) markers; in the stage 2 panel comprising 1,110 case subjects and 1,014 control subjects, we assessed 1,456 SNPs ($P < 0.0025$, stage 1); additionally to direct genotyping, 964 healthy control subjects formed the in silico control panel. Along with genome-wide exploration, we aimed to replicate the disease association of 17 SNPs from 16 candidate loci previously identified in Europeans. The associated and/or replicated loci (23 SNPs; $P < 7 \times 10^{-5}$ for genome-wide exploration and $P < 0.05$ for replication) were examined in the stage 3 panel comprising 4,000 case subjects and 12,569 population-based samples, from which 4,889 nondiabetic control subjects were preselected. The 12,569 subjects were used for overall risk assessment in the general population.

RESULTS—Four loci—1 novel with suggestive evidence (*PEPD* on 19q13, $P = 1.4 \times 10^{-5}$) and three previously reported—were identified; the association of *CDKAL1*, *CDKN2A/CDKN2B*, and *KCNQ1* were confirmed ($P < 10^{-19}$). Moreover, significant associations were replicated in five other candidate loci: *TCF7L2*, *IGF2BP2*, *SLC30A8*, *HHEX*, and *KCNJ11*. There was substantial overlap of type 2 diabetes susceptibility genes between the two populations, whereas effect size and explained variance tended to be higher in the Japanese population.

CONCLUSIONS—The strength of association was more prominent in the Japanese population than in Europeans for more than half of the confirmed type 2 diabetes loci. ***Diabetes* 58: 1690–1699, 2009**

The predisposition to and the course of type 2 diabetes vary according to ethnic group (1–3). In Japan, the incidence of type 2 diabetes has increased recently and is now comparable to that of other countries; this is supposedly attributable to the gradual spread of Western habits, such as consuming a high-fat diet, and the lower insulin secretory capacity of Japanese subjects (4,5). Recent technological developments have allowed the successful identification of gene regions involved in the development of type 2 diabetes in genome-wide association (GWA) studies (6–17). Several susceptibility gene loci identified by GWA studies to date have been used to obtain reproducible evidence of disease association in different populations of European descent and Asians, but not necessarily in African Americans (18–24). A number of GWA studies on type 2 diabetes have been conducted on populations of European descent (6–12). Two GWA scans in the Japanese population simultaneously reported the discovery of type 2 diabetes susceptibility gene (*KCNQ1*) variants in non-European populations; this result was also obtained in Scandinavian samples (25,26). Thus far, the replicated associations for a limited number of candidate genes have broadly indicated the tendency of interethnic similarity. Even though the common (or cosmopolitan) effect of type 2 diabetes risk variants is known, the extent to which the causation of this disease differs or overlaps between populations remains unknown. Here, besides comparing the genetic associations between European-descent and Japanese populations, we aimed to identify new genetic variants using a three-staged GWA study design.

RESEARCH DESIGN AND METHODS

Detailed characteristics of the subjects enrolled in each stage are described in the supplementary information and in supplementary Table S1, which is available in an online appendix at http://diabetes.diabetesjournals.org/cgi/content/full//db08-1494/DC1. Briefly, patients and unaffected control subjects analyzed in stages 1 and 2 were enrolled depending on whether they met certain uniform criteria. Type 2 diabetes was diagnosed according to 1999 World Health Organization criteria. All stage 1 and 2 control subjects (≥55

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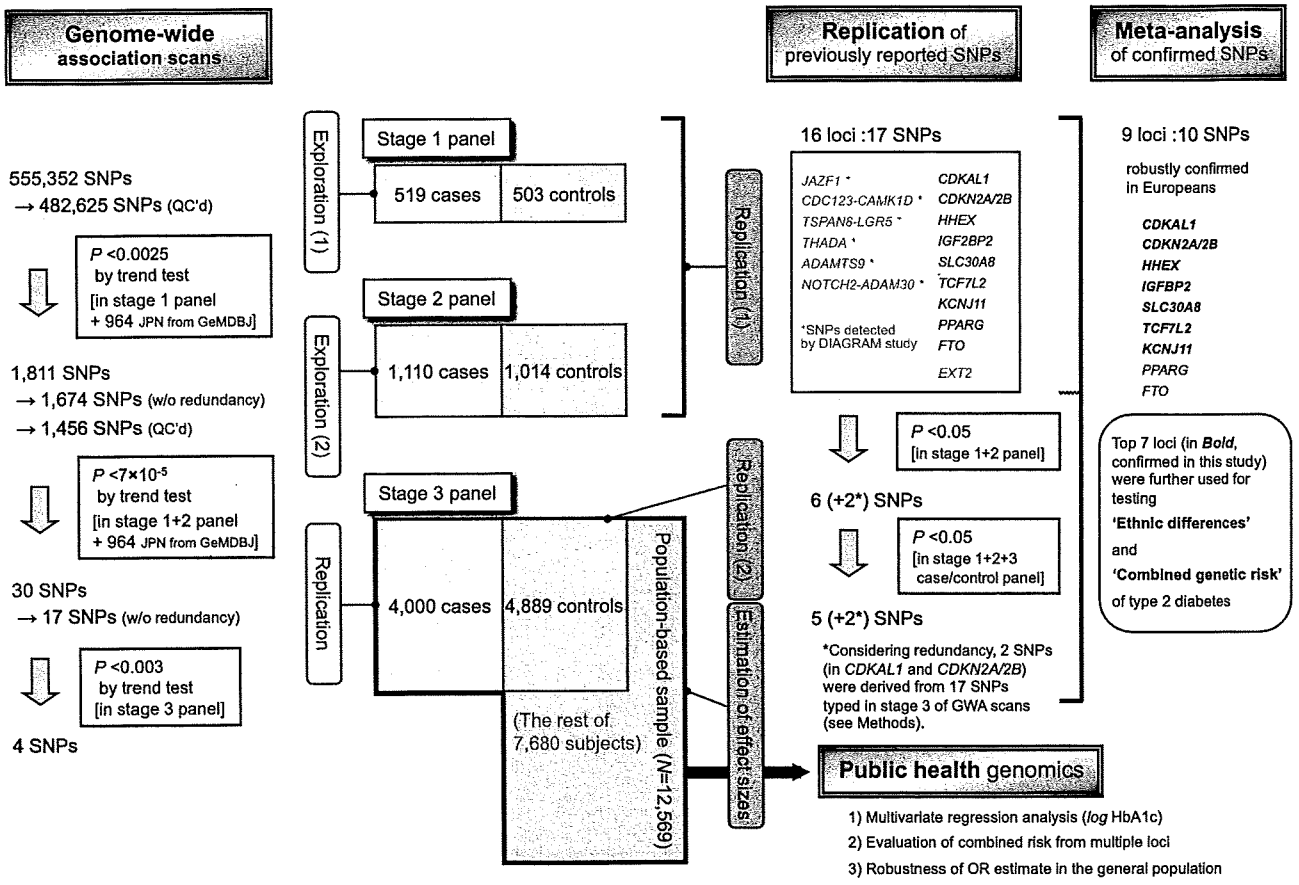

FIG. 1. Flow chart summarizing the multistage design and study aims. (A high-quality digital representation of this figure is available in the online issue.)

years of age at examination) had normal glucose tolerance. The stage 3 samples comprised 4,000 type 2 diabetes case subjects derived from the Biobank Japan project (http://biobankjp.org/) (27) and 12,569 subjects randomly selected from residents aged 50–74 years in the general population. The 12,569 subjects were used as a population panel; this panel contained 4,889 nondiabetic subjects who met the following conditions: age ≥55 years, A1C ≤5.0%, no previous and/or current treatment for diabetes, and absence of renal failure (serum creatinine <3.0 mg/dl). In stage 3, these 4,889 control subjects were used in a replication study wherein their genotypes were compared with those of 4,000 patients. In addition to the samples genotyped here, to boost the power of the GWA scan, we incorporated genotype frequencies in the general Japanese population (n = 964) derived from the Genome Medicine Database of Japan (GeMDBJ; http://gemdbj.nibio.go.jp), which was used as an in silico control panel. A flowchart summarizing the multistage design and study aims is shown in Fig. 1.

Stage 1 genome-wide scan and quality control. Genotyping was performed with the Infinium HumanHap550 BeadArray (Illumina), which interrogated 555,352 SNPs (supplementary information). The average call rate was 96.9% for the case and control subjects. Data cleaning and analysis were performed using PLINK software (28). Samples with a genotype call rate of <90% were excluded, as were outliers with respect to the number of heterozygous SNPs, duplicates or relatives of another sample, or ethnic outliers. We excluded SNPs for the following reasons: *1*) GenTrain genotype quality score <0.53, *2*) genotype call rate <0.95, *3*) genotype call rate <0.99 and minor allele frequency (MAF) <0.05, *4*) significant ($P < 10^{-6}$) deviation from the Hardy-Weinberg equilibrium in the control subjects, or *5*) MAF <0.001 (supplementary Table S2); the remaining 482,625 SNPs were analyzed.

Analysis of stage 1 genotype data. Ethnicity was verified for 1,022 samples (519 case and 503 control subjects) in the stage 1 panel with reference to data from HapMap populations (29) (see supplementary information). Type 2 diabetes association was tested with the Cochran-Armitage trend test in the stage 1 panel and an additional panel of 964 random control subjects. We pooled the genotype counts for combining multiple panels. To detect and correct population stratification and unnoticed differences in data processing between facilities, the test statistic was adjusted using Eigenstrat software (30) and the genomic-control method (31). The significance level for the first-stage scan was set to $P < 0.0025$; significant SNPs were additionally chosen using Fisher's χ^2 test ($P < 0.0025$) to combine the association results with the P value at the same locus in our previous affected sib-pair scan (32). A total of 1,811 SNPs surpassed the stage 1 threshold, and we removed redundant SNPs that were in mutual strong linkage disequilibrium ($r^2 > 0.9$) before proceeding to stage 2 (see supplementary information and supplementary Fig. S1 and Table S2 for detailed analysis).

Stage 2 genotyping and analysis. Stage 2 genotyping was performed with iPLEX (Sequenom) and GoldenGate (Illumina) assays. Quality control was conducted as described in stage 1, and 1,456 SNPs were successfully genotyped. We calculated P values with the trend test by combining 1,517 nondiabetic control subjects with 964 random control subjects similar to stage 1. The significance level for the second-stage scan was set to $P < 7 \times 10^{-5}$ in the comparison between 1,629 case subjects and 2,481 control subjects (i.e., the stage 1 + 2 panels and the 964 random control subjects). A total of 30 SNPs representing 17 unique loci remained significant.

Replication of previously reported SNPs. Along with genome-wide exploration, type 2 diabetes association was tested in the stage 1 and 2 panels using the HumanHap550 BeadArray, iPLEX assay, or TaqMan method (Applied Biosystems) for 17 SNPs from 16 candidate loci previously identified by GWA studies in populations of European descent (6–17). These included *IGF2BP2* (rs4402960), *PPARG* (rs1801282), *CDKAL1* (rs7754840 and rs7756992), *SLC30A8* (rs13266634), *CDKN2A/CDKN2B* (rs10811661), *HHEX* (rs1111875), *TCF7L2* (rs7903146), *EXT2* (rs3740878), *KCNJ11* (rs5219), *FTO* (rs8050136), *JAZF1* (rs864745), *CDC123-CAMK1D* (rs12779790), *TSPAN8-LGR5* (rs7961581), *THADA* (rs7578597), *ADAMTS9* (rs4607103), and *NOTCH2-ADAM30* (rs10923931). The significant SNPs (trend test, $P < 0.05$) were further analyzed in the stage 3 panel with the TaqMan method. Despite finding significant association for *CDKAL1* and *CDKN2A/CDKN2B* in the stage 1 and 2 panels, we proceeded with rs4712523 instead of rs7754840 and rs7756992 (*CDKAL1*) and with rs2383208 instead of rs10811661 (*CDKN2A/CDKN2B*) in the GWA scans from stage 1 to stage 3; this decision was made considering the

strong linkage disequilibrium between the SNPs in each of the corresponding loci.

Stage 3 genotyping and analysis. The stage 3 design involved the replication of association and the estimation of effect sizes in the GWA scan and/or replication study of previously reported SNPs. For an association to be considered significant in the case-control comparison (4,000 case vs. 4,889 nondiabetic control subjects), it had to involve the same risk allele as that in the previous stages, and it was accordingly assessed with a one-tailed test. For each SNP locus confirmed in stage 3, the association of additional independent SNPs or haplotypes in the locus was also tested (supplementary information). Moreover, to assess the risk of diabetes and pre-diabetes in the general population from the combination of SNPs robustly confirmed both in populations of European descent and in our panel, multiple regression analysis was performed with the logarithm of A1C (log A1C) as a response variable (supplementary information), using the entire 12,569-subject population-based sample.

Meta-analysis of other type 2 diabetes case-control studies in the Japanese population. In addition, for SNPs with robustly confirmed association in populations of European descent, we performed a meta-analysis by combining our stage 1 + 2 (rs1801282, rs7756992, and rs8050136) or stage 1 + 2 + 3 results (the remaining seven SNPs shown in supplementary Figure S2) with those of previous Japanese studies conducted by three other groups (19–21,33–36). According to Woolf's test (37), the heterogeneity among the studies in the Japanese population was insignificant ($P > 0.05$), with the exception of *PPARG* rs1801282 ($P = 0.0012$), for which the observed heterogeneity is supposedly attributable to low allele frequency. Thus, we pooled genotype counts across the studies to form a combined dataset for the Japanese population, and we estimated the effect sizes of individual loci. We used the rmeta package for R software (http://www.r-project.org) for the analysis.

Moreover, to compare the explained variance between the Japanese population and populations of European descent, we calculated the coefficient of determination R^2 for the loci confirmed in our replication study. Here, R^2 is the square of the correlation between the genotypes of an SNP coded by the number of risk alleles (0, 1, and 2) and the disease status (0 and 1) (supplementary information).

RESULTS

GWA scans. Of 482,625 SNPs that passed quality control in stage 1, genotypes were obtained for an average of 99.8% markers for each subject. The subjects were enrolled from regions of Japan with no strong population stratification (38), and although some variance inflation partly attributable to the subtle subpopulation structure was apparent, such confounding influences could be sufficiently removed using Eigenstrat (30) and genomic-control adjustment (31). A total of 1,456 markers were assessed in the stage 2 panel (Fig. 1 and supplementary Fig. S1 and Table S2).

After the second-stage scan, 30 SNPs representing 17 unique loci attained the arbitrarily defined statistical significance ($P < 7 \times 10^{-5}$) (supplementary Table S3). We used one SNP each from these 17 loci in the third-stage scan. Of 17 SNPs, 4 reached the significance threshold of $P < 0.003$ (= 0.05/17) with Bonferroni correction.

The current GWA study showed strong and highly consistent evidence for disease association of SNPs from *CDKAL1*, *CDKN2A/CDKN2B*, and *KCNQ1* (Fig. 2 and Table 1 and supplementary Tables S4 and S5). Although these three loci had already been reported in previous GWA studies (8,11,25,26), here they were identified as part of our genome-wide exploration. *CDKAL1* is among the best-replicated susceptibility loci. Significant association has also been detected in a region on chromosome 9p, near *CDKN2A/CDKN2B*. Moreover, strong association signals were observed in the intron of *KCNQ1* on chromosome 11p15.5, which is in agreement with the results of two previous GWA scans in the Japanese population (25,26).

Stage 2 genotyping provided evidence suggestive of a new association on chromosome 19q13. Several SNPs located in the vicinity of the *PEPD* (peptidase D) gene showed the tendency of replicated association in stages 1 and 2 (supplementary Table S3). Significant association was further replicated in a relatively large case-control study on the stage 3 panel (rs10425678, $P = 0.002$), but it did not attain genome-wide significance (i.e., $P = 1.4 \times 10^{-5}$ for all stages and $P = 2.1 \times 10^{-6}$ when the number of control subjects was increased by adding 964 random control subjects) (supplementary Table S4). Given the modest strength of association ($R^2 = 0.0017$, see below) assumed for this locus, the association still needs to be established.

Replication of previously reported SNPs. Of 16 candidate loci tested for replication in the Japanese population, 7 were found to be associated with type 2 diabetes (Table 1). However, no significant association was observed for SNPs from the remaining nine loci (*FTO*, *PPARG*, *EXT2*, *JAZF1*, *CDC123-CAMK1D*, *TSPAN8-LGR5*, *ADAMTS9*, and *NOTCH2-ADAM30* in the stage 1 + 2 panel and *THADA* in the stage 1 + 2 + 3 panel). Notably, the originally reported SNPs or those in complete linkage disequilibrium showed the strongest statistical evidence of association in the seven confirmed loci, where the linkage disequilibrium relations and haplotype patterns appear to be similar but not identical between European-descent and Japanese populations (supplementary Figs. S3 and S4).

Besides *KCNQ1* and the 16 candidate loci prioritized here, we investigated the disease association of two candidate gene SNPs—rs734312 in *WFS1* (39) and rs7501939 in *TCF2* (40)—based on the genotype data of our stage 1 panel (n = 1,022) and 964 random control subjects (supplementary Table S6). In some instances, it appeared that the sample size was not sufficient to detect the presumed odds ratio (OR) (supplementary Table S7). Nevertheless, except for rs12779790 in *CDC123-CAMK1D* and rs3740878 in *EXT2*, in the majority of instances, the ORs were consistent with those previously reported. Furthermore, we analyzed seven previously reported SNPs with suggestive evidence of an association in the Japanese population (25), but none attained nominal significance in our first-stage scan (supplementary Table S8).

Ethnic differences in genetic effects on type 2 diabetes. With regard to the candidate gene SNPs robustly confirmed in GWA studies conducted on Japanese and European-descent populations, we compared the risk allele frequency and OR between the meta-analysis dataset of the Japanese population and that of populations of European descent (8–10) (Fig. 3). The OR was consistently higher in the Japanese population for all SNPs except rs5219 in *KCNJ11*. Among the confirmed loci, *CDKAL1*, *CDKN2A/CDKN2B*, *SLC30A8*, and *HHEX* showed a significant difference in the ORs between European-descent and Japanese populations ($P < 0.05$, Woolf's test) (supplementary Table S6). However, the risk allele frequency fluctuated between the two ethnic groups, and the strength of association differed accordingly; this is because an SNP with an risk allele frequency of ~0.5 and a higher OR can give rise to stronger association signals. Thus, whereas *TCF7L2* was shown as the strongest susceptibility locus in populations of European descent (41), its association is estimated to be much weaker in the Japanese population because of the low risk allele frequency. In contrast, the results of the meta-analysis showed that the *CDKN2A/CDKN2B* and *CDKAL1* loci had the strongest associations in the Japanese population;

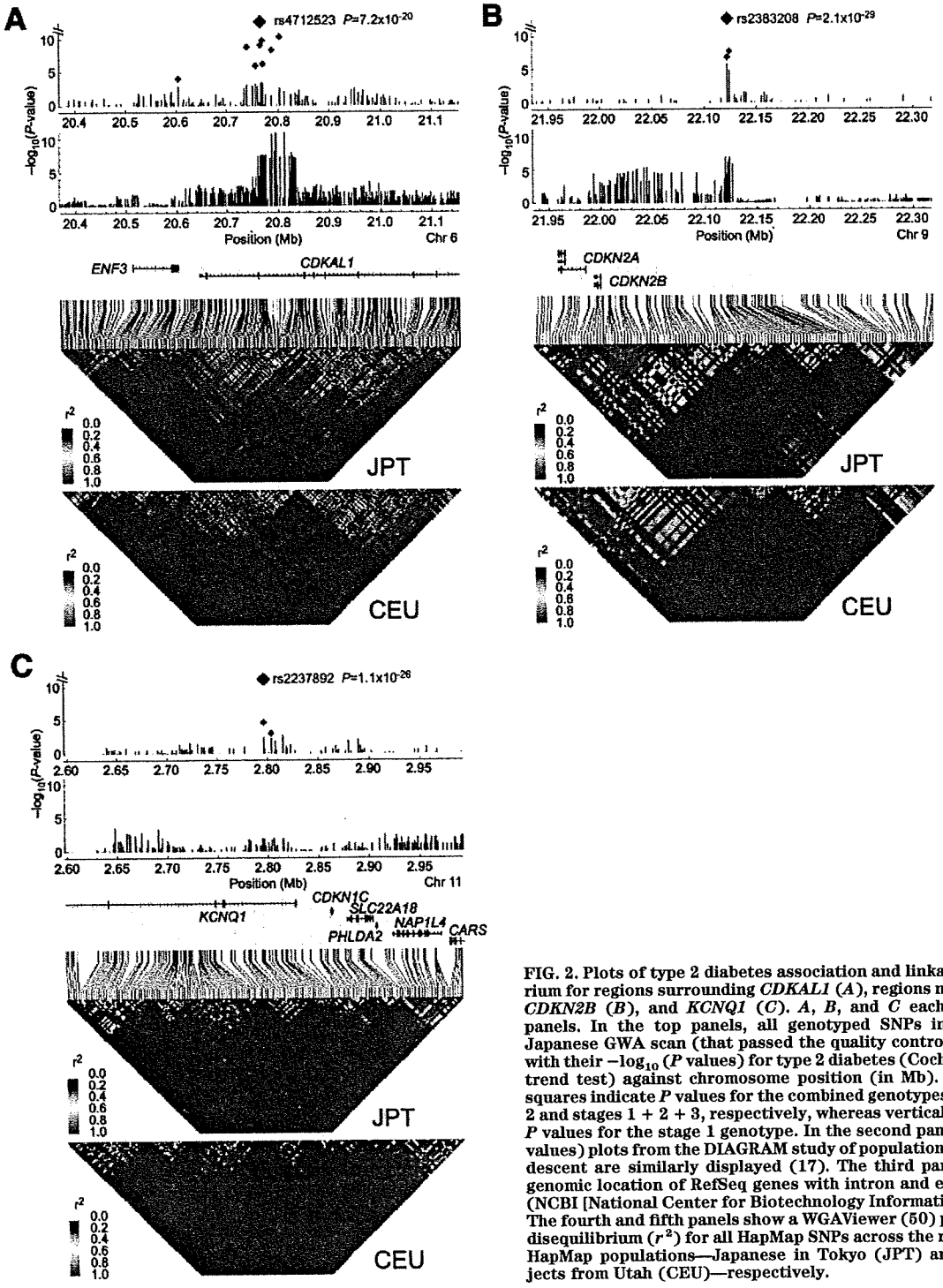

FIG. 2. Plots of type 2 diabetes association and linkage disequilibrium for regions surrounding *CDKAL1* (*A*), regions near *CDKN2A/CDKN2B* (*B*), and *KCNQ1* (*C*). *A*, *B*, and *C* each contain five panels. In the top panels, all genotyped SNPs in the current Japanese GWA scan (that passed the quality control) are plotted with their $-\log_{10}$ (*P* values) for type 2 diabetes (Cochran-Armitage trend test) against chromosome position (in Mb). Blue and red squares indicate *P* values for the combined genotypes of stages 1 + 2 and stages 1 + 2 + 3, respectively, whereas vertical bars indicate *P* values for the stage 1 genotype. In the second panels, $-\log_{10}$ (*P* values) plots from the DIAGRAM study of populations of European descent are similarly displayed (17). The third panels show the genomic location of RefSeq genes with intron and exon structure (NCBI [National Center for Biotechnology Information] Build 35). The fourth and fifth panels show a WGAViewer (50) plot of linkage disequilibrium (r^2) for all HapMap SNPs across the regions for the HapMap populations—Japanese in Tokyo (JPT) and CEPH subjects from Utah (CEU)—respectively.

indeed, we obtained the highest number of hits for these loci.

Next, we compared the strength of association for the seven confirmed loci between Japanese and European-descent populations and calculated R^2 as the proportion of phenotypic variance explained by an SNP (see RESEARCH DESIGN AND METHODS). In Fig. 3, we illustrate the curves corresponding to R^2 = 0.008, 0.004, and 0.002, for which the total sample size of case and control subjects required to attain 80% power is n = 4,300, 8,600, and 17,200 at a significance level of $P = 5 \times 10^{-7}$ (which is the significance threshold generally required in GWA tests), and n = 1,000, 2,000, and 3,900 at a level of P = 0.05. Based on R^2 measurements using the meta-analysis data, the associations of five of seven replicated loci are stronger in the Japanese population than in populations of European descent. For the *CDKAL1* locus, for example, one-fourth of the sample size necessary in populations of European descent is

TABLE 1
Type 2 diabetes susceptibility loci identified or tested for replication in the current Japanese study

rs no.*	Chromosome	Position (bp)	Region	Risk allele/ nonrisk allele	Control risk allele proportion	Stage 1 + 2 (1,629 case subjects/1,517 control subjects) OR (95% CI)	*P* trend
Identified in this GWA scan							
rs4712523	6	20,765,543	*CDKAL1*	G/A	0.407	1.38 (1.25–1.52)	8.0E-10
rs2383208	9	22,122,076	*CDKN2A/B*	A/G	0.553	1.31 (1.18–1.45)	1.6E-07
rs2237892	11	2,796,327	*KCNQ1*	C/T	0.594	1.25 (1.13–1.39)	2.3E-05
rs10425678	19	38,669,236	*PEPD*	C/T	0.261	1.23 (1.10–1.37)	3.6E-04
Replication of previously-reported SNPs							
rs10923931	1	120,230,001	*NOTCH2-ADAM30*	T/G	0.020	1.17 (0.83–1.65)	0.3821
rs7578597	2	43,644,474	*THADA*	T/C	0.990	1.95 (1.03–3.67)	0.0392
rs1801282	3	12,368,125	*PPARG*	C/G	0.969	1.00 (0.75–1.34)	0.9741
rs4607103	3	64,686,944	*ADAMTS9*	C/T	0.594	1.09 (0.99–1.21)	0.0902
rs4402960	3	186,994,389	*IGF2BP2*	T/G	0.310	1.15 (1.04–1.28)	0.0098
rs7754840	6	20,769,229	*CDKAL1*	C/G	0.392	1.42 (1.28–1.57)	1.7E-10
rs7756992	6	20,787,688	*CDKAL1*	G/A	0.448	1.35 (1.23–1.50)	4.6E-09
rs864745	7	27,953,796	*JAZF1*	T/C	0.789	1.08 (0.95–1.22)	0.2456
rs13266634	8	118,253,964	*SLC30A8*	C/T	0.570	1.18 (1.06–1.30)	0.0015
rs10811661	9	22,124,094	*CDKN2A/B*	T/C	0.555	1.35 (1.21–1.49)	2.2E-08
rs12779790	10	12,368,016	*CDC123-CAMK1D*	G/A	0.151	0.98 (0.85–1.13)	0.7984
rs1111875	10	94,452,862	*HHEX*	C/T	0.275	1.19 (1.07–1.33)	0.0011
rs7903146	10	114,748,339	*TCF7L2*	T/C	0.035	1.42 (1.10–1.84)	0.0073
rs5219	11	17,366,148	*KCNJ11*	T/C	0.355	1.22 (1.09–1.35)	2.5E-04
rs3740878	11	44,214,378	*EXT2*	A/G	0.633	1.01 (0.91–1.12)	0.8849
rs7961581	12	69,949,369	*TSPAN8-LGR5*	C/T	0.202	1.12 (0.99–1.27)	0.0751
rs8050136	16	52,373,776	*FTO*	A/C	0.203	1.11 (0.98–1.26)	0.0915

Continued on following page

sufficient to obtain the same level of statistical significance in the Japanese population. This is true for *CDKN2A/CDKN2B*, *HHEX*, and *SLC30A8*, in which <50% of the sample size seems to be sufficient for significance in the Japanese population. However, *TCF7L2* shows an opposite trend in this regard.

Combined genetic risk of type 2 diabetes. Despite the small value of explained variance (R^2) at each risk locus, it is assumed that knowledge about multiple-risk loci could allow us to identify individuals with accumulated genetic risk (42). To this end, a GWA study in Finns (10) investigated the combined risk of type 2 diabetes based on 10 associated loci by logistic regression analysis of the resampled dataset. The total variance explained by 10 loci in Finns is $R^2 = 0.030$, which is equivalent to the value for 7 loci obtained here (see DISCUSSION). Likewise, in a simulated population, we arranged the individuals in the order of the risk estimated by logistic regression, sorted them into 20 equal-sized groups (5% in each), and calculated the actual proportion of affected individuals in each group. We found a 3.7-fold variation in type 2 diabetes prevalence from the lowest to highest estimated risk groups for the combination of seven associated loci in our study (Fig. 4). The receiver operating characteristic curve was also depicted for the combined SNPs as a measure of sensitivity and specificity (supplementary Fig. S5).

Moreover, for risk assessment in the general population, we performed multiple regression analysis using A1C as a surrogate quantitative phenotype to estimate the unbiased effect size of individual loci (supplementary Table S9) and evaluated the combined risk from multiple loci in 12,569 population-based samples (Table 2 and supplementary information). Then, the estimated risk was compared with the actual A1C value and the disease classification of diabetes or pre-diabetes (supplementary information). In the multiple regression analysis, significant association ($P < 0.005$) was observed for all seven loci tested in accordance with the results for the case-control study (Table 1 and supplementary Table S4). As shown in Fig. 4, 5% of male subjects with the highest estimated risk are 2.3 times more likely to suffer from diabetes than those with the lowest estimated risk; the risk is 5.2 times in female subjects, indicating the potential existence of sex difference in the genetic risk of type 2 diabetes (supplementary Fig. S6). Moreover, notably, SNP genotypes alone exerted more exaggerated effects on the increase in genetic risk in diabetes compared with pre-diabetes (Table 2).

DISCUSSION

Conducting GWA studies on a wider range of populations, including East Asians, has recently gained importance because of the discovery of new type 2 diabetes susceptibility variants mapping to the *KCNQ1* gene simultaneously reported in two Japanese studies (25,26). Both studies were, however, initiated some years ago, and they are, by current standards, considered to be modest with regard to the coverage of common SNPs (21 and 56% in HapMap) and number of case subjects (187 and 194 subjects, respectively) in the first-stage scan. Therefore, we conducted another GWA study on the Japanese population with greater coverage of common SNPs (87% of all phase

TABLE 1
Continued

Stage 3 (4,000 case subjects/4,889 control subjects)†		All combined (5,629 case subjects/6,406 control subjects)†		OR (95% CI) reported in Europeans (14,586 case subjects/17,968 control subjects)
OR (95% CI)	*P* trend‡	OR (95% CI)	*P* trend	
1.23 (1.16–1.30)	4.0E-12	1.27 (1.21–1.33)	7.2E-20	1.12 (1.08–1.16)
1.33 (1.26–1.42)	4.8E-22	1.34 (1.27–1.41)	2.1E-29	1.20 (1.14–1.25)
1.36 (1.28–1.45)	8.0E-23	1.33 (1.27–1.41)	1.1E-26	1.18 (1.03–1.33)§
1.10 (1.03–1.18)	0.0020	1.14 (1.07–1.20)	1.4E-05	1.03 (0.97–1.09)§
—	—	—	—	1.13 (1.08–1.17)‖
0.98 (0.73–1.31)	0.55	1.13 (0.87–1.47)	0.35	1.15 (1.10–1.20)‖
—	—	—	—	1.14 (1.08–1.20)
—	—	—	—	1.09 (1.06–1.12)‖
1.14 (1.07–1.21)	2.5E-05	1.14 (1.08–1.21)	1.0E-06	1.14 (1.11–1.18)
—	—	—	—	1.12 (1.08–1.16)
—	—	—	—	1.26 (1.18–1.34)§
—	—	—	—	1.10 (1.07–1.13)‖
1.24 (1.17–1.31)	5.8E-13	1.22 (1.16–1.28)	1.8E-14	1.12 (1.07–1.16)
—	—	—	—	1.2 (1.14–1.25)
—	—	—	—	1.11 (1.07–1.14)‖
1.21 (1.13–1.29)	2.6E-09	1.21 (1.15–1.28)	6.7E-12	1.13 (1.09–1.17)
1.59 (1.38–1.83)	5.3E-11	1.54 (1.36–1.74)	7.6E-12	1.37 (1.31–1.43)
1.02 (0.96–1.08)	0.3008	1.07 (1.01–1.13)	0.0149	1.14 (1.10–1.19)
—	—	—	—	1.20 (1.11–1.30)¶
—	—	—	—	1.09 (1.06–1.12)‖
—	—	—	—	1.17 (1.12–1.22)

Results for one SNP each selected from the individual chromosomal regions in the GWA scans are shown in the table (see supplementary Table S4 for details and supplementary Table S5 for the results of logistic regression adjusted for BMI). The final *P* value was assessed by pooling genotype counts for each SNP from all stages tested (without including 964 random control subjects from GeMBDJ). In two regions, chromosome 6p22.3 (*CDKAL1*) and 19p13 (*PEPD*), the haplotype class showed more significant association than the individual SNP (see supplementary Information). *In the stage 3 panel, we genotyped rs4712523 instead of rs7754840 (r^2 = 0.96) or rs7756992 (r^2 = 0.65) in CDKAL1, and rs2383208 instead of rs10811661 (r^2 = 0.89) near CDKN2A/B, with the aim of determining the SNP(s) with the strongest association in the Japanese population. †In stage 3 of the replication study on previously reported SNPs, after the confirmation of significant association in 4,000 case subjects and 4,889 preselected control subjects, we further characterized 7,680 subjects (who comprised the rest of the 12,569 population-based samples) (see RESEARCH DESIGN AND METHODS and Fig. 1). Thus, for the corresponding SNPs, 5,395 control subjects were reselected from the entire population-based samples and used for the final association analysis in stage 3, which increased the total number of control subjects across the three stages to 6,912. ‡One-tailed test for association was performed in the direction consistent with stage 1 + 2 data; §for 4,549 case and 5,579 control subjects derived from the DIAGRAM consortium of Zeggini et al. (17); ‖for ~60,000 total samples from Zeggini et al. (17); ¶for 3,278 case and 3,508 control subjects from Sladek et al. (6).

1 + 2 HapMap variants [MAF ≥0.05] in CHB (Chinese in Beijing) + JPT (Japanese in Tokyo) and a larger number of case subjects (519 subjects) and unaffected control subjects (503 subjects) in addition to random control subjects in the first-stage scan. Four loci (three previously reported and one novel) were identified via the multistage scans. For the top three loci (*KCNQ1*, *CDKN2A/CDKN2B*, and *CDKAL1*) the OR (>1.25) and MAF (0.41–0.45 in the control subjects) were higher in the Japanese population than in populations of European descent. In addition to the nomination of four susceptibility loci (*KCNQ1*, *CDKN2A/CDKN2B*, *CDKAL1*, and *PEPD*), the current study replicated the significant association of five other loci (*TCF7L2*, *IGF2BP2*, *SLC30A8*, *HHEX*, and *KCNJ11*) previously reported in populations of European descent (6–17) and provided an unbiased estimate of the risk from the confirmed disease genotype.

Empirical studies suggest that the genetic effects of individual causal risk alleles underlying complex genetic diseases such as type 2 diabetes are modest, with most genotype relative risks in the range of 1.1–2.0 (43). Indeed, we observed this to be true for loci that were robustly implicated in the development of type 2 diabetes by GWA scans and/or extensive candidate gene approaches in populations of European descent. Currently, the number of loci has increased to almost 20 (as listed in supplementary Table S6), and in most cases, except for *TCF7L2* and *KCNQ1*, the OR is estimated to be between 1.09 and 1.20.

The current study provides, via genome-wide exploration and replication analysis of some a priori selected loci, significant evidence for the overall tendency toward a stronger association in Japanese rather than European-descent populations at least for alleles with a cosmopolitan effect. The tendency for higher OR in Asians than in Europeans was previously reported for the *CDKAL1* locus (22). Currently, it remains unknown whether the penetrance for a genotype of interest differs considerably between Japanese and European-descent populations. With regard to genetic effects, four of seven confirmed loci have demonstrated significantly higher OR in the Japanese population ($P = 4.1 \times 10^{-5}$ to 0.024) (supplementary Table S6). To simplify the situation, we have further assessed the strength of association for individual SNPs by measuring R^2, which is scaled against OR and risk allele frequency in

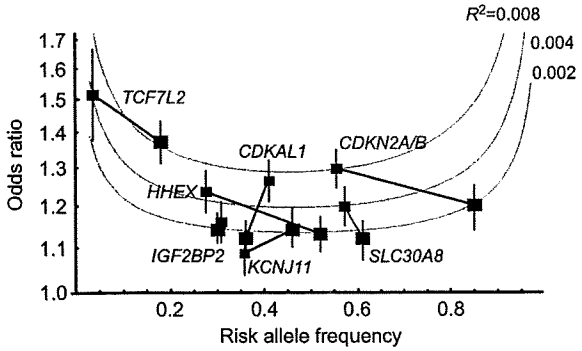

FIG. 3. Comparison of the strength of association for seven confirmed type 2 diabetes loci between Japanese and European-descent populations. For the Japanese population, we estimated ORs and their 95% CIs (red solid squares and vertical lines, respectively) for each locus based on our meta-analysis involving four Japanese case-control studies (supplementary Fig. S2). For populations of European descent, on the other hand, the corresponding values (blue solid squares and vertical lines) were derived from the published data (8–10). The association of an SNP with type 2 diabetes is measured by the coefficient of determination (R^2), which represents the ability to detect association signals using the Cochran-Armitage trend test.

Fig. 3. We found that despite the limited number of SNPs tested here, the same level of statistical significance is often detectable in the Japanese population with a much smaller sample size than that in populations of European descent (supplementary Table S7). Theoretically, the stringency of ascertaining control subjects could lead to some bias in effect size (44). In this respect, in addition to the multistage case-control study, an extensive analysis of associated loci in the general population was conducted, which is the strength of the current study. We used the population-based samples (n = 12,569) in stage 3 to

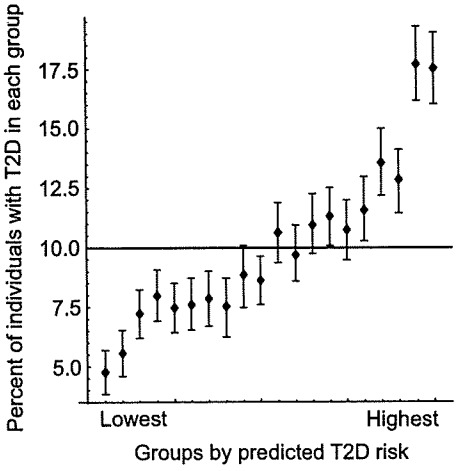

FIG. 4. Estimation of the increase in type 2 diabetes risk from the combination of seven susceptibility variants previously identified and robustly replicated in the current study. We used case and control subjects with complete data from all stages of our study (n = 12,105). First, the risk for the genotypes of an SNP was estimated by logistic regression. Then, the multilocus risk for an individual was assessed as the sum of the risks for his/her genotype at seven SNPs. We simulated a population with 10% prevalence by bootstrap sampling. In the simulated population, we arranged the individuals in the order of their multilocus risk, sorted them into 20 equal-sized groups, and calculated the actual prevalence in each group. Means and 95% CIs of the groupwise prevalence were estimated based on 1,000 bootstrap sampling trials and are plotted in the figure. No significant gene-gene interaction was observed between the seven SNPs by multiple logistic regression analysis. T2D, type 2 diabetes.

investigate the effect of control selection criteria on OR in a case-control comparison and found that the ORs in our meta-analysis were almost comparable to those estimated in the general Japanese population (supplementary Table S10). Moreover, with regard to ethnic diversity, linkage disequilibrium in *CDKAL1* and *KCNJ11* is stronger in East Asians (JPT + CHB), whereas linkage disequilibrium in *IGFBP2* and *HHEX* tends to be stronger in Europeans (CEU [Centre d'Etude du Polymorphisme Humain (CEPH) subjectsfrom Utah]) (Supplementary Figure S4); thus, besides the issue of power, the results of the GWA scans in the Japanese population (or East Asians) seem to be useful in terms of interethnic comparison of association signals, which may enhance the power of fine-mapping efforts designed to identify the causal variants (45).

The tendency of stronger genetic association in the Japanese population is also supported by the concomitant evaluation of multilocus effects. When assuming an additive model, the combined risk of type 2 diabetes can be measured by the sum of the R^2 values of individual loci. For example, the total variance explained by the seven loci depicted in Fig. 3 is 0.030 in the Japanese population and 0.018 in populations of European descent. It remains unknown whether these findings reflect higher heritability of type 2 diabetes in Japanese than in European-descent populations. Because little data are available on the estimation of heritability in the Japanese or East Asian populations, further studies are required to obtain the standardized measures of heritability across different populations by taking into account potential sources of heterogeneity, such as the degree of westernization of lifestyle.

Suggestive evidence of association was identified for SNPs in the *PEPD* gene. *PEPD* plays an important role in collagen metabolism, and some extracellular matrix constituents such as collagen IV have been shown to have a profound impact on insulin secretion (46). Moreover, enhanced collagen degradation via *PEPD* activity has been reported in diabetic patients (47). Although there is evidence suggestive of association at *PEPD* in all three stages, the current GWA study by itself could not confirm or refute the evidence; no significant association was found in the previously reported Diabetes Genetics Replication and Meta-Analysis (DIAGRAM) data from Europeans (risk allele frequency = 0.52, OR = 1.03) (Table 1) and in the initial screening data of the JSNP (Japanese Single Nucleotide Polymorphisms) scan in the Japanese population (187 casevs. 752 random control subjects; P = 0.18 at rs2241380, which is in complete linkage disequilibrium with rs10425678 in *PEPD*; r^2 = 1.0) (25).

The number of genes that could account for an appreciable population-attributable fraction of common diseases is under debate (48). Although the current study detected and/or replicated a total of nine susceptibility loci, including *PEPD* in the Japanese population, a substantial number of SNPs showing some extent of association signals in the first-stage scan remain to be investigated, as reflected by the wide distribution of replicated SNPs with unexamined "gaps" in the lower-left part of the Q-Q plot (supplementary Fig. S7). The ORs corresponding to such unexamined SNPs mostly fall in the range of 1.10–1.25. To assess the statistical power in our GWA scan, we simulated the frequency at which a disease-associated SNP could surpass the cutoff level of the first two stages (stages 1 and 2) (supplementary information and supplementary Table S11). In the current experimental setting, it is likely that >50% of the susceptibility loci

TABLE 2
Combined risk of diabetes and pre-diabetic status based on seven confirmed loci, age, BMI, and sex in the general Japanese population

		Diabetes		Pre-diabetes		Diabetes + Pre-diabetes	
	A1C (%)	RR versus population average (95% CI)	Prevalence	RR versus population average (95% CI)	Prevalence	RR versus population average (95% CI)	Prevalence
Male							
Whole population	5.29 ± 0.88	1.00	0.16	1.00	0.07	1.00	0.23
Highest risk group (5%) assessed by							
All predictors	5.48 ± 0.87	1.65 (1.29–1.97)	0.27	1.34 (0.73–1.83)	0.09	1.56 (1.26–1.78)	0.36
SNP genotypes	5.57 ± 1.12	1.67 (1.32–2.06)	0.27	0.92 (0.44–1.40)	0.07	1.45 (1.16–1.73)	0.34
Age and BMI*	5.44 ± 0.78	1.16 (0.87–1.46)	0.19	1.95 (1.39–2.60)	0.14	1.40 (1.16–1.65)	0.33
Lowest risk group (5%) assessed by							
All predictors	4.98 ± 0.73	0.46 (0.26–0.74)	0.08	0.50 (0.20–0.90)	0.04	0.47 (0.33–0.70)	0.11
SNP genotypes	5.11 ± 0.74	0.72 (0.39–0.92)	0.12	0.71 (0.30–1.10)	0.05	0.72 (0.46–0.86)	0.17
Age and BMI*	4.98 ± 0.77	0.46 (0.30–0.73)	0.08	0.40 (0.10–0.60)	0.03	0.44 (0.27–0.63)	0.10
Female							
Whole population	5.17 ± 0.60	1.00	0.07	1.00	0.06	1.00	0.13
Highest risk group (5%) assessed by							
All predictors	5.55 ± 0.96	3.09 (2.36–3.73)	0.22	2.05 (1.37–2.60)	0.13	2.61 (2.10–2.96)	0.35
SNP genotypes	5.37 ± 0.88	2.30 (1.60–2.78)	0.17	1.17 (0.73–1.80)	0.07	1.78 (1.41–2.10)	0.24
Age and BMI*	5.42 ± 0.78	2.26 (1.71–2.78)	0.16	1.95 (1.34–2.53)	0.12	2.12 (1.73–2.46)	0.28
Lowest risk group (5%) assessed by							
All predictors	4.91 ± 0.43	0.16 (0.00–0.32)	0.01	0.14 (0.00–0.28)	0.01	0.15 (0.04–0.26)	0.02
SNP genotypes	5.02 ± 0.45	0.45 (0.16–0.73)	0.03	0.80 (0.38–1.22)	0.05	0.61 (0.35–0.83)	0.08
Age and BMI*	4.94 ± 0.36	0.24 (0.08–0.64)	0.02	0.19 (0.00–0.37)	0.01	0.22 (0.09–0.47)	0.03

Data are the means ± SD, unless otherwise indicated. Relative risk (RR) is calculated as the ratio of the prevalence in 5% of people with the highest or lowest risk to the prevalence in the whole population. In this study, the combined disease risk for each individual was assessed using the regression for A1C (see supplementary information). Subjects with self-reported diabetes or with A1C ≥6.1 were classified as diabetic, and those who were not under antidiabetic medication and with 5.6 ≤ A1C < 6.1 were classified as pre-diabetic. The actual A1C level and the distribution by diabetic status for each 5% subgroup of the risk group are illustrated in supplementary Fig. S6. *For reference, diabetes and/or pre-diabetes risk was assessed using the participant's age and BMI alone as predictors.

with modest but substantial effects (OR = 1.2–1.3) were unidentified. For example, though not statistically significant, the association of *PPARG* in the Japanese population showed an OR ($P = 0.06$, OR = 1.18 at rs1801282) similar to that in populations of European descent in a meta-analysis, including the current study (supplementary Table S4). Increasing the sample size of the stage 1 panel and/or the number of SNPs genotyped in the second-stage scan would allow us to discover more susceptibility variants, including new population-specific loci, in the Japanese population.

The incidence of type 2 diabetes is escalating to epidemic proportions globally, with a higher acceleration rate in non-European populations (49). The integration of GWA study results, i.e., a meta-analysis (17), for both European-descent and non-European populations is necessary for a comprehensive understanding of the genetics of type 2 diabetes, and it will lead to the efficient use of genomic information based on ethnic diversity in clinical research.

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No potential conflicts of interest relevant to this article were reported.

The GWA study conducted by NIBIO GWA Study Group has been organized to clarify the pathogenesis of diabetes and associated metabolic disorders as well as cardiovascular complications. The collaborating institutions that constitute the NIBIO GWA Study Group are as follows: International Medical Center of Japan; Kyushu University; Osaka University; Nagoya University; Kinki University; Shimane University; Tohoku University; the Institute for Adult Diseases, Asahi Life Foundation; Chubu Rosai Hospital; Amagasaki Health Medical Foundation; collaborating groups in the Amagasaki Medical Association; and collaborating groups in the Kyushu region [see details in Nawata et al. (32)].

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Role of Advanced Glycation End Products in Diabetic Neuropathy

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Abstract: Diabetic neuropathy is the commonest form of peripheral neuropathy in the developed countries of the world. In diabetic patients, the presence of peripheral neuropathy increases their risks for developing foot ulceration and subsequent necrosis that necessitates lower limb amputation. Although the precise mechanisms underlying diabetic neuropathy remain unclear, there is evidence that hyperglycemia-induced formation of advanced glycation end products (AGEs) is related to diabetic neuropathy; AGE-modified peripheral nerve myelin is susceptible to phagocytosis by macrophages and contributes to segmental demyelination; modification of major axonal cytoskeletal proteins such as tubulin, neurofilament, and actin by AGEs results in axonal atrophy/degeneration and impaired axonal transport; and glycation of extracellular matrix protein laminin leads to impaired regenerative activity in diabetic neuropathy. Recently, the receptor for AGEs (RAGE) has been found to colocalize with AGEs in diabetic peripheral nerves. This suggests that, in diabetic neuropathy, AGEs and AGE/RAGE interactions induce oxidative stress, result in upregulation of nuclear factor (NF)-kappaB and various NF-kappaB-mediated proinflammatory genes, and exaggerate neurological dysfunction, including altered pain sensation. Additionally, AGE/RAGE-induced oxidative stress further accelerates formation of glycoxidation products such as Nepsilon-(carboxymethyl)lysine and pentosidine. Although new drugs that inhibit the formation of AGEs and block the AGE-RAGE interaction are being studied, no effective treatment modalities against AGE-induced nerve injury are currently available clinically. A therapeutic strategy to prevent and ameliorate diabetic neuropathy using anti-AGE agents needs to be established. In this review, the current issues involved in the role of the glycation process and the potential treatment options for diabetic neuropathy are explored.

Key Words: Diabetic neuropathy, AGEs, oxidative stress, RAGE, CML.

INTRODUCTION

Diabetic neuropathy is the commonest complication of long-term hyperglycemia and the leading cause of nontraumatic lower limb amputations in diabetic patients [1]. Although the pathogenesis of diabetic neuropathy remains enigmatic [2], there are emerging data from animal and clinical studies suggesting that hyperglycemia-induced formation of advanced glycation end products (AGEs) may play a key role in the pathogenesis of diabetic neuropathy [3], and that it is linked with other pathogenic mechanisms, such as the presence of an increased flux through the polyol pathway [4], increased oxidative stress [5], and activation of the diacylglycerol-protein kinase C (PKC) pathway [6,7]. All of these mechanisms contribute to increased oxidative stress, which in turn further increases the formation of glycoxidation products such as N-epsilon-(carboxymethyl)lysine (CML) and pentosidine. Recently, the receptor for AGEs (RAGE) has been cloned; it was found to colocalize with AGEs in the perineurium, as well as the endoneurial and epineurial vessels, of patients with diabetic neuropathy [8,9]. The formation of AGEs and their interaction with RAGE activate intracellular signaling pathways that induce transcription of proinflammatory genes and therefore cellular oxidative stress, as has been shown in peripheral nerves [8,10]. As well, hyperglycemia *per se* and subsequent activation of the polyol pathway result in increased production of intracellular dicarbonyl AGE precursors such as methylglyoxal and 3-deoxyglucosone; subsequently, these molecules modify proteins to form AGEs intracellularly and cause abnormal interactions with other matrix proteins, which eventually leads to functional and structural abnormalities in diabetic peripheral nerves. The recent development of a noninvasive method for specifically measuring tissue AGE levels showed an association between tissue AGE levels and the severity of peripheral and autonomic dysfunction, as well as the occurrence of foot ulceration in diabetic patients [11]. Thus, this review outlines the current knowledge of the interrelated biochemical mechanisms that have been hypothesized to account for AGE-mediated progressive damage and loss of diabetic nerve fibers. Furthermore, potential therapeutic approaches by which AGE formation can be inhibited or the pathogenic effects of AGE formation limited in experimental and human diabetic neuropathy are discussed.

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INTERRELATIONSHIPS BETWEEN AGES AND OTHER METABOLIC PATHWAYS

(1) AGEs and the Polyol Pathway

Increased production of fructose *via* activation of the polyol pathway enhances generation of glycated proteins. In the polyol pathway, glucose is reduced to sorbitol by the first enzyme, aldose reductase; sorbitol is then converted to fructose by the second enzyme, sorbitol dehydrogenase. Fructose is further metabolized to fructose-3-phosphate and fructose-1-phosphate by 3-phosphokinase [12] and fructokinase [13], respectively. Additionally, fructose is also converted to fructose-6-phosphate by hexokinase; fructose-6-phosphate lies within the glycolysis metabolic pathway and is converted to glucosamine-6-phosphate by glutamine:fructose-6-phosphate amidotransferase and then finally converted to uridine diphosphate *N*-acetylglucosamine in the hexosamine pathway [14] (Fig. 1). One of the reactive alpha-oxoaldehydes and a potent AGE precursor, 3-deoxyglucosone, is formed by nonoxidative rearrangement from fructose [15] and fructose-3-phosphate [16]; it rapidly reacts with protein amino groups to form AGEs such as imidazolone, pyrraline, CML, and pentosidine [17]. Methylglyoxal, another reactive alpha-oxoaldehyde, is formed by the fragmentation of glyceraldehyde-3-phosphate in anaerobic glycolysis [18,19], as well as the oxidative decomposition of polyunsaturated fatty acids [20]. Exposure of rat Schwann cells to methylglyoxal decreases intracellular reduced glutathione (GSH) content, activates p38 mitogen-activated protein kinase (MAPK), and induces apoptosis; these findings suggest that methylglyoxal has a potential role in Schwann cell injury through oxidative stress-mediated p38 MAPK activation [21]. Glyceraldehyde-derived AGEs have been found to be the most common toxic AGEs in the serum of diabetic patients, and they are formed from glyceraldehyde-3-phosphate and fructose-1-phosphate [22] (Fig. 1). Interestingly, in isolated rat Schwann cells, AGEs derived from glyceraldehyde and glycolaldehyde, but not glucose,

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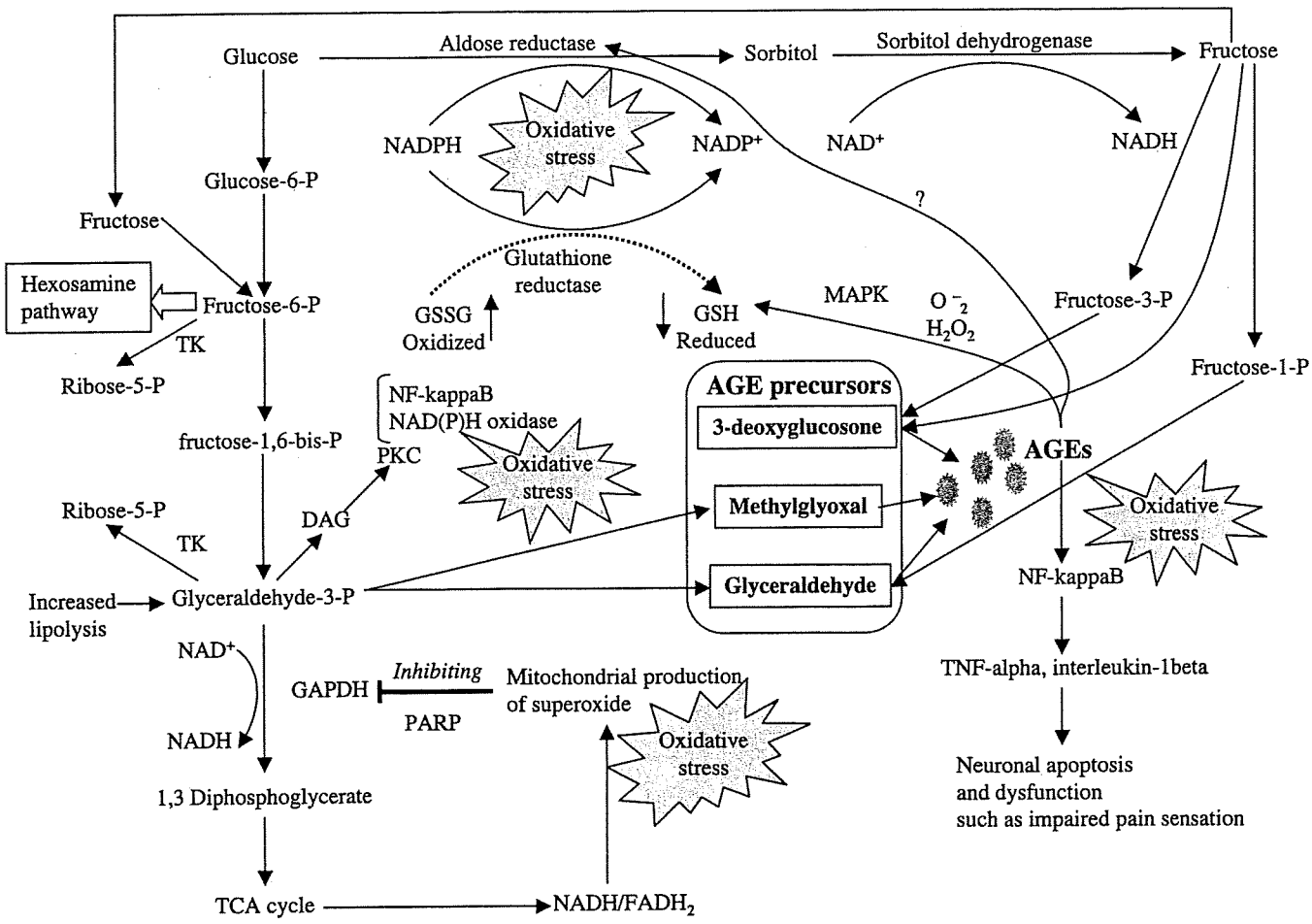

Fig. (1). Interrelationship between nonenzymatic glycation and other metabolic pathways.

In the polyol pathway, the aldose reductase consumes the cofactor NADPH [117], which is also the essential cofactor for regenerating a critical intracellular antioxidant, GSH. Increased flux through the polyol pathway promotes intracellular oxidative stress. The neurotoxic intracellular AGE precursors methylglyoxal and glyceraldehyde are formed from glyceraldehyde-3-phosphate, which also activates the classical protein kinase C (PKC) pathway, since the activator of PKC, diacylglycerol (DAG), is also formed from glyceraldehyde-3-phosphate. Hyperglycemia-induced generation of superoxide inhibits glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity by activating poly(ADP-ribose) polymerase (PARP). In the unifying hypothesis, it is postulated that impaired GAPDH activity activates the polyol pathway, the PKC pathway, and the hexosamine pathway, and increases AGE formation [118]. Oxidative stress induced by AGE formation, as well as other pathways activated by hyperglycemia, enhances generation of glycoxidation products such as Nepsilon-(carboxymethyl)lysine and pentosidine. Thiamine and benfotiamine activate transketolase (TK), which stimulates the conversion of glyceraldehyde-3-phosphate and fructose-6-phosphate to ribose-5-phosphate, thereby inhibiting the hexosamine pathway, the PKC pathway, and AGE formation [119]. AGEs, advanced glycation end products; FADH, reduced flavin adenine dinucleotide; GSSG, oxidized glutathione; MAPK, mitogen-activated protein kinase, NAD(P)H, reduced nicotinamide adenine dinucleotide (phosphate); NF-kappaB, nuclear factor-kappaB; RAGE, receptor for advanced glycation end products; TCA, trichloroacetic acid; TNF, tumor necrosis factor.

increase the release of tumor necrosis factor (TNF)-alpha and interleukin-1beta, activate nuclear factor (NF)-kappaB, decrease the mitochondrial membrane potential, and induce apoptosis [23]. It has also been demonstrated that, under high glucose conditions, NF-kappaB expression is upregulated in rat Schwann cells *via* activation of the polyol pathway [24]. It has been suggested that long-term upregulation of NF-kappaB, along with PKC-beta activation linked to the polyol pathway activity, contributes to the development of peripheral nerve dysfunction, including altered pain sensation in diabetic mice [25,26]. Low TNF-alpha concentrations applied along the nerve elicit an increase in ectopic discharges of single nociceptive primary afferent fibers, whereas higher concentrations lead to reduced firing rates [27]. Thus, it is possible that, in experimental diabetic neuropathy models, along with increased flux from the polyol pathway, AGE-dependent intracellular signaling in Schwann cells leads to neurological dysfunction, including altered nociception *via* oxidative stress-induced activation of NF-kappaB and increased levels of proinflammatory cytokines such as TNF-alpha (Fig. **1**).

The role of fructose as a glycator is further exemplified by the fact that diabetic patients and animals treated with aldose reductase inhibitors develop significant reductions in glycated protein levels in the aorta, lens, and erythrocytes [28-30]. Conversely, methylglyoxal has been reported to increase aldose reductase mRNA/protein expression and activity in rat aortic smooth muscle cells by inducing oxidative stress [31]. Enhanced activation of the polyol pathway by AGEs has also been demonstrated in mouse aortic smooth muscle cells and mesangial cells overexpressing the human aldose reductase gene [32,33]; this enhancement may be mediated by AGE-induced activation of the extracellular signal-regulated kinase pathway under oxidative stress [34,35]. Thus, these results suggest that increased production of fructose through activation of the polyol pathway enhances generation of glycated proteins, which in turn activate the polyol pathway in tissues that are the targets of