of G-CSFR is not well understood (Stratos et al., 2007; Naito et al., 2009). We proved that BM-derived cells were not directly involved in skeletal muscle regeneration by G-CSF; however, BM-derived cells expressing G-CSF ligand can stimulate skeletal muscle proliferation through myoblast-specific expression of G-CSFR. This study demonstrates for the first time that the factors involved in the inflammatory process switch on the process of skeletal muscle regeneration.

Clinically, G-CSF is used to treat patients with neutropenia resulting from immunosuppressive chemotherapy, severe congenital neutropenia, life-threatening infections, and stem cell harvesting (Hammond et al., 1989; Molineux et al., 1990; Welte et al., 1996). Interestingly, myalgia is one of the main side effects of G-CSF administration in humans (Taylor et al., 1989). We may speculate that innate skeletal muscle regenerates itself to some extent to adapt the physiological turn over, that G-CSF injection stimulates small population of these skeletal myoblasts, and that the burst of skeletal myocyte proliferation gives rise to myalgia. The safety and side effects of G-CSF have been studied in several clinical settings (Anderlini and Champlin, 2008). Therefore, a clinical trial of G-CSF for human skeletal muscle injury may be warranted. The results of this study underline the importance of G-CSF in skeletal muscle development and regeneration and strengthen the case for using G-CSF as a skeletal muscle regeneration therapy.

## MATERIALS AND METHODS

**Whole-mount in situ hybridization.** Mouse embryos were removed from wild-type Institute of Cancer Research pregnant mice on E10.5. Whole-mount in situ hybridization was performed as described previously (Yuasa et al., 2005). The full-length cDNAs for mouse *c-met*, *pax3*, *myoD*, and *mrf4* (available from GenBank/EMBL/DDBJ under accession numbers NM_008591, NM_001159520, NM_010866, and NM_008657 [listed as *myf6*], respectively) were provided by M.E. Buckingham (Pasteur Institute, Paris, France). The full-length cDNA for mouse *csf3r* (GenBank accession number NM_007782) was provided by S. Nagata (Osaka University, Suita, Osaka, Japan; Fukunaga et al., 1990). The probes were generated using T3 or T7 RNA polymerase.

**Animals.** The *myf5 nlacZ* mice were a gift from S. Tajbakhsh (Pasteur Institute; Tajbakhsh et al., 1996). The $csf3r^{-/-}$ mice were a gift from D.C. Link (Washington University School of Medicine, St. Louis, MO; Richards et al., 2003). All the experimental procedures and protocols were approved by the Animal Care and Use Committee of Keio University and conformed to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

**Immunofluorescence.** Mouse embryos on E8.5, E9.5, E10.5, and E11.5 were fixed in 4% paraformaldehyde for 3 h and embedded in Tissue-Tek OCT (Sakura) for frozen sectioning. The samples were incubated with Triton X-100 for 5 min at room temperature, washed, and incubated with the following primary antibodies: anti–G-CSFR (1:50; Santa Cruz Biotechnology, Inc.), anti-Pax3 (1:200; American Type Culture Collection), anti-Pax7 (1:50; R&D Systems), anti-MyoD (1:50; Dako), antimyogenin (1:50; Santa Cruz Biotechnology, Inc.), antidesmin (Dako), anti–G-CSF (1:50; Santa Cruz Biotechnology, Inc.), anti–α-actinin (1:1,000; Sigma-Aldrich), and anti-GAPDH (1:200; Santa Cruz Biotechnology, Inc.). After overnight incubation, bound antibodies were visualized with a secondary antibody conjugated to Alexa Fluor 488 or 546 (Invitrogen). Nuclei were stained with DAPI (Invitrogen). For BrdU staining, a BrdU labeling kit (Roche) was used. After antigen retrieval using HistoVT One (L6F9587; Nacalai Tesque) and blocking, BrdU staining was performed as described in the manufacturer's protocol.

**Myoblast culturing.** C2C12 mouse myoblasts (American Type Culture Collection) were cultured in DME/10% FBS (Invitrogen). The medium was replaced with DME/2% horse serum (Invitrogen) to induce differentiation. Recombinant mouse G-CSF (R&D Systems) was added on the indicated days. Inhibition of G-CSF signaling was analyzed by administering an anti–G-CSFR neutralizing antibody (R&D Systems).

**Western blotting.** C2C12 cells were treated with G-CSF. Cell extracts were prepared at 0, 5, 10, 15, 30, 45, and 60 min after G-CSF stimulation. Protein lysates were resolved by SDS-PAGE and transferred to a polyvinylidene fluoride membrane, followed by immunoblotting with anti–phospho-STAT3, anti–phospho-AKT, anti–phospho-ERK, anti–phospho-JNK, and anti–phospho-p38MAPK antibodies (all from Cell Signaling Technology) and horseradish peroxidase–conjugated anti IgG, followed by development with the SuperSignal West Pico Chemiluminescent reagent (Thermo Fisher Scientific). The same membrane was retrieved and reblotted with anti-STAT3, anti-AKT, anti-ERK, anti-JNK, and anti-p38MAPK antibodies (all from Cell Signaling Technology), respectively.

**Luciferase analysis.** C2C12 cells plated in DME were transfected with Lipofectamine (Invitrogen) according to the manufacturer's instructions. The APRE luciferase plasmid was provided by A. Yoshimura (Keio University, Shinjuku, Tokyo, Japan) and used at a dosage of 100 ng. The administered dosages of G-CSF were 37.5, 125.0, and 375.0 pg/ml. CMV-*Renilla* luciferase was used as an internal control to normalize for variations in transfection efficiency. All of the proteins were expressed at similar levels, as confirmed by Western blotting.

**Skeletal muscle injury model.** 10 µM cardiotoxin (*Naja mossambica mossambica*; Sigma-Aldrich) diluted in 100 µl PBS was injected into the rectus femoris muscles of BL6/J mice using a 27-gauge needle and a 1-ml syringe. The needle was inserted deep into the rectus femoris longitudinally to the knee. Cardiotoxin was injected along the length of the muscle. The mice in the control group were injected with 100 µl PBS. Mice (treated and control groups) were sacrificed at various time points after cardiotoxin injection, and blood samples (1.0–1.5 ml from each mouse) were collected in heparin-rinsed syringes.

**Handgrip strength testing.** 10 µM cardiotoxin (Sigma-Aldrich) diluted in 100 µl PBS was injected into forearm muscles of BL6/J mice. Five training sessions were performed during which the animals were held, facing the bar of the grip strength meter (Muromachi Kikai), while the forearm was gently restrained by the experimenter. When the unrestrained forepaw is brought into contact with the bar of the grip strength meter, the animal grasps the bar, after which the animal is gently pulled away from the device. The grip strength meter measures the maximal force applied before the animal released the bar.

**BM transplantation.** BM cells were harvested from 8-wk-old enhanced GFP (EGFP)–transgenic mice. After irradiation with a single dose of 9.0 Gy, the unfractionated $EGFP^+$ BM cells ($1 \times 10^6$ cells) were injected via the tail vein, as described previously (Kawada et al., 2006). To assess chimerism, peripheral blood cells were collected from the recipient mice 60 d after BM transplantation, and the frequency of $EGFP^+$ cells in the population of peripheral nucleated blood cells was determined in a FACS sorter (BD) after hemolysis was induced with ammonium chloride to eliminate erythrocytes.

**Statistical analysis.** The data were analyzed using the StatView J-4.5 software (SAS Institute, Inc.). Values are reported as means ± SD. Comparisons among groups were performed by one-way analysis of variance. Scheffe's F test was used to determine the level of significance. The probability level accepted for significance was $P < 0.05$.

**Online supplemental material.** Fig. S1 shows the effect of G-CSF on myoblast differentiation in C2C12 cells and myoblasts harvested from $csf3r^{+/+}$ and wild-type mice. Fig. S2 shows histological analysis of cardiotoxin-injured skeletal muscle from day 1 to 28. Fig. S3 shows the chimerism of hematopoietic cells before and after BM cell transplantation and quantitative analysis of the areas of the skeletal myocyte sections in the $csf3r^{-/-}$ and $csf3r^{+/+}$ mice with BM transplantation. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20101059/DC1.

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**Comparison Between Valsartan and Amlodipine Regarding Cardiovascular Morbidity and Mortality in Hypertensive Patients With Glucose Intolerance : NAGOYA HEART Study**

Takashi Muramatsu, Kunihiro Matsushita, Kentaro Yamashita, Takahisa Kondo, Kengo Maeda, Satoshi Shintani, Satoshi Ichimiya, Miyoshi Ohno, Takahito Sone, Nobuo Ikeda, Masato Watarai, Toyoaki Murohara and for the NAGOYA HEART Study Investigators

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# Comparison Between Valsartan and Amlodipine Regarding Cardiovascular Morbidity and Mortality in Hypertensive Patients With Glucose Intolerance

## NAGOYA HEART Study

Takashi Muramatsu, Kunihiro Matsushita, Kentaro Yamashita, Takahisa Kondo, Kengo Maeda, Satoshi Shintani, Satoshi Ichimiya, Miyoshi Ohno, Takahito Sone, Nobuo Ikeda, Masato Watarai, Toyoaki Murohara, for the NAGOYA HEART Study Investigators

***Abstract***—It has not been fully examined whether angiotensin II receptor blocker is superior to calcium channel blocker to reduce cardiovascular events in hypertensive patients with glucose intolerance. A prospective, open-labeled, randomized, controlled trial was conducted for Japanese hypertensive patients with type 2 diabetes mellitus or impaired glucose tolerance. A total of 1150 patients (women: 34%; mean age: 63 years; diabetes mellitus: 82%) were randomly assigned to receive either valsartan- or amlodipine-based antihypertensive treatment. Primary outcome was a composite of acute myocardial infarction, stroke, coronary revascularization, admission attributed to heart failure, or sudden cardiac death. Blood pressure was 145/82 and 144/81 mm Hg, and glycosylated hemoglobin was 7.0% and 6.9% at baseline in the valsartan group and the amlodipine group, respectively. Both of them were equally controlled between the 2 groups during the study. The median follow-up period was 3.2 years, and primary outcome had occurred in 54 patients in the valsartan group and 56 in the amlodipine group (hazard ratio: 0.97 [95% CI: 0.66–1.40]; $P=0.85$). Patients in the valsartan group had a significantly lower incidence of heart failure than in the amlodipine group (hazard ratio: 0.20 [95% CI: 0.06–0.69]; $P=0.01$). Other components and all-cause mortality were not significantly different between the 2 groups. Composite cardiovascular outcomes were comparable between the valsartan- and amlodipine-based treatments in Japanese hypertensive patients with glucose intolerance. Admission because of heart failure was significantly less in the valsartan group. **(*Hypertension.* 2012;59:580-586.)** • **Online Data Supplement**

**Key Words:** angiotensin II type 1 receptor blocker ■ calcium channel blocker ■ cardiovascular disease ■ diabetes mellitus ■ hypertension ■ impaired glucose tolerance

Hypertension and type 2 diabetes mellitus (T2DM) are major risk factors for cardiovascular diseases (CVDs), and a combination of those further increases CVD.[1–3] Activation of the renin-angiotensin system exacerbates not only hypertension but also insulin resistance and diabetic vascular complications.[4–6] Indeed, various renin-angiotensin system blockers (ie, angiotensin-converting enzyme inhibitor [ACEI] or angiotensin II type 1 receptor blocker [ARB]) have been shown to suppress new onset of T2DM and to reduce the progression of diabetic nephropathy.[7–10] Hence, many guidelines worldwide recommend ACEI/ARB as the first-line antihypertensive medications for diabetic hypertensive patients.[3,11–14]

Several clinical trials previously assessed head-to-head comparisons between ACEI/ARB and calcium channel blocker (CCB) regarding the efficacies on CVD.[15–21] In diabetic hypertensive patients, some small-sample trials showed that ACEI significantly reduced the risk of CVD compared with CCB,[15,16] whereas another large-scale trial showed no difference.[17] The Valsartan Antihypertensive Long-Term Use Evaluation (VALUE) and Candesartan Antihypertensive Survival Evaluation in Japan (CASE-J) trials, which recruited 5250 (34%) and 2018 patients (43%) with T2DM, respectively,[18,19] showed that ARB significantly reduced new onset of T2DM but failed to reduce total CVD compared with CCB. The Irbesartan Diabetic Nephropathy Trial (IDNT) also compared ARB and CCB in

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diabetic patients with nephropathy.[20,21] Although the IDNT revealed better renal protection by ARB than CCB as a primary outcome,[20] ARB and CCB had similar efficacies on composite CVD as a secondary outcome.[21] Among the components, ARB was more protective against heart failure (HF), whereas CCB tended to be more protective against myocardial infarction (MI) and stroke.

Epidemiologically, CV events in East Asia are different from those in Western countries. Age-adjusted incidence of ischemic heart disease is ≈80% lower, but cerebrovascular mortality is 2- to 3-fold higher in Japan compared with those in the United States.[22,23] In addition, mean body mass indices in East Asians are lower than that of Western population.[24] ARB is less protective against new onset of T2DM in lean patients compared with the obese,[25] and CCB has a beneficial property for preventing stroke.[26] Thus, CCBs are still frequently used in hypertensive patients with T2DM in East Asia.

Taken together, it is still unknown whether ACEI/ARB should be the first-line medication for diabetic hypertensive patients in East Asia for the CVD protection. Accordingly, we carried out the NAGOYA HEART Study (NHS) to compare the efficacies of an ARB valsartan and a CCB amlodipine on cardiovascular morbidity and mortality as a primary outcome in Japanese hypertensive patients with glucose intolerance.

## Materials and Methods

### Study Design

The rationale and design of the NHS have been described previously.[27] The NHS is an investigator-initiated trial which used a prospective, randomized, open-labeled, blinded endpoints design.[28] Participants were recruited by 171 cardiologists only from 46 board-certified medical centers and hospitals. All of the patients provided their written informed consent. This study was approved by the ethical review committee of the Nagoya University School of Medicine and of participating institutions.

### Inclusion Criteria

Eligible participants were men and women aged between 30 and 75 years with both hypertension and glucose intolerance (ie, T2DM or impaired glucose tolerance [IGT]). We enrolled hypertensive patients with not only T2DM but also IGT, because IGT has a similarly elevated risk for CVD compared with T2DM.[29,30] Hypertension was defined as having received any antihypertensive drugs already or blood pressure ≥140/90 mm Hg. T2DM was defined as having received any antidiabetic agents or plasma glucose level ≥7.0 mmol/L in fasting state, ≥11.1 mmol/L in nonfasting state, or 2 hours after glycemic load in an oral glucose tolerance test. IGT was defined by plasma glucose level <7.0 mmol/L in fasting state and 7.8 to 11.0 mmol/L as the 2-hour value in an oral glucose tolerance test.[31] For exclusion criteria, please see the online-only Data Supplement.

### Study Outcomes Measure

Primary outcome was a composite of acute MI (ECG changes, elevation of cardiac enzymes more than twice as high as upper limit of normal levels, and culprit lesion detected by coronary angiogram), stroke (neurological deficit persisting for >24 hours and relevant findings in computed tomography or MRI), admission because of HF (new or worsening typical clinical symptoms including dyspnea, shortness of breath, and peripheral edema, together with pulmonary congestion in chest roentgenogram, echocardiographic left ventricular dysfunction according to the guidelines of the American Heart Association/American College of Cardiology, and increased plasma brain natriuretic peptide levels), coronary revascularization (percutaneous coronary intervention or coronary bypass graft surgery unplanned at randomization), or sudden cardiac death (unexpected intrinsic death within 24 hours after the onset of symptoms). All-cause mortality was included as the secondary outcome. All of the reported adverse events were analyzed, and outcomes were strictly adjudicated by an independent End point Evaluation Committee in a blinded manner as for the assigned treatments.

### Procedures and Follow-Up

Patients were randomly assigned to the valsartan- or the amlodipine-based treatment group. Random allocation was performed by a minimization method with 5 factors of baseline characteristics, such as age, sex, medication for dyslipidemia, current smoking status, and the T2DM/IGT ratio.

As an initial dose, either valsartan 80 mg or amlodipine 5 mg once daily was administered to patients in a respective group. For patients already taking antihypertensive drugs at the enrollment, all of the ACEI/ARB and CCB were once discontinued and the allocated drug was started without a run-in period. During the follow-up, target blood pressure was ≤130/80 mm Hg.[3,11–14] Physicians could increase the respective dose until 160 mg or 10 mg daily after 4 weeks, and other antihypertensive drugs, such as diuretics, β-blockers, or α-blockers could be added after 8 weeks as needed. Blood glucose control was performed according to the treatment guidelines issued from the Japan Diabetes Society.[32] For additional information, please see the online-only Data Supplement.

### Sample

Sample size calculation was described previously.[27] Patient enrollment began in October 2004 on the assumption that 1500 patients for each group were enrolled and anticipated to be finished by the end of 2006. Finally, we recruited total 1168 patients by the end of January 2009, when the Data and Safety Monitoring Board suggested stopping the recruitment because a longer recruitment period might be required to complete the enrollment. Consequently, the steering committee decided not to recruit patients thereafter. Follow-up was continued until July 31, 2010, and available data were fixed on November 5, 2010.

### Interim Analyses and Data Monitoring

The interim analyses were assessed immediately after closing the enrollment and every 6 months (4 times in total). For additional information, please see the online-only Data Supplement.

### Statistical Analysis

Data were analyzed on the basis of the intention-to-treat principle. Only the first cardiovascular event was analyzed as a primary outcome in case of multiple events observed in a single patient. Cumulative incidence of cardiovascular events was estimated by the Kaplan-Meier method. The crude hazard ratios (HRs) and 95% CIs were calculated by the Cox proportional hazard model to compare the treatment group differences. The Levene test and repeated-measure ANOVA were used to compare the changes of blood pressure and glycosylated hemoglobin (HbA1c) levels throughout the follow-up. All of the statistical analyses were performed by an independent statistical analysis board, and a $P$ value <0.05 was considered statistically significant.

## Results

### Patient Characteristics

A total of 1168 patients were considered to be eligible for the present study, but 6 patients who met exclusion criteria and 12 patients who withdrew their consent were excluded. Consequently, 1150 patients were randomly assigned to the valsartan group (n=575) or the amlodipine group (n=575), and a total of 1117 patients (97%) completed the follow-up throughout the study (Figure S1). Please see the online-only Data Supplement.

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**Table 1. Baseline Characteristics of Enrolled Patients**

| Variables | Valsartan (n=575) | Amlodipine (n=575) | *P* |
|---|---|---|---|
| Age, y | 63 (8) | 63 (8) | 0.62 |
| Women, n (%) | 197 (34) | 199 (34) | 0.90 |
| Body mass index, kg/m$^2$ | 25 (4) | 25 (4) | 0.21 |
| Current smoker, n (%) | 106 (18) | 104 (18) | 0.88 |
| Dyslipidemia, n (%) | 245 (43) | 253 (44) | 0.63 |
| Previous cardiovascular diseases, n (%) | 150 (26) | 156 (27) | 0.69 |
| Previous cerebrovascular diseases, n (%) | 24 (4) | 30 (5) | 0.40 |
| Systolic blood pressure, mm Hg | 145 (18) | 144 (19) | 0.44 |
| Diastolic blood pressure, mm Hg | 82 (13) | 81 (13) | 0.71 |
| Heart rates, bpm | 70 (11) | 71 (12) | 0.31 |
| Antihypertensive use, n (%) | 327 (57) | 327 (57) | 1.00 |
| Treatment for hypertension, n (%) | | | |
| Angiotensin II receptor blockers | 171 (30) | 168 (29) | 0.85 |
| Angiotensin converting enzyme inhibitors | 54 (9) | 44 (8) | 0.29 |
| Calcium channel blockers | 258 (45) | 275 (48) | 0.32 |
| $\beta$-blockers | 125 (22) | 147 (26) | 0.13 |
| $\alpha$-blockers | 12 (2) | 17 (3) | 0.35 |
| Antialdosterone agents | 15 (3) | 10 (2) | 0.31 |
| Thiazides | 17 (3) | 13 (2) | 0.46 |
| Other diuretics | 20 (4) | 25 (4) | 0.45 |
| Status of glucose intolerance, n (%) | | | |
| Type 2 diabetes mellitus | 470 (82) | 472 (82) | 0.88 |
| Impaired glucose tolerance | 105 (18) | 103 (18) | |
| Treatment for glucose intolerance, n (%) | | | |
| Sulfonylurea | 141 (25) | 134 (23) | 0.58 |
| Insulin | 40 (7) | 36 (6) | 0.64 |
| Others | 196 (34) | 198 (34) | 0.80 |
| Other medication, n (%) | | | |
| Aspirin | 157 (27) | 162 (28) | 0.74 |
| Statins | 227 (40) | 217 (38) | 0.55 |
| Laboratory measurements | | | |
| Glycosylated hemoglobin, %* | 7.0 (1.4) | 6.9 (1.1) | 0.08 |
| Fasting plasma glucose, mmol/L | 8.2 (3.0) | 7.9 (2.6) | 0.08 |
| Triglycerides, mmol/L | 1.7 (1.1–2.3) | 1.6 (1.2–2.3) | 0.76 |
| HDL cholesterol, mmol/L | 1.6 (0.4) | 1.6 (0.4) | 0.49 |
| LDL cholesterol, mmol/L | 3.5 (1.0) | 3.6 (1.0) | 0.12 |
| Uric acid, $\mu$mol/L | 328 (83) | 334 (84) | 0.28 |
| Blood urea nitrogen, mmol/L | 5.6 (1.5) | 5.6 (1.6) | 0.79 |
| Serum creatinine, $\mu$mol/L | 60 (18) | 60 (17) | 0.71 |

HDL indicates high-density lipoprotein; LDL, low-density lipoprotein. Continuous variables are shown as mean (SD) or median (interquartile range).

*Presented as National Glycohemoglobin Standardization Program value.

Baseline characteristics of enrolled patients are shown in Table 1. All of the patients were diagnosed as hypertensive, and 82% and 18% had T2DM and IGT, respectively. In overall samples, mean age was 63 years, and 66% were men. Mean blood pressure and HbA1c were 145/82 mm Hg and 7.0%, respectively. There were no significant differences in baseline characteristics between the 2 groups. Table 2 shows the prescribed medications during the follow-up. Immediately after the random allocation, 77% of patients in both groups were prescribed only the assigned regimen as an antihypertensive medication. The concomitant antihypertensive drugs were mainly $\beta$-blockers in both groups. Antihypertensive and hypoglycemic agents showed no differences in use between the 2 groups.

### Blood Pressure Changes and HbA1c Levels During the Study

Changes in blood pressure and HbA1c levels are shown in Figure 1. Blood pressure was reduced to 131/73 mm Hg in the valsartan group and 132/74 mm Hg in the amlodipine group at 54 months. The Levene test for equality of variances showed no differences between the 2 groups. Blood pressure did not differ between the 2 groups throughout the trial ($P$=0.653 in systolic BP and $P$=0.658 in diastolic BP by repeated-measure ANOVA). HbA1c levels were shown to decrease steadily to 6.7% in both groups, and the changes did not differ between the 2 groups.

### Clinical Outcomes

The median follow-up period reached 3.2 years (interquartile range: 2.6–4.7 years), and the Data and Safety Monitoring Board suggested finishing the follow-up at that point. A total of 202 clinical adverse events from 148 patients (26%) in the valsartan group and 204 events from 162 patients (28%) in amlodipine group were reported to the end point evaluation committee.

Figure 2 shows the incidence of adjudicated primary composite cardiovascular outcomes. A total of 56 events from 54 patients (9.4%) in the valsartan group and 64 events from 56 patients (9.7%) in the amlodipine group were adjudicated as primary outcomes, and time-to-event curves did not significantly differ between the 2 groups (HR: 0.97 [95% CI: 0.66–1.40]; $P$=0.85). Table 3 shows HRs for each component ascertained in this study, and there were no significant differences in the risk of MI, stroke, coronary revascularization, or sudden cardiac death between the 2 groups. However, incidence of admission because of HF was significantly less in the valsartan group than in the amlodipine group (3 versus 15 patients; HR: 0.20 [95% CI: 0.06–0.69]; $P$=0.012). Figure S2 shows the time-dependent curves of the incidence of admission attributed to worsening of HF. Please see the online-only Data Supplement. All-cause mortality, as a secondary outcome, did not significantly differ between the 2 groups (22 versus 16 patients; HR: 1.37 [95% CI: 0.72–2.61]; $P$=0.34; Table 3).

### Adverse Events

With respect to the safety outcome, we confirmed 106 adverse events of 94 patients in the valsartan group and 112 events of 94 patients in the amlodipine group during the follow-up (Table S1). However, any serious adverse events were not observed. There were no significant differences in the incidence of each adverse event, including the definite solid cancer (22 in the valsartan group and 23 in the amlodipine group) between the 2 groups. Please see the online-only Data Supplement.

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**Table 2. Prescribed Medications During the Follow-Up**

| Medications | After Allocation | | Month 12 | | Month 24 | | Month 36 | | Month 48 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Valsartan (n=575) | Amlodipine (n=575) | Valsartan (n=549) | Amlodipine (n=555) | Valsartan (n=492) | Amlodipine (n=493) | Valsartan (n=343) | Amlodipine (n=336) | Valsartan (n=206) | Amlodipine (n=197) |
| Concomitant antihypertensives | | | | | | | | | | |
| β-blockers | 107 (19) | 110 (19) | 138 (25) | 142 (26) | 126 (26) | 134 (27) | 83 (24) | 98 (29) | 48 (23) | 57 (29) |
| α-blockers | 13 (2) | 14 (2) | 36 (7) | 18 (3) | 33 (7) | 19 (4) | 20 (6) | 13 (4) | 10 (5) | 10 (5) |
| Aldosterone blockers | 14 (2) | 8 (1) | 14 (3) | 15 (3) | 10 (2) | 14 (3) | 10 (3) | 8 (2) | 5 (2) | 4 (2) |
| Thiazides | 15 (3) | 5 (1) | 64 (12) | 24 (4) | 68 (14) | 29 (6) | 57 (17) | 26 (8) | 33 (16) | 17 (9) |
| Other diuretics | 19 (3) | 22 (4) | 23 (4) | 24 (4) | 23 (5) | 25 (5) | 12 (4) | 18 (5) | 8 (4) | 12 (6) |
| Discontinuation | 14 (2) | 15 (3) | 77 (14) | 66 (12) | 65 (13) | 65 (13) | 62 (18) | 49 (15) | 40 (19) | 35 (18) |
| Monotherapy | 441 (77) | 442 (77) | 328 (60) | 347 (63) | 280 (57) | 286 (58) | 185 (54) | 180 (54) | 108 (52) | 113 (57) |
| Hypoglycemic agents | | | | | | | | | | |
| Sulfonylurea | 141 (25) | 133 (23) | 133 (24) | 126 (23) | 118 (24) | 127 (26) | 89 (26) | 82 (24) | 43 (21) | 52 (26) |
| Insulin | 40 (7) | 37 (6) | 30 (6) | 28 (5) | 24 (5) | 23 (5) | 14 (4) | 14 (4) | 7 (3) | 10 (5) |
| Other medications | 216 (38) | 211 (37) | 241 (44) | 252 (45) | 229 (47) | 252 (51) | 201 (59) | 208 (62) | 102 (50) | 123 (62) |
| Other medication | | | | | | | | | | |
| Aspirin | 157 (27) | 162 (28) | 149 (27) | 160 (29) | 136 (28) | 153 (31) | 89 (26) | 94 (28) | 44 (21) | 59 (30) |
| Statins | 227 (40) | 217 (38) | 230 (42) | 231 (42) | 220 (45) | 225 (46) | 148 (43) | 159 (47) | 90 (44) | 91 (46) |

Data are presented as n (%).

## Discussion

The NHS is a randomized, prospective clinical trial comparing the efficacies on cardiovascular outcomes between ARB and CCB in hypertensive patients with glucose intolerance. The present study has a novelty to evaluate the cardiovascular events as a primary outcome exclusively in non-Western patients with glucose intolerance. East Asians are generally less obese than Western population, although they had similar prevalence of T2DM.[24] This epidemiological data suggest that East Asians could have some different substrates in glucose intolerance. Furthermore, the incidence of CVD in East Asia is much different from Western countries. However, there was little clinical evidence that supports therapeutic guidelines for the treatment of diabetic hypertensive patients in East Asia. In this study, both blood pressure and glycemic status were equally controlled between the 2 treatment groups, and there was no difference in a primary composite cardiovascular outcome. Our result was generally in line with the data of the Western IDNT Trial for which the number of patients were almost similar to ours, whereas the cardiovascular outcome was measured as a secondary outcome.[21] The VALUE and CASE-J trials enrolled a larger number of diabetic patients than ours, but they also showed no difference in composite cardiovascular outcomes between the ARB- and CCB-based treatments.[18,19] Consequently, any evidence in clinical advantage of ARB against CCB regarding composite CVD has not been yielded regardless of the race.

In the present study, the valsartan-based treatment significantly reduced the risk of HF as compared with the amlodipine-based treatment. One may argue that more frequent use of thiazides in the valsartan group possibly attributed to better protection against HF. However, this effect on HF in valsartan-

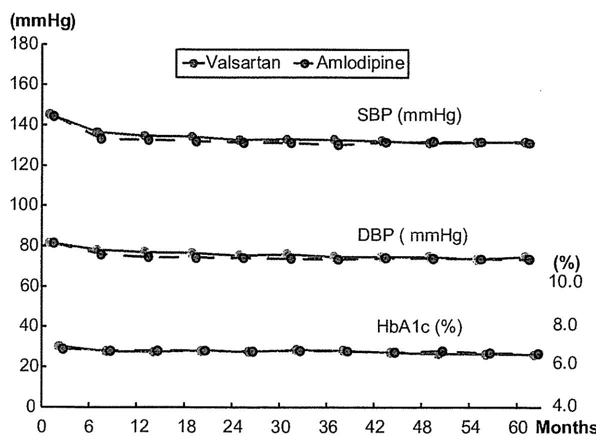


**Figure 1.** Changes in blood pressure and glycemic control throughout the study. SBP indicates systolic blood pressure; DBP, diastolic blood pressure.

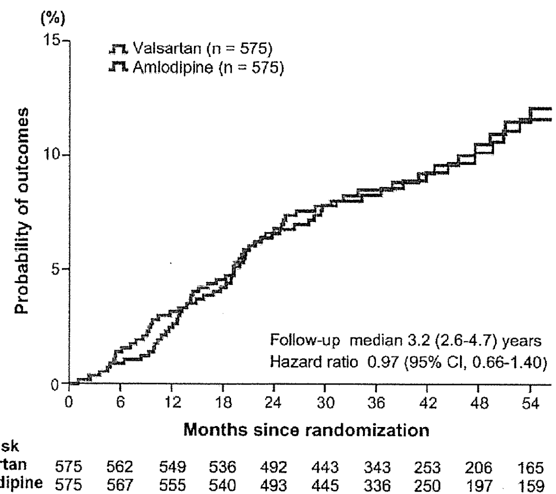


**Figure 2.** Kaplan-Meier curves for the incidence of primary composite outcome. Time to the first cardiovascular event was used for the analysis.

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**Table 3. Primary Outcome and Overall Cardiovascular Event**

| | Valsartan Group (n=575) | | Amlodipine Group (n=575) | | | |
|---|---|---|---|---|---|---|
| Outcomes | n of Events (%) | Incident Rate | n of Events (%) | Incident Rate | HR (95% CI) | *P* |
| Primary outcome | | | | | | |
| Composite cardiovascular event | 54 (9.4) | 27.4 | 56 (9.7) | 28.5 | 0.97 (0.66–1.40) | 0.85 |
| Components | | | | | | |
| Acute myocardial infarction | 7 (1.2) | 3.6 | 3 (0.5) | 1.5 | 2.33 (0.60–9.01) | 0.22 |
| Stroke | 13 (2.3) | 6.6 | 16 (2.8) | 8.1 | 0.81 (0.39–1.68) | 0.57 |
| Ischemic stroke | 10 (1.7) | 5.1 | 11 (1.9) | 5.6 | 0.90 (0.38–2.12) | 0.81 |
| Intracerebral hemorrhage | 2 (0.3) | 1.0 | 4 (0.7) | 2.0 | 0.50 (0.09–2.74) | 0.43 |
| Subarachnoid hemorrhage | 1 (0.2) | 0.5 | 1 (0.2) | 0.5 | 1.00 (0.06–16.1) | 0.997 |
| Coronary revascularization | 29 (5.0) | 14.7 | 26 (4.5) | 13.2 | 1.12 (0.66–1.90) | 0.68 |
| Admission because of heart failure | 3 (0.5) | 1.5 | 15 (2.6) | 7.6 | 0.20 (0.06–0.69) | 0.012 |
| Sudden cardiac death | 4 (0.7) | 2.0 | 4 (0.7) | 2.0 | 1.00 (0.25–3.99) | 0.997 |
| Secondary outcome | | | | | | |
| All-cause death | 22 (3.8) | 11.2 | 16 (2.8) | 8.1 | 1.37 (0.72–2.61) | 0.34 |

HR indicates hazard ratio. Incident rates are presented as per 1000 patient-years.

based treatment was statistically significant even in those who never received thiazides throughout the study (n=967; HR: 0.21; [95% CI: 0.05–0.96]; $P$=0.04), and test of heterogeneity in thiazides use showed no statistical significance ($P$=0.50). Thus, our study suggested the more protective efficacy of valsartan against HF regardless of the use of thiazides, and our findings confirm the results of the IDNT Trial, as well as a meta-analysis in diabetic patients in Western countries.[21,33] In contrast, both the original VALUE Trial and CASE-J Trial showed significant difference of blood pressure throughout the follow-up and no difference in the risk of HF.[18,19] However, a modified analysis of the VALUE Trial indicated that, when blood pressure effects of valsartan and amlodipine were adjusted equally, the only difference in outcomes between the 2 groups was a lower incidence of HF in the valsartan group.[34] Our study showed considerably lower HR (0.20) for HF than previous trials because of the small number of cases, whereas there may be several possible explanations for this finding. First, given the fact that T2DM leads to renal damage and sodium retention, our patients could be more likely to develop HF than those with hypertension only.[5] Indeed, a subanalysis of the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial suggested that ACEI was more protective against HF compared with CCB in diabetic patients,[17] and a meta-analysis in diabetic patients showed that ARB showed a greater protection against HF than other antihypertensive drugs.[33] In addition, the progression of insulin resistance and diabetic vascular complications are enhanced by the renin-angiotensin system.[5] It is then conceivable that the complicated glucose intolerance unmasked the beneficial effect of ARB compared with hypertension alone. Second, our study patients are all Japanese. A subanalysis of the Reduction of Endpoints in Non–Insulin-Dependent Diabetes Mellitus With the Angiotensin II Antagonist Losartan Study showed that ARB was particularly effective against diabetic nephropathy in Japanese patients compared with other ethnicities.[35] Taken together, ARB may have a greater potential to reduce the risk of HF compared with CCB, especially in Japanese hypertensive patients with glucose intolerance.

We showed that CCB had a tendency to reduce the risk of MI compared with ARB similar to the IDNT findings.[21] The VALUE Trial demonstrated that MI was significantly lower in the CCB group compared with the ARB group.[18] However, it requires careful interpretation, because this finding might be yielded by the difference in blood pressure control (≈4/2 mm Hg) during the follow-up in the VALUE Trial. However, when the effect of blood pressure was adjusted, the difference of MI risk disappeared.[34] Furthermore, an updated meta-analysis demonstrated that ARB and CCB showed equivalent MI risk.[36]

In the risk of stroke, we found no difference between ARB and CCB. This finding is also in line with a previous meta-analysis in diabetic patients[33] and head-to-head comparison trials not only in Asians[19] but also in whites.[18,21] However, it is quite in contrast to the 2 recent Japanese trials, the JIKEI HEART and KYOTO HEART Studies that clearly showed reduced risk of stroke in the add-on group with valsartan compared with the non-ARB group, who mainly received CCB.[37,38] In these 2 trials, >50% of patients in the ARB add-on group also received CCB, so that these trials might suggest the superiority of combined therapy with ARB and CCB against the control group (mainly CCB-based without ARB). Thus, the discrepancy between the NHS and the JIKEI/KYOTO HEART Studies may partially be explained by the difference in the concomitant medications and the study design.

We applied the prospective, randomized, open-labeled, blinded endpoints method to assess outcomes. The prospective, randomized, open-labeled, blinded endpoints design is relatively vulnerable to reporting bias, because allocated drugs are open to both patients and physicians. In this regard, softer end points should be adjudicated with a special care. In the present study, an independent clinical research nurse coordinator group managed to follow up patient records and to collect the data in >90% of patients, and all of the reported adverse events were strictly adjudicated by an independent end point evaluation committee under a blinded manner as to the drug assignment. In fact, among 386 provisional reports, only 120 (31.1%) were adjudi-

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cated as the primary end point by the committee. Therefore, we believe that the bias would be highly unlikely to account for the differences. Rather, prospective, randomized, open-labeled, blinded endpoints design is close to daily clinical practice and less stressful to patients.[28]

Our patients were relatively well controlled in both blood pressure and glycemic status. A recent international cohort study reconfirmed that the incidence of CVD was significantly lower in Japan compared with other countries.[39] These underlying conditions might result in quite lower incidence of primary outcomes (3.1% per year) than we anticipated. In addition, the sample size (n=1150) was less than the initially planned number of samples (n=3000), and that the present study was underpowered to determine our initial hypothesis that ARB might be more effective in preventing major CV events than CCB. However, postcensored analysis indicated acceptable statistical power (84.9%), the risk of primary outcome in each group was almost even (HR: 0.97), and our results were consistent with previous clinical evidence.[18,19,21,33,34]

### Perspectives

The NHS is the first randomized, controlled trial comparing the clinical efficacies of ARB and CCB in Japanese hypertensive patients with glucose intolerance. Composite major CV events were similarly observed between ARB-based and CCB-based antihypertensive treatment. However, HF was more significantly reduced by ARB regimen. The NHS results echo those of the IDNT CV event trial and confirm the efficacy of ARB in this patient population in diabetic hypertensive patients in East Asia. Our results will highlight the safety and efficacy of ARB and support the current therapeutic guidelines for the treatment of diabetic hypertensive patients.

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# Online Supplement

## Comparison between valsartan and amlodipine regarding cardiovascular morbidity and mortality in hypertensive patients with glucose intolerance: NAGOYA HEART Study

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**Expanded Materials and Methods**

**Exclusion criteria**

Patients with the following conditions were excluded from the study: prior CVD within 6 months; taking CCB for angina pectoris; left ventricular ejection fraction < 40%; atrioventricular block; secondary or severe hypertension ( ≥ 200/110 mmHg); serum creatinine ≥ 221 μmol/L; pregnant women; life expectancy less than 3 years; or other conditions for which physicians judged it inappropriate to enroll patients.

**Procedures and follow up**

Patients were followed up every month in the first 3 months and then every 1 to 3 months. Pre-specified measurements, prescribed medications, and clinical events were reported to the Data Management Center every 6 months. We also made clinical research coordinators (CRC) visit regularly to collect and reconfirm the reported data. Even when patients had stopped visiting institutions, CRC checked up their health status by either letter or phone call. Finally, the CRC group managed 40/46 institutions (87%) and 1043/1150 patients (91%).

**Interim analyses and data monitoring**

The interim analyses were assessed immediately after closing the enrollment and every 6 months (four times in total). The Data and Safety Monitoring Board (DSMB) independently monitored every updated result and suggested to continue or to close this study. The prespecified conditions to close this study were as follows: the difference of effects in the two treatment groups were shown to be statistically significant (O'Brien-Fleming stopping boundary); the number of incident CVD reached more than we anticipated (321 cases in total); median follow-up period reached more than 3 years; or any serious adverse events (grade 3 or more) that might threaten the safety of study continuation were observed.

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**Table S1. Adverse events in safety analysis (n of cases ≥ 2)**

| Adverse events | Valsartan group (n = 575) | Amlodipine group (n = 575) |
|---|---|---|
| Solid cancer | 22 | 23 |
| Dizziness | 14 | 10 |
| Liver dysfunction | 4 | 5 |
| Aortic aneurysm | 4 | 4 |
| Headache | 3 | 5 |
| Rashes / Zoster | 4 | 2 |
| Benign tumor | 3 | 3 |
| Fracture | 2 | 2 |
| Face flush | 1 | 3 |
| Fatigue | 1 | 3 |
| Hyperkalemia | 3 | 0 |
| Atrioventricular block | 0 | 3 |
| Gastric ulcer | 0 | 3 |
| Pruritis | 0 | 3 |
| Dry cough | 1 | 1 |
| Angioedema | 0 | 2 |
| Gingival hypertrophy | 0 | 2 |
| Total events | 106 | 112 |

n indicates number.

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**Figure S1.**

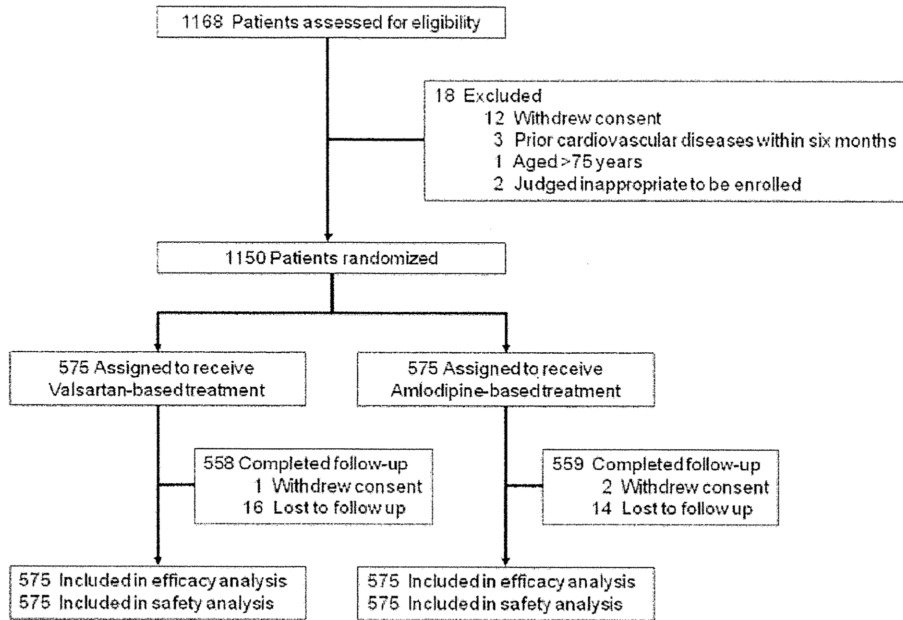


**Figure S1.** Flow diagram of the NAGOYA HEART Study (NHS).

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Figure S2.

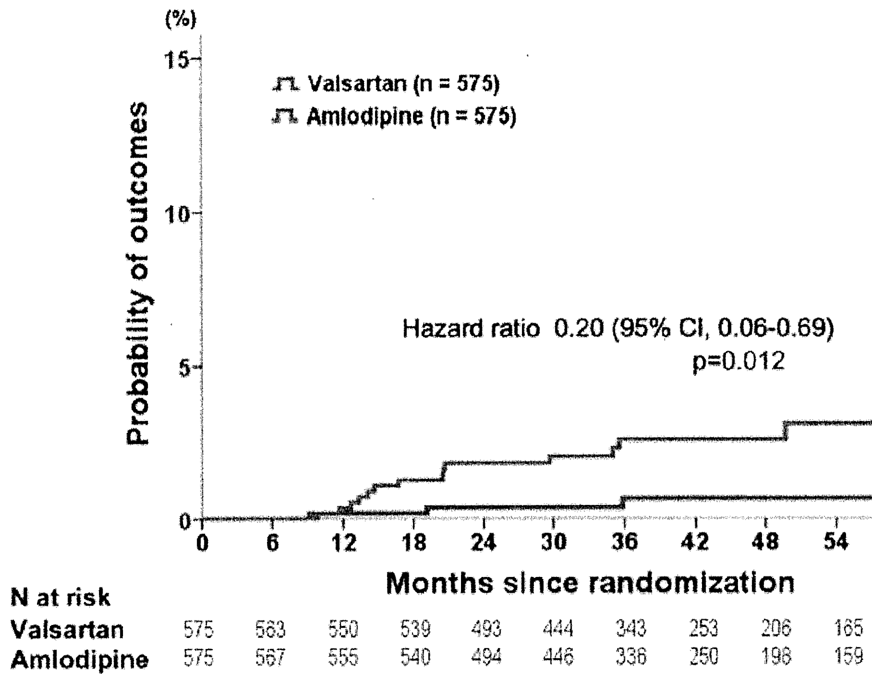


**Figure S2.** Kaplan-Meier curves for the incidence of admission due to worsening of heart failure. Time to the first event was used for the analysis. CI indicates confidence interval.

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# Gene and Protein Expression Analysis of Mesenchymal Stem Cells Derived From Rat Adipose Tissue and Bone Marrow

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***Background:*** Mesenchymal stem cells (MSC) are multipotent and reside in bone marrow (BM), adipose tissue and many other tissues. However, the molecular foundations underlying the differences in proliferation, differentiation potential and paracrine effects between adipose tissue-derived MSC (ASC) and BM-derived MSC (BM-MSC) are not well-known. Therefore, we investigated differences in the gene and secretory protein expressions of the 2 types of MSC.

***Methods and Results:*** ASC and BM-MSC were obtained from subcutaneous adipose tissue and BM of adult Lewis rats. ASC proliferated as rapidly as BM-MSC, and had expanded 200-fold in approximately 2 weeks. On microarray analysis of 31,099 genes, 571 (1.8%) were more highly (>3-fold) expressed in ASC, and a number of these genes were associated with mitosis and immune response. On the other hand, 571 genes (1.8%) were more highly expressed in BM-MSC, and some of these genes were associated with organ development and morphogenesis. In secretory protein analysis, ASC secreted significantly larger amounts of growth factor and inflammatory cytokines, such as vascular endothelial growth factor, hepatocyte growth factor and interleukin 6, whereas BM-MSC secreted significantly larger amounts of stromal-derived factor-1$\alpha$.

***Conclusions:*** There are significant differences between ASC and BM-MSC in the cytokine secretome, which may provide clues to the molecule mechanisms associated with tissue regeneration and alternative cell sources.

**Key Words:** Cell therapy; Mesenchymal stem cells; Microarray; Secretory protein

Mesenchymal stem cells (MSC) are multipotent cells that reside within various tissues, including bone marrow (BM), adipose tissue and many other tissues,[1,2] and can differentiate into a variety of cell types of mesodermal lineage.[1,3] MSC can be expanded in vitro over the short term, and they are thought to be an attractive tool for cell therapy. It has been demonstrated in animal and human studies of cardiovascular disease that transplanted BM-MSC induce neovascularization and differentiate into functional cells.[4–8] In addition, recent studies suggest that MSC exert tissue regeneration, secreting various kinds of angiogenic and cytoprotective factors.[6,9,10]

**Editorial p ????**

Subcutaneous adipose tissue can be harvested more safely and noninvasively than BM, and ASC have emerged as a possible alternative cell source to BM-MSC.[9,11] We and others have demonstrated that ASC transplantation induces neovascularization in animal models of myocardial infarction and hindlimb ischemia.[12,13] ASC are similar to BM-MSC in terms of morphology and surface marker expression.[14] However, few data exist regarding their differences in biological activity, such as proliferative activity, differentiation potential and productive ability. Using microarray and enzyme-linked immunosorbent

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assay (ELISA), we have performed a comprehensive analysis to evaluate both the differences between ASC and BM-MSC, and their usage as an effective transplanted cell source from the point of view of the gene and protein expression profile of the 2 MSC sources.

## Methods

### Isolation and Culture of ASC and BM-MSC

All protocols were performed in accordance with the guidelines of the Animal Care Committee of the National Cardiovascular Center Research Institute and Kanazawa University. MSC isolation and culture were performed according to previously described methods.[15] In brief, we harvested BM from male Lewis rats (Japan SLC, Hamamatsu, Japan) weighing 200–250 g by flushing their femoral cavities with phosphate-buffered saline. Subcutaneous adipose tissue was harvested from the inguinal region and minced with scissors, then digested with 0.1% type I collagenase (300 U/ml; Worthington Biochemical, Lakewood, NJ, USA) for 1 h at 37°C in a water-bath shaker. After filtration with 100-$\mu$m filter mesh (Cell Strainer; Becton Dickinson, Bedford, MA, USA) and centrifugation at 1,240 g for 5 min, MSC were cultured in complete culture medium: $\alpha$-minimal essential medium ($\alpha$-MEM; Invitrogen, Carlsbad, CA, USA), 10% fetal bovine serum (FBS, Invitrogen), 100 U/ml penicillin and 100 $\mu$g/ml streptomycin (Invitrogen). A small number of cells developed visible symmetric colonies by days 5–7. Nonadherent hematopoietic cells were removed, and the medium was replaced. The adherent, spindle-shaped MSC population expanded to >5×$10^7$ cells within 3–5 passages after the cells were first plated.

### Cell Proliferation

We compared the proliferative activity of ASC and BM-MSC in cell culture, as reported previously.[16] In brief, cells (3×$10^5$ cells/dish) at passage 1 were cultured in a 10-cm dish with complete culture medium, and harvested at 70–90% confluency at each passage. Cell number was counted with a hemocytometer (n=5).

### Differentiation of ASC and BM-MSC Into Adipocytes and Osteoblasts

MSC (1×$10^5$ cells/well) were seeded onto 12-well plates, and differentiation into adipocytes and osteocytes was induced when MSC were 70–80% confluent. MSC were cultured in $\alpha$-MEM with MSC osteogenesis supplements (Dainippon Sumitomo Pharma, Osaka, Japan) according to the manufacturer's instructions. After 14–17 days of differentiation, cells were fixed and stained with Alizarin Red S (Sigma-Aldrich, St Louis, MO, USA). To induce differentiation into adipocytes, MSC were cultured with adipocyte differentiation medium: 0.5 mmol/L 3-isobutyl-1-methylxanthine (Wako Pure Chemical Industries, Osaka, Japan), 1 $\mu$mol/L dexamethasone (Wako Pure Chemical Industries), 50 $\mu$mol/L indomethacin (Wako Pure Chemical Industries), and 10 $\mu$g/ml insulin (Sigma-Aldrich) in Dulbecco's modified Eagle medium (DMEM, Invitrogen) containing 10% FBS. After 21 days of differentiation, adipocytes were stained with Oil Red O (Sigma-Aldrich). In order to measure lipid accumulation, isopropyl alcohol was added to the stained culture plate, the extracted dye was immediately collected, and the absorbance was measured spectrophotometrically at 490 nm (Bio-Rad, Hercules, CA, USA).

### Microarray Analysis of ASC and BM-MSC

To compare the gene expression of ASC and BM-MSC, microarray analysis was performed according to previously reported methods.[17] Total RNA was extracted from cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was quantified by spectrometry, and its quality was confirmed by gel electrophoresis. Double-stranded cDNA was synthesized from 10 $\mu$g of total RNA, and in-vitro transcription was performed to produce biotin-labeled cRNA using GeneChip One-Cycle Target Labeling and Control Reagents (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. After fragmentation, 10 $\mu$g of cRNA was hybridized with a GeneChip Rat Genome 230 2.0 Array (Affymetrix) containing 31,099 genes. The GeneChips were then scanned in a GeneChip Scanner 3000 (Affymetrix). Normalization, filtering and Gene Ontology analysis of the data were performed with GeneSpring GX 7.3.1 software (Agilent Technologies, Palo Alto, CA, USA). The raw data from each array were normalized as follows: each CEL file was preprocessed with RMA, and each measurement for each gene was divided by the $80^{th}$ percentile of all measurements. Genes showing at least a 3-fold change were then selected.

### Quantitative Real-Time Reverse-Transcription–Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cultured BM-MSC and ASC as described, and 5 $\mu$g of total RNA was reverse-transcribed into cDNA using a QuantiTect reverse-transcription kit (Qiagen) according to the manufacturer's instructions. PCR amplification was performed in 50 $\mu$l containing 1 $\mu$l of cDNA and 25 $\mu$l of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, amplified from the same samples, served as an internal control. After an initial denaturation at 95°C for 10 min, a 2-step cycle procedure was used (denaturation at 95°C for 15s, annealing and extension at 60°C for 1 min) for 40 cycles in a 7700 sequence detector (Applied Biosystems). Gene expression levels were normalized according to that of GAPDH.

### ELISA

To investigate differences in protein secretion between ASC and BM-MSC, we measured the levels of various bioactive proteins, including proliferative and anti-apoptotic factors such as hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) and adrenomedullin (AM); chemokines such as stem cell-derived factor-1$\alpha$ (SDF-1$\alpha$); inflammatory cytokines such as tumor necrosis factor-$\alpha$ (TNF-$\alpha$) and interleukin-6 (IL-6); and adipokines such as leptin and plasminogen activator inhibitor-1 (PAI-1). Protein levels were measured in conditioned medium 24 h after medium replacement. MSC (1×$10^6$ cells/dish) were plated in 10-cm dishes and cultured in complete culture medium. After 24 h, conditioned medium (n=6) was collected and centrifuged at 2,000 g for 10 min, and the supernatant was filtered through a 0.22-$\mu$m filtration unit (Millipore, Bedford, MA, USA). Angiogenic and growth factors were measured by ELISA according to each of the manufacturer's instructions (VEGF, TNF-$\alpha$: R&D Systems, Minneapolis, MN, USA; HGF: Institute of Immunology, Tokyo, Japan; AM: Phoenix Pharmaceuticals, Burlingame, CA, USA; IL-6: Pierce, Rockford, IL, USA; adiponectin: AdipoGen, Seoul, Korea; PAI-1, Oxford Biomedica Reseach, Oxford, CT, USA).

### Statistical Analysis

Data are expressed as mean ± standard error of the mean. Comparisons of parameters among groups were made by 1-way ANOVA, followed by Newman-Keuls' test. Differences were

A B C D E F

**Figure 1.** Morphology and differentiation of adipose tissue-derived mesenchymal stem cells (ASC) and bone marrow-derived mesenchymal stem cells (BM-MSC). Cultured ASC (**A**) and BM-MSC (**B**) at passage 2 (×100). Their typical morphology is a similar spindle shape, but ASC are slightly longer than BM-MSC. ASC (**C**) and BM-MSC (**D**) differentiated into adipocytes (×100; Oil Red O). ASC (**E**) and BM-MSC (**F**) differentiated into osteoblasts (×100; Alizarin Red S).

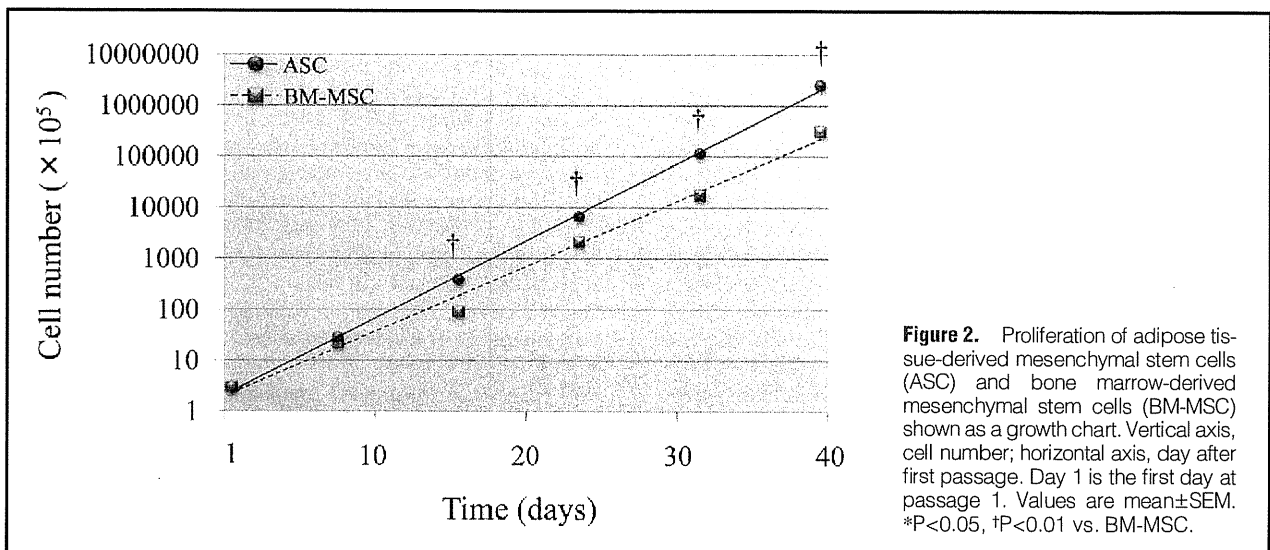


**Figure 2.** Proliferation of adipose tissue-derived mesenchymal stem cells (ASC) and bone marrow-derived mesenchymal stem cells (BM-MSC) shown as a growth chart. Vertical axis, cell number; horizontal axis, day after first passage. Day 1 is the first day at passage 1. Values are mean±SEM. *$P<0.05$, †$P<0.01$ vs. BM-MSC.

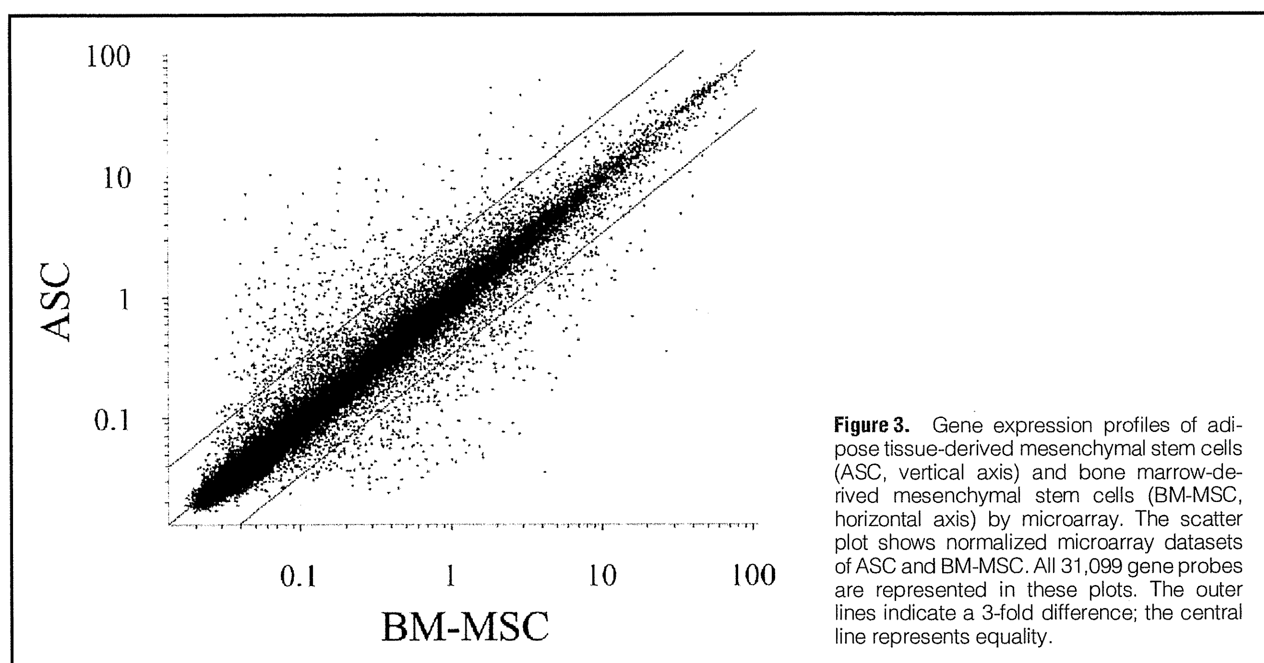


**Figure 3.** Gene expression profiles of adipose tissue-derived mesenchymal stem cells (ASC, vertical axis) and bone marrow-derived mesenchymal stem cells (BM-MSC, horizontal axis) by microarray. The scatter plot shows normalized microarray datasets of ASC and BM-MSC. All 31,099 gene probes are represented in these plots. The outer lines indicate a 3-fold difference; the central line represents equality.

**Table 1. Genes Upregulated in ASC in Comparison With BM-MSC (>10-Fold Upregulation)**

| Gene name | GenBank Acc. no. | Fold change |
|---|---|---|
| Interleukin 1α (Il1a) | NM017019 | 38.1 |
| Interleukin 1 receptor, type II (Il1r2) | NM053953 | 21.7 |
| Chemokine (C-X-C motif) ligand 1 (Cxcl1) | NM030845 | 21.6 |
| Lipocalin 2 (Lcn2) | NM130741 | 21.5 |
| Fast myosin alkali light chain (Rgd:620885) | NM020104 | 20.6 |
| Interleukin 6 (Il6) | NM012589 | 20.5 |
| Chemokine (C-C motif) ligand 20 (Ccl20) | AF053312 | 17.6 |
| Twist homolog 2 (Twist2) | NM021691 | 17.5 |
| RAS, dexamethasone-induced 1 (Rasd1) | AF239157 | 17.1 |
| Complement component 3 (C3) | NM016994 | 16.9 |
| NADPH oxidase 1 (Nox1) | NM053683 | 16.3 |
| Matrix metallopeptidase 9 (Mmp9) | NM031055 | 15.2 |
| Colony-stimulating factor 3 (Csf3) | NM017104 | 14.5 |
| Prostaglandin E synthase (Ptges) | AB048730 | 12.8 |
| Adenosine A2B receptor (Adora2b) | NM017161 | 12.5 |
| Oxidized low-density lipoprotein receptor 1 (Oldlr1) | NM133306 | 12.4 |
| Uterine sensitization-associated gene 1 protein (Sostdc1) | AA892798 | 12.1 |
| Chemokine (C-X-C motif) ligand 5 (Cxcl5) | NM022214 | 11.9 |
| Neuregulin 1 (Nrg1) | U02315 | 11.8 |
| CD24 antigen (Cd24) | BI285141 | 11.6 |
| Cathepsin c (Ctsc) | AA858815 | 11.2 |
| Lymphocyte antigen 68 (C1qr1) | BI282932 | 11.2 |
| Interleukin 1 receptor antagonist (Il1rn) | NM022194 | 11.1 |
| Chemokine (C-C motif) ligand 2 (Ccl2) | NM031530 | 10.8 |

ASC, adipose tissue-derived mesenchymal stem cells; BM-MSC, bone marrow-derived mesenchymal stem cells.

considered significant at $P<0.05$.

## Results

### Proliferation and Differentiation of ASC and BM-MSC

Both ASC and BM-MSC could be expanded on a plastic dish, and they exhibited a similar fibroblast-like morphology (**Figures 1A,B**). To examine the potential of ASC and BM-MSC to differentiate into adipocytes, the cells were cultured in adipogenesis medium for 21 days (**Figures 1C,D**). Although lipid droplets were not observed in undifferentiated ASC or BM-MSC, ASC and BM-MSC cultured in adipogenesis

Table 2. Genes Upregulated in BM-MSC in Comparison With ASC (>10-Fold Upregulation)

| Gene name | GenBank Acc. no. | Fold change |
|---|---|---|
| WNT1 inducible signaling pathway protein 2 (Wisp2) | NM031590 | 202.5 |
| Complement component factor H (Cfh) | NM130409 | 81.9 |
| Osteomodulin (Omd) | NM031817 | 67.4 |
| Solute carrier organic anion transporter family, member 2a1 (Slco2a1) | AI407489 | 65.8 |
| Dynein, cytoplasmic, intermediate chain 1 (Dncic1) | NM019234 | 64.8 |
| 3-$\alpha$-hydroxysteroid dehydrogenase (RGD:708361) | BF545626 | 37.7 |
| Preproenkephalin, related sequence (Penk-rs) | NM017139 | 29.3 |
| Fc receptor, IgG, low affinity Iib (Fcgr2b) | X73371 | 29.3 |
| Actin, $\gamma$ 2 (Actg2) | NM012893 | 25.9 |
| $\alpha$–2-macroglobulin (A2m) | NM012488 | 23.2 |
| Lysozyme (Lyz) | L12458 | 22.2 |
| Jagged 1 (Jag1) | NM019147 | 19.3 |
| Phospholamban (Pln) | BI290034 | 17.6 |
| Procollagen, type XI, $\alpha$ 1 (Col11a1) | BM388456 | 16.2 |
| Gamma sarcoglycan (RGD:1359577) | AA850867 | 15.3 |
| Pleiomorphic adenoma gene-like 1 (Plagl1) | NM012760 | 15.0 |
| Matrix metallopeptidase 12 (Mmp12) | NM053963 | 14.7 |
| Cyclin D2 (Ccnd2) | L09752 | 14.4 |
| Transforming growth factor, $\beta$ 2 (Tgfb2) | NM031131 | 14.3 |
| Solute carrier family 29, member 1 (Slc29a1) | NM031684 | 14.1 |
| Tissue inhibitor of metalloproteinase 3 (Timp3) | AA893169 | 13.2 |
| Procollagen, type XI, $\alpha$ 1 (Col11a1) | BM389291 | 13.1 |
| Down syndrome critical region gene 1-like 1 (Dscr1l1) | AI138048 | 12.8 |
| Bone morphogenetic protein 4 (Bmp4) | NM012827 | 12.7 |
| Matrix metallopeptidase 13 (Mmp13) | M60616 | 11.8 |
| Macrophage galactose N-acetyl-galactosamine specific lectin 1 (Mgl1) | NM022393 | 11.2 |
| Glycoprotein nmb (Gpnmb) | NM133298 | 10.7 |
| Aquaporin 1 (Aqp1) | AA891661 | 10.6 |
| Cadherin 13 (Cdh13) | NM138889 | 10.5 |
| Selenoprotein P, plasma, 1 (Sepp1) | AA799627 | 10.5 |
| Secreted frizzled-related protein 4 (Sfrp4) | AF140346 | 10.4 |
| Cellular retinoic acid binding protein 2 (Crabp2) | U23407 | 10.2 |

ASC, adipose tissue-derived mesenchymal stem cells; BM-MSC, bone marrow-derived mesenchymal stem cells.

medium stained positively with Oil Red O in 3 weeks. To quantify lipid accumulation, the absorbance of the extracted cells was measured; however, there was no difference in the absorbance between differentiated ASC and BM-MSC. In addition, both ASC and BM-MSC differentiated identically into osteocytes (**Figures 1E,F**). ASC proliferated more rapidly than BM-MSC; the number of ASC was approximately 10-fold higher than that of BM-MSC at the $40^{th}$ day (**Figure 2**). In approximately 2 weeks, ASC had expanded almost 200-fold, whereas BM-MSC had expanded nearly 30-fold.

### Differences in the Gene Expression of ASC and BM-MSC

Of 31,099 genes analyzed, 571 (1.8%) were more highly (>3-fold) expressed in ASC, whereas 571 genes (1.8%) were more highly (>3-fold) expressed in BM-MSC (**Figure 3**). The genes showing the most enriched expression (>10-fold) in ASC and BM-MSC are listed in **Table 1**. Of note, the genes that were highly expressed in ASC included various types of molecules involved in inflammation, such as IL-1$\alpha$ and IL-6, and chemotaxis, such as chemokine (C-C motif) ligand 20 and chemokine (C-X-C motif) ligand 5 (**Table 1**). The genes that were highly expressed in BM-MSC included differentiation-associated genes, such as WNT1-inducible signaling pathway protein 2 (Wisp2), osteomodulin and jagged1 (**Table 2**). Furthermore, the differential expression patterns of 5 representative genes in ASC and BM-MSC obtained by microarray were confirmed by qRT-PCR, which gave the relative expression of IL-1$\alpha$ as 438.2±560.9 (ratio ASC/BM-MSC, n=5), IL-6 as 54.0±26.6, MMP9 as 3.9±2.2, VEGF 1.8±0.4, and Wisp2 as 7.0±2.2.

To evaluate the genes upregulated in ASC, 571 genes that were more highly expressed in ASC were classified by functional annotation using gene ontology terms (**Table 3**). The 31 terms listed had a P-value <0.00001, and included mitosis (eg, pituitary tumor-transforming 1, cyclin B1, cyclin-dependent kinase 2), immune response (eg, chemokine (C-C motif) ligand 20, cathepsin C and IL-1$\alpha$) and response to stress (glutathione peroxidase 2, superoxide dismutase 2 and metallothionein). In BM-MSC, 22 terms were listed for the 571 enriched genes, and included regulation of organ development (eg, Wisp2, osteomodulin and bone morphogenetic protein 4), morphogenesis (cadherin 13, elastin and Neuropillin 2) and cell migration (chemokine (C-X3-C motif) ligand 1 and chemokine (C-X-C motif) receptor 4) (**Table 4**).

### Differences Between ASC and BM-MSC in Secretory Proteins Determined by ELISA

In previous reports, MSC evoked a cell protective effect and induced angiogenesis via secretion of various cytokines, includ-