ASK family kinases mediate cellular stress and redox signaling to circadian clock

Kiyomichi Imamura a,1, Hikari Yoshitane a,1, Kazuki Hattori b, Mitsuo Yamaguchi a, Kento Yoshida a, Takenori Okubo a, Isao Naguro b, Hidenori Ichijo a, and Yoshitaka Fukada a,1

*Department of Biological Sciences, School of Science, The University of Tokyo, Tokyo 113-0033, Japan and bLaboratory of Cell Signaling, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113-0033, Japan

Edited by Joseph S. Takahashi, Howard Hughes Medical Institute and University of Texas Southwestern Medical Center, Dallas, TX, and approved March 1, 2018 (received for review November 5, 2017)

Daily rhythms of behaviors and physiologies are generated by the circadian clock, which is composed of clock genes and the encoded proteins forming transcriptional/translational feedback loops (TTFLs). The circadian clock is a self-sustained oscillator and flexibly responds to various time cues to synchronize with environmental 24-h cycles. However, the key molecule that transmits cellular stress to the circadian clockwork is unknown. Here we identified apoptosis signal-regulating kinase (ASK), a member of the MAPKKK family, as an essential mediator determining the circadian period and phase of cultured cells in response to osmotic changes of the medium. The physiological impact of ASK signaling was demonstrated by a response of the clock to changes in intracellular redox states. Intriguingly, the TTFLs drive rhythmic expression of ASK genes, indicating ASK-mediated association of the TTFLs with intracellular redox. In behavioral analysis, Ask1, Ask2, and Ask3 triple-KO mice exhibited compromised light responses of the circadian period and phase in their activity rhythms. LC-MS/MS–based proteomic analysis identified a series of ASK-dependent and osmotic stress-responsive phosphorylations of proteins, among which CLOCK, a key component of the molecular clockwork, was phosphorylated at Thr843 or Ser845 in the carboxyl-terminal region. These findings reveal the ASK-dependent stress response as an underlying mechanism of circadian clock flexibility.

Circadian rhythms of gene expression and physiological functions are generated by a cell-autonomous time-measuring system termed the circadian clock, which is driven by transcriptional/translational feedback loops (TTFLs) composed of a subset of clock genes (1, 2). The circadian clock is robust in oscillation under constant conditions, but can respond flexibly to a variety of signals and adjust its period and phase to environmental 24-h cycles (2–4). It is well known that a series of protein kinases are required for the two defining properties of the circadian clock, i.e., robustness and flexibility (3, 5, 6). In mammals, a central clock resides in the hypothalamic suprachiasmatic nucleus (SCN) and is synchronized to 24-h light–dark cycles (2, 3). In contrast, peripheral clocks are distributed among most of the peripheral tissues and even in cultured cells, and are controlled by various nonphotic stimuli (7–10). These stimuli potentially evoke a cellular stress response through protein kinase signaling (11). The cellular stress response and the circadian clock system are the most fundamental functions conserved in almost all organisms for adaptation to changes in environmental conditions. However, whether the circadian clock responds to cellular stress through protein kinase signaling and, if so, the key molecule(s) transmitting the cellular stress to the circadian clockwork, remain elusive.

Results

Apoptosis Signal-Regulating Kinases Are Responsible for Osmolarity-Dependent Period Change. We first noticed that the circadian period of cellular rhythms in culture is lengthened day by day not only in PER2::LUC mouse embryonic fibroblasts (MEFs) (Fig. L4) but also in NIH 3T3 and U2OS cells (Fig. S1A and B) after synchronization with dexamethasone (Dex) pulse treatment. When the media were collected at day 7 and reused for monitoring the circadian rhythms of freshly plated PER2::LUC MEFs (Fig. S1C), the period was significantly longer than that recorded in the fresh media (25.13 ± 0.09 h vs. 23.91 ± 0.06 h) (Fig. S1D). This period-lengthening effect of the reused media was blunted when the reused media were diluted with distilled water to isotonicity (Fig. S1 C and D), suggesting that the osmolarity of the culture media determines the circadian cellular period. Indeed, the circadian period was markedly lengthened when the cells were chronically exposed to hypertonic media by adding final concentrations of 200 mM sorbitol (Hyper 480; Fig. 1B and Fig. S1 E and F) or 100 mM NaCl (Fig. S1G) to the fresh media (isotonic, approximately 280 mOsm). In contrast, the period was shortened when the cells were exposed to hypotonic media (Hypo 210; Fig. 1C and Fig. S1H). Taken together, these results indicate that the oscillation speed of the cellular clock is regulated bidirectionally by the increases and decreases in extracellular osmolarity that evoke cellular stress responses (12–14).

In many cases, cellular stress activates a variety of mitogen-activated protein kinase kinase kinases (MAPKKKs), which trigger the MAPK cascade conserved from yeast to humans (15). Among the MAPKKKs, apoptosis signal-regulating kinases (MAP3Ks), which activate protein kinase kinase kinases (MAPKKKs), which trigger the MAPK cascade conserved from yeast to humans (15). Among the MAPKKKs, apoptosis signal-regulating kinases (MAP3Ks), which trigger the MAPK cascade conserved from yeast to humans (15). Among the MAPKKKs, apoptosis signal-regulating kinases (MAP3Ks), which trigger the MAPK cascade conserved from yeast to humans (15). Among the MAPKKKs, apoptosis signal-regulating kinases (MAP3Ks), which trigger the MAPK cascade conserved from yeast to humans (15). Among the MAPKKKs, apoptosis signal-regulating kinases (MAP3Ks), which trigger the MAPK cascade conserved from yeast to humans (15). Among the MAPKKKs, apoptosis signal-regulating kinases (MAP3Ks), which trigger the MAPK cascade conserved from yeast to humans (15). Among the MAPKKKs, apoptosis signal-regulating kinases (MAP3Ks), which trigger the MAPK cascade conserved from yeast to humans (15). Among the MAPKKKs, apoptosis signal-regulating kinases (MAP3Ks), which trigger the MAPK cascade conserved from yeast to humans (15). Among the MAPKKKs, apoptosis signal-regulating kinases (MAP3Ks), which trigger the MAPK cascade conserved from yeast to humans (15). Among the MAPKKKs, apoptosis signal-regulating kinases (MAP3Ks), which trigger the MAPK cascade conserved from yeast to humans (15).
ASK family members are essential for the osmotic stress response of the circadian period of cellular rhythms. (A) The change in the circadian period length in PER2::LUC MEFs. The period length was calculated (n = 22) as a period between the trough (or peak) of the day and the next trough (or peak) as shown at the left. (B, C, E, and F) The effects of chronic hypertonic (B and E; final 200 mM sorbitol to approximately 480 mOsm) and chronic hypotonic (C and F; diluted by distilled water to approximately 210 mOsm) treatment on the circadian period of the cellular rhythms in PER2::LUC MEFs (B and C) and Ask-TKO/PER2::LUC MEFs (E and F). Data are reported as mean ± SEM. n = 4 (B and C) or n = 3 (E and F). Student’s t test, **P < 0.01; ***P < 0.001; not significant (n.s.), P ≥ 0.05 vs. mock treatment. (D) Immunoblot analysis of the phosphorylation state of ASKs in response to the hyperosmotic (final 200 or 600 mM sorbitol to approximately 480 or 880 mOsm) and hypoosmotic (diluted by distilled water to approximately 210 or 187 mOsm) stress. HEK293A cells were collected 30 min after treatment with the indicated stimuli.

(ASKs) ASK1, ASK2, and ASK3 play pivotal roles in response to various stress stimuli, such as oxidative stress and ultraviolet radiation (16). Notably, phosphorylation levels of cellular ASKs are increased in hypotonic media and decreased in hypertonic media (Fig. 1D), while their phosphorylation in the activation loop causes phosphorylation signaling of the downstream MAPK pathway (17).

We hypothesized that ASKs are key players in the bidirectional clock responses to the osmolarity changes. To test this hypothesis, triple-KO (TKO) mice deficient for Ask1 (18), Ask2 (19), and Ask3 (17) were generated (Ask1-TKO) and further crossed with PER2::LUC knockin mice (20) to prepare MEFs. The circadian period of the PER2::LUC rhythms in control MEFs was gradually lengthened during the culture, whereas the period of Ask-TKO MEFs was kept mostly constant during the long culture time (Fig. S2A–C). The period changes by the chronic hypertonic (200 mM sorbitol) or hypotonic treatment were also blunted in the Ask-TKO MEFs (Fig. 1E and F and Fig. S2 D and E). These results demonstrate that ASKs are indispensable for the osmotic stress response of the oscillation speed of the cellular clock.

**ASKs Are Essential for the Osmotic Stress-Dependent Circadian Phase Shift.** We found that osmotic stress regulates not only the circadian period, but also the circadian phase as a time cue in a manner similar to photic stimuli (21–23). As illustrated in Fig. S3A, when the MEFs were exposed to a 30-min sorbitol pulse (hyperosmotic stress) at 66 h after the Dex synchronization [time after Dex (TAD), 66 h], the phase of the cellular rhythms was markedly shifted compared with that after the mock treatment (Fig. 2A). The phase response curve (PRC) and phase transition curve (PTC) after the hypertonic pulse treatment demonstrated type 0 resetting, which is characterized by phase shifts of the oscillator to a common new phase after the sorbitol pulse given at various time of the day (Fig. 2B). Remarkably, no significant phase shift was observed in the Ask-TKO MEFs when they received the hypertonic pulse at any time of day (Fig. 2C and D), while the Dex pulse synchronized the mutant MEFs in a manner indistinguishable from the control PER2::LUC MEFs (Fig. S5B). These results reveal that hyperosmotic stress resets the cellular clock through ASK signaling, which is dispensable for Dex-induced resetting.

Molecularly, we found rapid induction of Dec1, Dec2, and E4bp4 mRNA levels within 30 min after the 30-min sorbitol pulse treatment (Fig. S4A). The induction of these clock(-related) genes was abrogated by treatment with a transcription inhibitor, actinomycin D, but not by a protein synthesis inhibitor, cycloheximide, indicating immediate early responses of Dec1, Dec2, and E4bp4 to the hyperosmotic stress (Fig. S4B). The hyperosmolarity-induced transcriptions of the three genes were blunted in the Ask-TKO MEFs (Fig. S4C), suggesting that the immediate early responses of these genes may be involved in ASK-dependent clock resetting.

**Ask1 and Ask2 Expression Is Rhythmically Controlled by the Circadian Clock.** Our previously reported RNA-Seq data revealed potential circadian changes of Ask1 and Ask2 mRNAs in the mouse liver (24) (Fig. 3A). Here we performed quantitative RT-PCR (qRT-PCR) analysis and found circadian rhythms of Ask1 and Ask2 expression in the liver of mice reared in the constant dark (DD) condition (Fig. 3B). Ask1 and Ask2 transcripts were kept at low levels throughout the day in Bmn1l-KO livers, in which expression of clock-controlled genes, such as Bmn1l, Cry1, and Dhp, became arrhythmic (Fig. 3B). Intriguingly, our CLOCK-ChIP-Seq data in the mouse livers (25) showed rhythmic binding of CLOCK to the first intron region of Ask1 (Map3k5) loci, where we found canonical (CAGCGT) and noncanonical (CACAGT) E-box sequences (25) in the CLOCK-binding peak region (Fig. 3C). In a promoter analysis using a luciferase reporter, CLOCK and BMAL1 activated transcription of Ask1 from the first intronic region, and introduction of mutations in the E-box sequences caused marked reduction of transcriptional activation (Fig. 3D). Collectively, these data indicate that ASK signaling regulates the TTFLs (Figs. 1 and 2) and, vice versa, Ask1 (Ask1) expression is controlled by the TTFLs.
Physiological Roles of ASK Signaling in Clock Input. ASKs are phosphorylated in response to reactive oxygen species (ROS) (16), and we verified that ASKs are phosphorylated by H$_2$O$_2$ treatment (Fig. S5A). A 30-min pulse treatment of PER2::LUC MEFs with 0.4 mM H$_2$O$_2$ at TAD 66 h caused a remarkable phase shift of cellular rhythms, and the phase shift was significantly attenuated in the Ask-TKO MEFs (Fig. 4A and Fig. S5B). Then PER2::LUC MEFs were exposed to an intrinsic oxidative stress by treatment with 3-amino-1,2,4-aminotriazole (ATZ), a cell-permeable inhibitor of catalase that is a key enzyme catalyzing the conversion of H$_2$O$_2$ to water and oxygen. We found that the circadian period of the cellular rhythm was lengthened by the chronic ATZ treatment, and the period-lengthening effect was significantly attenuated in Ask-TKO MEFs (Fig. 4B and Fig. S5C). Taken together, these results indicate that the intracellular redox state provides input to the TTFLs through ASK signaling (Fig. S5D).

These effects of stress signals on the circadian period and phase of the cellular clock are reminiscent of photic input to the central clock in the SCN (22, 23). Physiological relevance of the cellular stress to the photic regulation of the clock system has been implicated by pioneering studies on melatonin secretion patterns of the clock (-related) genes at protein (Fig. 4C) and mRNA (Fig. S6D) levels in the mouse liver under the LD condition. When transferred to DD, Ask-TKO mice showed a stable circadian expression of clock genes in the liver, determined by RT-PCR analysis in WT (black) and Bmal1-KO (blue) mice. n = 3. One-way ANOVA, **p < 0.001; not significant (n.s.), p ≥ 0.05. (C) CLOCK Chip-Seq data at the Ask1 locus from our previous study (25). Canonical (black) and noncanonical E-box sequences (gray) near the CLOCK-binding peak region are highlighted. CT, circadian time; ZT, zeitgeber time. (D) CLOCK/Bmal1 (CL/B1)-dependent transactivation through the E-box sequences in the Ask1 intron 1, determined by a dual luciferase reporter assay in HEK293T17 cells.

Intriguingly, this period-lengthening effect of LL was significantly reduced in the Ask-TKO mice, which showed a shorter period compared with WT mice under LL (Fig. 4D and E and Fig. S6).
Fig. S6E). The circadian period of Ask1 single KO in LL was significantly shorter than that in WT mice but longer than that in Ask-TKO mice, suggesting that each ASK member contributes cooperatively to circadian behavioral rhythms (Fig. S6F). In phase-response analysis of the behavioral rhythms by a 30-min light pulse, we found significant effects of Ask deficiency on both the phase delay (WT: −3.03 ± 0.21 h, KO: −1.64 ± 0.10 h) by the light at circadian time (CT) 14 and the phase advance (WT: 1.53 ± 0.22 h, KO: 0.54 ± 0.11 h) at CT22 (Fig. 4F and G). These results demonstrate that ASKs play pivotal roles not only in the cultured cells, but also in the central clock in the SCN for regulation of the light-regulated circadian period and phase.

Molecular Mechanism for ASK-Dependent Clock Input. To understand the molecular mechanisms of how ASKs mediate the clock input, we investigated correlations between the kinase activities of ASKs and the cellular circadian period. The circadian period in NIH 3T3 cells was markedly shortened by overexpression of ASK3 (Fig. 5A), which is autophosphorylated in the transfected cells (17). Importantly, the period-shortening effect was significantly suppressed by chronic treatment of an ASK inhibitor, K811 (29) (Fig. 5A), and overexpression of ASK3 KM, a kinase-dead mutant (17), had no obvious effect on the circadian period (Fig. 5B). These results demonstrate that the kinase activity of ASK3 is important in determining the cellular circadian period. In the MAPK cascade, c-Jun N-terminal kinase (JNK) and p38 are widely known as major stress-activated kinases (17, 30, 31). In our experimental condition, however, hypertonic treatment resulted in the dephosphorylation of endogenous ASKs (Fig. 1D) and phosphorylation (activation) of endogenous JNK and p38 (Fig. S7A and B) (14, 17). Furthermore, a JNK inhibitor, SP600125, and/or a p38 inhibitor, SB203580, had no obvious effect on the hyperosmolarity-induced resetting (Fig. S7C–E), indicating atypical signaling downstream of ASKs as the clock input. In addition, the hyperosmotic stress caused ASK dephosphorylation and input to the clock, whereas the clock input was abolished by Ask deficiency (Figs. 1 and 2). Based on the apparent inconsistency, we now propose a model in which ASK dephosphorylation triggers atypical ASK signaling in terms of clock input (Fig. 5F). This model is based on the previous observation that Ask1 forms a high molecular mass (HMM) complex (>1,500 kDa) including Ask1 regulatory proteins, which are changed in response to the phosphorylation levels of ASK1 (32).

To reinforce this model, we searched for ASK-dependent and hyperosmotic stress-induced changes in protein phosphorylation by performing LC-MS/MS–based proteomic analysis (Fig. 5C). We identified 2,821 phosphopeptides (Dataset S1), among which the amounts of 65 peptides were significantly changed by the hyperosmotic treatment in control MEFs but were kept constant in the Ask-TKO MEFs (Fig. S8 and Dataset S2). Importantly, 21 of the 65 phosphopeptides were up-regulated by the osmotic treatment in control MEFs but were constantly low in the Ask-TKO MEFs (Fig. S9), and this group included a phosphopeptide derived from CLOCK protein. The MS/MS spectrum identified Thr843 or Ser845 as an ASK-dependent phosphorylation site in
Discussion

In the present study, we have demonstrated that osmotic stress has prominent effects on the period and phase of the mammalian circadian clock, and that these effects were completely blocked by deficiencies of ASK family members (Figs. 1 and 2). Similarly, extracellular and intracellular oxidative stress, akin to osmotic stress, provides input to the circadian clockwork through ASK signaling (Fig. 4 and Fig. S5). Our behavioral analysis revealed in vivo roles of ASK signaling to regulate the oscillation speed and phase in response to light (Fig. 4 and Fig. S6). It is probable that the cellular stress acts as a time cue through a mechanism mediated by ASK signaling and that cellular stress and light may share, at least in part, a common input pathway to the circadian clockwork.

We previously reported a strong impact of ASK3 on water/salt reabsorption in renal tubular epithelial cells directly exposed to massive osmotic changes in body fluids (17). Osmotic stress is regulated rhythmically, peaking at night in the inner medulla of the kidney (33), and such a rhythm of cellular stress may be physiologically important for the peripheral clock. The circadian clock is known to control many renal functions, and dysfunction of the clock is associated with several diseases, including hypertension and type 2 diabetes (34).

Osmotic stress is known to stimulate stress-activated protein kinases, JNK and p38, downstream of the MAPK cascade (14, 16). We previously found that JNks phosphorylate BMAL1 on exposure to hyperosmotic stress and regulate the circadian clock by controlling the oscillation speed and phase in response to light (28). In the present study, however, no remarkable alterations were observed by inhibition of JNK and p38 in the phase responses to acute hyperosmotic stimuli (Fig. S7 C–E). Therefore, hyperosmotic stress-induced phosphorylation/activation of p38/JNK is not involved in ASK-mediated clock input; rather, we propose that an HMM complex is important for the clock input (Fig. 5F).

Changes in the ASK HMM components in response to hyperosmotic stress should trigger atypical ASK signaling accompanied by dephosphorylation of ASKs. As evidence supporting this model, we found increases or decreases in ASK-dependent phosphorylation in response to hyperosmotic stress (Figs. S8 and S9 and Dataset S2), and identified T843 or S845 as the phosphorylation site of CLOCK protein (Fig. 5 D and E). It is notable that a phosphopeptide database (www.phosphosite.org) includes a human pS836-CLOCK peptide corresponding to mouse pS845 as an in vivo phosphorylation site induced by cold ischemia in tumors (35). In this model (Fig. 5F), we consider that a balance between phosphorylated and dephosphorylated forms of ASKs should determine the circadian period under various osmotic conditions. Even in the isotonic condition, clock input signal from immediately dephosphorylated ASKs lengthens the circadian period, and the hypoosmotic treatment causes ASK phosphorylation, which further weakens the period-lengthening signal (Fig. 1F). In the Ask-TKO MEFs, the period-lengthening signal is completely lost, resulting in its short period phenotype (Fig. 1F). The model may need refinement when we take into account the fact that ASK1, ASK2, and ASK3 form a heteromeric complex (16), because various cellular stress signals received differently by the isoforms may be integrated into a change in the components of the HMM complex.

Aerobic organisms use oxygen to obtain energy efficiently, and as a result, they are inevitably exposed to oxidative stress, such as H$_2$O$_2$ (36). The intracellular level of the redox-related metabolite NAD$^+$ and redox state of the antioxidant enzyme peroxiredoxin (PRX) are reported to have daily rhythms in mouse liver (37, 38). We emphasize that the PRX redox rhythms also have been identified in human red blood cells, in which transcription is arrested (39). The identification of the redox rhythms has led to a new idea of a redox oscillator (37, 39), in parallel with the TTFL oscillator. Nevertheless, the mechanism of coupling between the redox rhythms and the TTFLs remains largely unknown. The present study indicates that ASKs mediate redox
signaling to the TTFILs (Fig. 4 A and B and Fig. S5), and, vice versa, Ask expression rhythms are controlled by the TTFILs (Fig. 3). The expression rhythms of *Ask1* and *Ask2* in the mouse liver exhibited peaks at CT10–14 (Fig. 3 A and B), a time of peak urine (cortisol) oxidation levels, as measured by PRX redox and NAD*/NADH* levels (37–39). These lines of evidence support the fascinating idea that the TTFILs and intracellular redox state are interlocked with each other via ASK signaling (Fig. S5).

**Materials and Methods**

Further elaboration of the methods used in this study is provided in SI Materials and Methods.

**Real-Time Monitoring of Cellular Rhythms and Osmotic Stress Treatments.** Real-time monitoring of the cellular bioluminescence rhythms was performed as described previously (10) with minor modifications. In brief, cells were treated with 0.1 μM (final concentration) dexamethasone for 2 h, after which the media were replaced by recording media. The bioluminescence signals were continuously recorded for 5–10 d at 37 °C in air with a Kronos AB-2500 or AB-2550 (Atto), Lumicycle (Actimetrics), or CL24A (Churitsu) luminometer. For chronic hypertonic stimuli, NaCl (final concentration 100 mM) or sorbitol (final concentration 200 mM) was added to the cultured media. For pulse hypertonic stimuli, the cultured media (2.5 mL) were removed, and 0.88 mL of the used media was rapidly returned back to the original wells after mixing with 0.12 mL of 5 M sorbitol in the fresh media (final concentration 600 mM). After a 30-min incubation, the hyperosmotic media were replaced by the residual used media that had been kept at 37 °C (Fig. S3A). For hypotonic stimuli, the cultured media were diluted by mixing with distilled water (hypotonic) or 140 mM NaCl (mock).

**Materials and Behavioral Rhythms.** The mice used in this study (C57BL/6 background) were handled according to approved institutional Animal Care and Use Committees of The University of Tokyo (14-2). Ask-TKO mice were generated by crossing Ask-F1 KO (18), Ask2-KO (19), and Ask3-KO (17) mice. Behavioral rhythms of mice were recorded as described previously (24), with minor modifications. In brief, male mice were individually housed in a polycarbonate cage equipped with a running wheel in a light-dark chamber. The locomotor activity rhythms of mice were measured by wheel revolutions in 5-min bins and analyzed using ClockLab software (Actimetrics). The circadian periods were analyzed by a *χ*2 periodogram with a P value < 0.001, based on locomotor activity at days 4–17 after the start of each condition.

**LC-MS/MS-Based Phosphoproteomic Analysis.** Enzymatic digestion by trypsin was performed according to a phase-transfer surfactant (PTS) protocol (40) with modifications. The peptides were desalted using a MonoSpin C18 column (GL Sciences) and applied to a high-select Fe-NTA phosphopeptide enrichment kit (Thermo Fisher Scientific). The enriched phosphopeptides were subjected to LC-MS/MS analyses using a mass spectrometer (Q Exactive Plus Thermo Fisher Scientific) equipped with a nano ultra-HPLC system (Dionex-UltraHigh; Thermo Fisher Scientific). The raw spectra were extracted using Proteome Discoverer 2.2 (Thermo Fisher Scientific) and searched against the mouse SwissProt database (v2017-06-07), with phosphorylation (+79.966 Da) at Ser, Thr, and Tyr set as a dynamic (nonfixed) modification for peptide. The amount of each peptide was semiquantified using peak area with the Precursor Ions Quantifier mode in Proteome Discoverer 2.2.

**ACKNOWLEDGMENTS.** We thank Dr. Hiroshi Kyotada, Dr. Kuniyoshi Niwa, and Miho Yoshimura for their help with experiments, and Hiroto Fukuwa and Hiroaki Watatsuki for mouse breeding. K.I. is supported by a Japan Society for the Promotion of Science research fellowship for young scientists. This work was partially supported by Grants-in-Aid for Scientific Research (to H.Y., K.H., I.N., H.I., and Y.F.) from the Ministry of Education, Culture, Sports, Science and Technology–Japan and by PRIME (to H.Y.) and JT17gmS010001 (to H.I.) from the Japan Agency for Medical Research and Development.