



## Original

# A simplified enriched environment increases body temperature and suppresses cancer progression in mice

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**Abstract:** Mice housed in an enriched environment (EE) have inhibited tumor development because of eustress (positive stress) stimulation. However, the mechanisms underlying increased cancer resistance in EEs remain unclear; this may be due to poor reproducibility of the results because of the complicated EE assembly requirements. In this study, we examined the effects of a simplified EE (sEE) model, consisting only of a nesting shelter and a running wheel, on cancer development in mice. We found that, similar to the complex EE, the sEE promoted motor function and alleviated anxiety in mice. Moreover, the mice housed in the sEE showed inhibited tumor growth and metastasis in addition to a higher average body temperature (especially at the point of transition from light to darkness). Furthermore, mice in the sEE had a decreased brown adipose tissue (BAT) mass, with a significant upregulation of the *Ucp1* and *Adrb3* genes (which encode uncoupling protein 1 and  $\beta$ -adrenergic receptor, respectively) observed in the BAT at the point of transition from light to darkness. An antibody against the immune checkpoint protein programmed cell death 1 was also found to have an additive effect with the sEE against tumor development. Our findings indicate that the established sEE model may be a useful tool for studying the antitumor effects of eustress and can potentially introduce new avenues for cancer prevention and treatment.

**Key words:** animal models of cancer, body temperature, cancer prevention, enriched environment, eustress

## Introduction

Environmental factors including physical living conditions and social stimulation can regulate neuronal activity in the brain and have also been suggested to influence the development of cancer. Psychosocial distress (negative stress) is associated with a higher cancer incidence and poorer survival [6], whereas eustress (positive stress) inhibits cancer development [23]. Housing of rodents in an enriched environment (EE) is a classical *in vivo* eustress model used to study environmental effects [18]. The EE typically consists of tunnels, nesting material, toys, and running wheels; these components positively affect animal physiology and social behavior, including the reduction of anxiety levels; stimulation of

motor functions, learning, and memory; and improved recovery from brain injury [29]. Furthermore, previous studies suggested that EEs were associated with antitumor phenotypes and also inhibited tumor growth in animal models of colon, breast, lung, and pancreatic cancers [5, 13, 15, 27]. However, another study found that the EE did not influence tumor growth rates in mice [32]. The discordant findings regarding the anticancer effects of EEs may be attributed to the complex equipment used to create these environments, which makes them difficult to standardize. Therefore, it is necessary to develop a simpler easy-to-reproduce EE model to investigate EE-induced anticancer mechanisms.

It was previously suggested that the EE's impact on cancer development is mediated by sympathetic nerve

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activation. Therefore, living in an EE led to significant inhibition of cancer growth through the upregulation of brain-derived neurotrophic factor in the hypothalamus and sympathoneural stimulation, which reduced blood leptin levels [5]. Furthermore, the EE enhanced the anticancer activity and tumor infiltration of natural killer cells in pancreatic and lung cancers via sympathetic activation of the lectin-like receptor NKG2D and C-C chemokine receptor CCR5 [27].

The EE is also known to decrease adiposity, stimulate energy metabolism, and induce brown-like (beige) cells in white fat [8], which increases body temperature. Hypothermia during surgery enhanced postoperative tumor growth [16], whereas physiologic responses to high body temperature can enhance the tumor microenvironment's ability to resist tumors [20]. Our own research indicated that the intake of *Inonotus obliquus* (Chaga mushroom) extract, which is used as a traditional anti-cancer medicine, could prevent cancer development in mice through the maintenance of their body temperature [1]. These data suggest that the induction of lipid metabolism and thermogenesis may be critical for cancer prevention; therefore, we hypothesized that the anticancer benefits of EEs could be associated with thermogenesis.

More recent research into immune checkpoint proteins such as programmed cell death 1 (PD-1), which negatively regulate immune activation and thereby limit antitumor responses, has led to the development of PD-1 pathway inhibitors that have revolutionized treatments for patients with various types of cancers [21]. It is well known that cancer immunotherapy is effective, although certain proportions of patients do not show complete responses and may also develop adverse events [25]. Based on this background, we considered that eustress modeled by an EE could have beneficial effects as a hindrance to tumor progression and an enhancer of PD-1 checkpoint blockade via sympathetic activation and thermogenesis. Hence, the aim of this study was to establish a simplified EE (sEE) model and investigate its effect on cancer development in mice. The EE in our study consisted only of a shelter and exercise equipment, as this EE can be easily reproduced.

## Materials and Methods

### Animal housing and behavioral testing

C57BL/6 mice obtained from Japan SLC Inc. (Shizuoka, Japan) were bred and housed in rooms with constant temperature ( $23 \pm 2^\circ\text{C}$ ) and humidity ( $50 \pm 5\%$ ) under a 12 h light/dark cycle (lights on at 08:00) and with *ad libitum* access to water and standard rodent chow. Control mice were housed in TM-TPX-5 cages

( $16.8 \times 29.9 \times 13.3$  cm; Tokiwa Kagaku Kikai Co., Ltd., Tokyo, Japan) with clean paper bedding (Sankyo Lab. Co., Tokyo, Japan). sEE-conditioned mice were housed in TM-TPX-10 cages ( $21.8 \times 32.0 \times 13.3$  cm; Tokiwa Kagaku Kikai Co., Ltd.) with clean paper bedding and a Mouse Igloo and Fast-Trac (Animec, Tokyo, Japan) as a shelter and exercise equipment. Under both conditions, breeding was performed with one male and two female mice, and the pups were weaned at 4–5 weeks of age and housed under the same conditions (control or sEE) throughout the experiment. For behavior experiments (Figs. 1B–I), eight to fourteen male pups from 14 births by 3 dams per condition were randomly assigned to each experimental group. In the other experiments (Figs. 2–6), four to six female pups from 2–3 dams per condition were randomly assigned to each experimental group. All animal experiments were approved by the Institutional Animal Care and Use Committee of Showa University (Permit Number: 56011), which operates in accordance with the Japanese Government guidelines for the care and use of laboratory animals. All surgical procedures were performed under 4% isoflurane anesthesia, and all efforts were made to minimize suffering.

### Behavioral tests

C57BL/6 male mice housed under control or sEE conditions for 10–14 weeks were used for behavioral tests. The open field and novel object tests were performed to assess anxiety-like behavior for two days [2]. The active avoidance test was performed to evaluate memory function on days 2–4 after the novel object test [19]. Motor function was analyzed using the rotarod test [26] one week after the active avoidance test.

### Open field test

The aim of the open field test was to assess anxiety and exploratory drive in animals based on their natural tendency for exploration and self-protection as described previously [31]. Each mouse was placed in the center of a peripheral zone within a  $40 \times 40$  cm square arena facing a 40 cm wall and allowed to explore the apparatus for 10 min. The movement of the mouse in the open field arena was continuously recorded using a computerized SMART video-tracking system (version 2.5, Panlab, Barcelona, Spain). The arena was virtually subdivided into a central zone as well as peripheral zones located within 10 cm from the wall, and the time spent in the central zone was measured as an indicator of anxiety. The total distance traveled by the mouse was also measured to assess animal activity.

### Novel object test

The novel object test examined the ability of the mice to explore a novel object in a familiar environment, as their behavior when doing so reflects the extent of their anxiety. One day after the open field test, the mouse was again positioned in the center of the open field arena that now contained a single novel object (a white container) located in the center of the box, and it was allowed to explore the object for 8 min. The mouse was considered to have approached the novel object when it touched it and to have spent time around the object when it was within 10 cm of it.

### Active avoidance test

In the active avoidance test, mice were examined for their ability to associate conditioned and unconditioned stimuli. The experiment was performed using an active avoidance apparatus (Panlab) that consisted of a sound-proof box (Le26), programmer/shocker (LE2708), and software (SHUTAVOID). Each trial consisted of 10 s of exposure to a conditioned stimulus (light) followed by a 5 s unconditioned stimulus (electrical shock with 60 V delivered through the grid floor). Crossing to the other compartment during the conditioned stimulus was considered an act of avoidance, while failure to cross until the unconditioned stimulus (the shock) was considered an error. Each test was followed by a 20–40 s intertrial interval, with each session consisting of 100 tests. The number of avoidance responses within the 100 tests reflected the mouse's memory function.

### Rotarod test

Motor performance was evaluated using a rotarod apparatus (Panlab). Mice were placed for 1 min on rod of the apparatus, which was rotated starting at 4 rpm. The rotation was then accelerated over 2 min to reach a final speed of 40 rpm. The animals were allowed to stay on the rod for a maximum of 2 min, and the time and speed at falling were recorded. The test was repeated five times, and scores from three trials were averaged after excluding the maximum and minimum scores.

### Cancer cell line

The Lewis lung carcinoma cell line (3LL) was obtained from the National Institutes of Biomedical Innovation, Health and Nutrition (Osaka, Japan) and maintained in RPMI 1640 medium supplemented with glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% (v/v) heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA).

### Cancer models

Murine cancer models were established as described previously [1]. Briefly, mice living under control or sEE conditions for 14 weeks were injected with 3LL tumor cells ( $5 \times 10^4$ ) suspended in 0.2 ml of serum-free MEM (Sigma, St. Louis, MO, USA) subcutaneously in the right flanks to develop solid intra-abdominal tumors (tumor-bearing model). Mice were sacrificed on day 16, and solid tumors were collected and weighed. Alternatively, 3LL cells ( $1 \times 10^5$ ) were injected into the tail vein to form colonies of metastatic cells in the lung (metastasis model). On day 9 post injection, mice were anesthetized with isoflurane and examined for pulmonary metastatic nodules using computed tomography (CT). To analyze the effect of PD-1 blockade in the sEE model, 3LL cells ( $5 \times 10^4$ ) were injected into the tail veins of mice, and 0.1 mg of anti-mouse PD-1 antibody (clone 4H2) was administered intraperitoneally 7 and 14 days post tumor transplantation. The anti-mouse PD-1 antibody was kindly provided from Ono Pharmaceutical Co., Ltd. (Osaka, Japan) [24]. The nodules were visualized and counted in 3D lung micro-CT images (R\_mCT2 micro-CT, Rigaku Corp., Tokyo, Japan) obtained with the following CT scanning parameters: field of view, 24 (φ24 mm × H19 mm); tube voltage, 90 kV; and tube current, 160 µA. Humane endpoints were established if tumors had reached 2,000 mm<sup>3</sup> or more, or if pulmonary metastatic nodules had reached 50 or more, or if mice had presented piloerection, stereotyped or aggressive behaviors, pain indicative postures, lack of activity, and tremors or convulsions. Animals were monitored every day on weekdays to assess room temperature and humidity, and their health was assessed by scientists and vivarium technicians with more than 5 years of experience through evaluation of their behavior and general appearance. Once animals reached endpoint criteria, they were euthanized by cervical dislocation within 24 h. All surviving animals were euthanized 36 days after tumor transplantation.

### Body temperature measurement

Mouse body temperature was monitored using DST nano-T temperature loggers (17 mm × 6 mm, 1 g; Star-Oddi, Gardabaer, Iceland). The loggers were implanted in the abdomen, and body temperature was continuously measured at 30 min intervals.

### Fat tissue collection and real-time polymerase chain reaction (RT-PCR)

Fat tissues including interscapular brown adipose tissue (iBAT), retroperitoneal white adipose tissue (rp-WAT), and gonadal white adipose tissue (gWAT) were

**Table 1.** Primers used for real-time polymerase chain reaction

Gene symbol	Species	Forward (5' to 3')	Reverse (5' to 3')	Product size (bp)
<i>Ucp1</i>	Mouse	GGCATTTCAGAGGCAAATCAGCT	CAATGAACACTGCCACACCTC	151
<i>Adrb3</i>	Mouse	TCGACATGTTCTCCACCAA	GATGGTCCAAGATGGTGCTT	144
<i>Ppargc1a</i>	Mouse	CCCTGCCATTGTAAAGACC	TGCTGCTGTTCTGTTTC	161
<i>Gapdh</i>	Mouse	GCTACACTGAGGACCAGGTTGT	CTCCTGTTATTATGGGGGTCTG	306

collected from 24- to 35-week-old mice housed in control and sEE conditions. Tissues were weighed, photographed, snap-frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until analysis. Total RNA was isolated from fat tissues using RNA-BEE (Tel-Test, Friendswood, TX, USA) and an RNeasy Mini Kit (Qiagen, Hilden, Germany), and was then reverse transcribed into cDNA using a PrimeScript RT reagent Kit (Takara Bio Inc., Shiga, Japan). RT-PCR was performed with primers specific for *Ucp1* (which codes for uncoupling protein 1 [UCP1]), *Adrb3* ( $\beta$ -adrenergic receptor 3), *Ppargc1a* (peroxisome proliferator-activated receptor  $\gamma$  coactivator 1- $\alpha$ ), and *Gapdh* (glyceraldehyde 3-phosphate dehydrogenase); the primer sequences are shown in Table 1. The reactions were performed with SYBR Premix Ex Taq II reagent (Takara Bio. Inc.) in an ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA). Relative gene expression was calculated using the comparative  $\Delta\text{Ct}$  method after normalization to the Ct levels of the housekeeping gene (*Gapdh*) and then to the levels of each gene in the control group.

### Statistical analysis

The significance of the differences between the sEE and control groups was evaluated using the unpaired t-test based on at least two independent experiments. Survival rate differences between the sEE and sEE+anti-PD-1 antibody group were evaluated using the log rank test. The BellCurve for Excel software (Social Survey Research Information Co., Ltd., Tokyo, Japan) was used for statistical analyses. *P*-values  $<0.05$  were considered statistically significant.

## Results

### Establishment of an sEE

For good reproducibility of mechanistic studies, we established sEE conditions in lieu of the conventional and more complex EEs; the sEE included only a shelter and exercise equipment (Fig. 1A). First, we tested whether the sEE had the same beneficial effects on motor, emotional, and cognitive functions as the complex EE. Mouse pups housed in our sEE gained significantly more weight than control pups (Fig. 1B), suggesting that dams in the sEE had better nursing abilities. After being housed

under the control or sEE conditions, mice were examined using a battery of behavioral tests. The rotarod test demonstrated that the sEE improved motor function (Figs. 1C and D), and the open field and object exploration tests showed that the sEE reduced anxiety-like behavior (Fig. 1E–H), similar to results obtained in complex EEs [29]. However, active avoidance tests indicated that the sEE did not improve memory. These data collectively showed that the sEE exerted the same beneficial effects as the complex EE, with the exception of memory function, suggesting that our sEE model could be a good tool for studying the anticancer influence of eustress. Because we found that male mice often fight in the sEE (similar to their behavior in complex EEs) [28], we used female mice in the subsequent experiments.

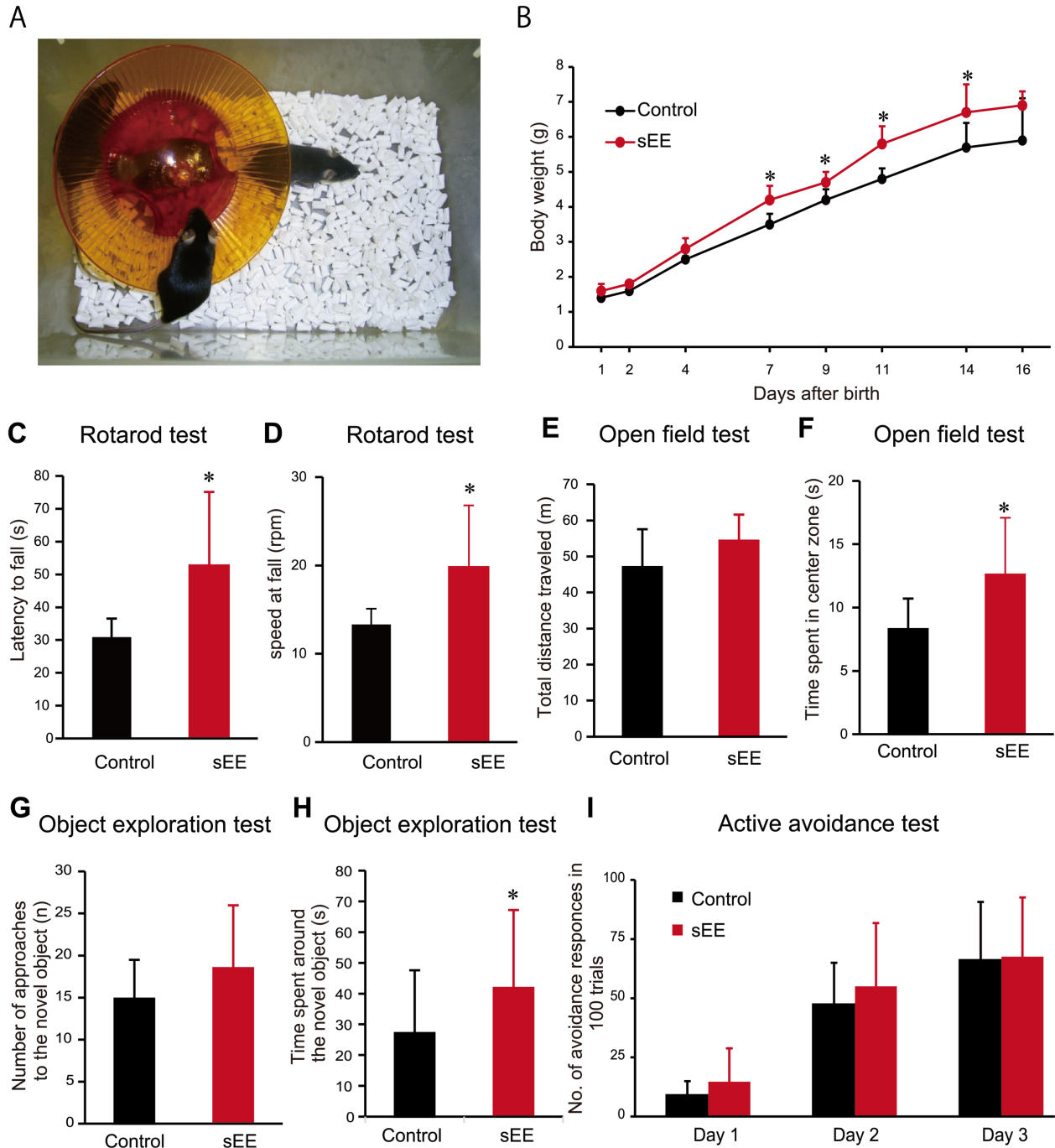
### Living in an sEE slowed tumor progression and suppressed metastasis in mice

To explore whether the sEE could suppress tumor growth, 3LL cells were injected subcutaneously to induce solid intra-abdominal carcinomas. Mice living in the sEE exhibited significantly slower tumor growth, with tumor weights reduced by 70.7% compared with the control group (Figs. 2A and B). These results indicated that the sEE inhibited carcinogenesis.

To further explore the cancer-suppressing effects of the sEE, we analyzed the development of spontaneous metastases in the lungs. Micro-CT analysis indicated that the sEE significantly decreased the number of tumor nodules in the lungs (Fig. 2C), suggesting that the sEE (similar to the complex EE) prevented tumor spreading.

### The sEE increased body temperature in mice during the transition from darkness to light

To test whether the maintenance of body temperature could be affected by the sEE as in the complex EE, we monitored the body temperature in real time. As expected, tumor-free mice living in an sEE showed higher body temperatures than control mice (Fig. 3A). Increased thermogenesis was also observed in tumor-bearing sEE mice compared with control mice during the transition from light to darkness and from darkness to light (Figs. 3B and C).

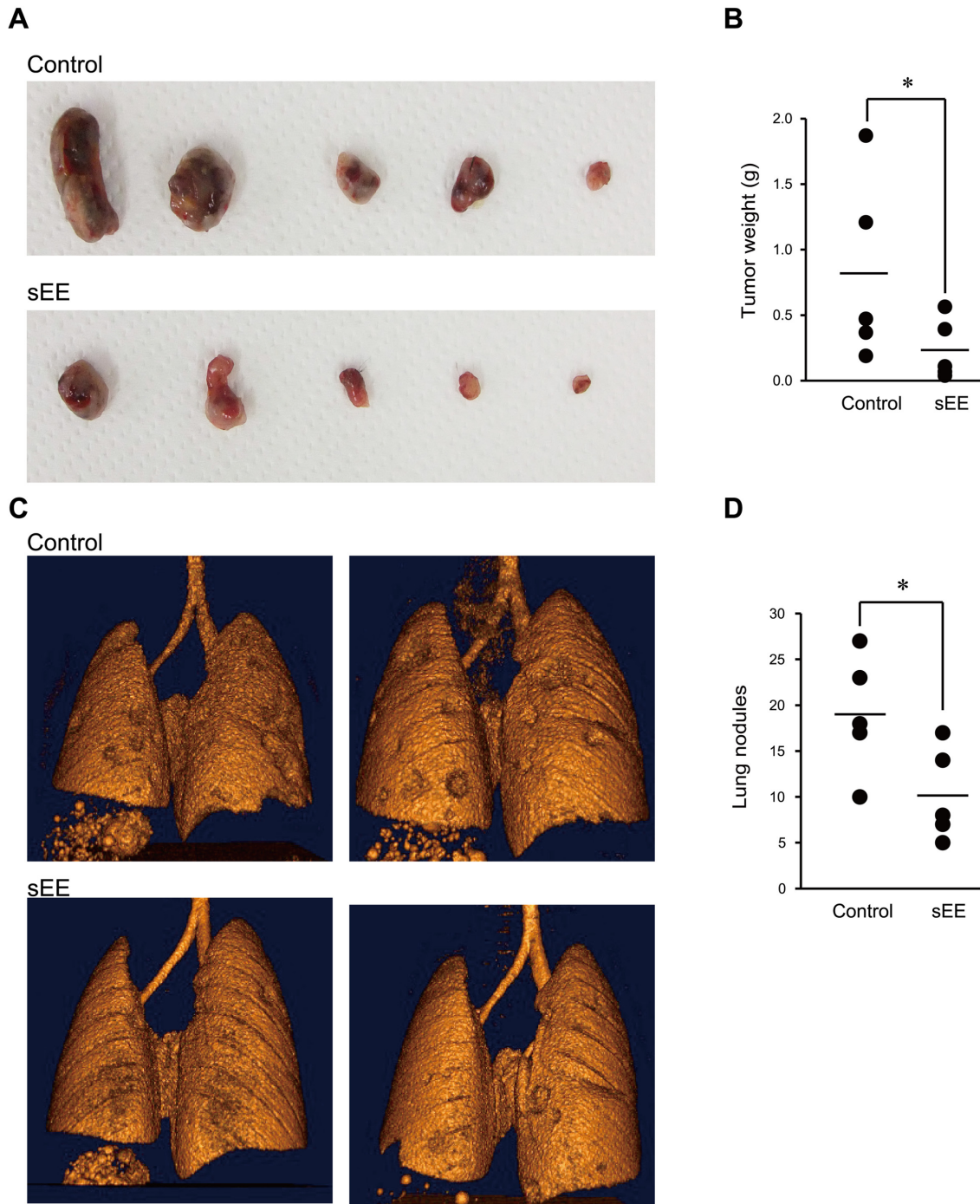


**Fig. 1.** Effects of the simplified enriched environment (sEE) on mouse behavior. A, A representative image of mice housed in the sEE. B, Mean body weights of newborn mice housed under control or sEE conditions ( $n=12-13$ ). C-I, Behavioral tests ( $n=8-14$ /group): rotarod tests to measure motor functions (C, D), open field test (E, F) and novel object test (G, H) to assess anxiety-like behavior, and the active avoidance test (I)-to assess recognition memory. The data are presented as the mean  $\pm$  standard deviation;  $*P<0.05$  versus the control group.

### sEE-housed mice had a reduced BAT mass at the point of transition from light to darkness

To examine whether the sEE affected energy metabolism, we assessed fat deposition in sEE and control mice. As shown in Figs. 4A and B, sEE mice had a significant (36.6%) reduction in their iBAT mass at the point of transition from light to darkness compared with control mice. However, there was no statistically significant dif-

ference between the groups in rpWAT (Figs. 4C and D) and gWAT; gWAT tended to increase in sEE mice (Figs. 4E and F). These data suggest that sEE-housed mice may consume iBAT to generate heat at the point of transition from light to darkness, which increases body temperature.

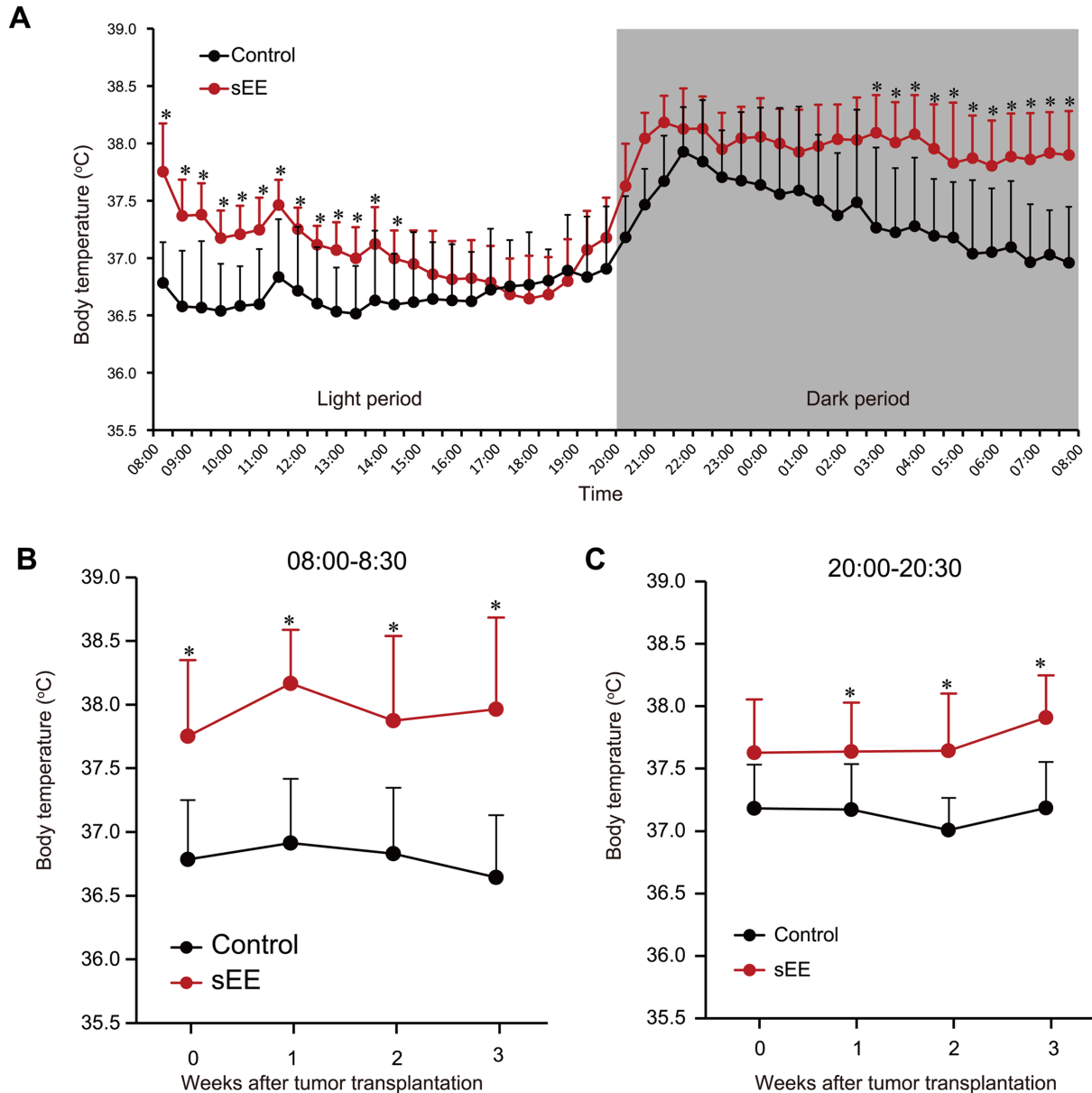


**Fig. 2.** Housing in a simplified enriched environment (sEE) suppresses tumor development and metastasis in mice implanted with Lewis lung carcinoma (3LL) cells. Mice housed under control or sEE conditions were injected with 3LL cells subcutaneously in their right flanks, and the resulting tumors were excised and weighed. A, Representative images of the excised carcinomas. B, Mean weights of the solid tumors on day 16 after 3LL cell implantation. C, Representative computed tomography (CT) images of lungs containing carcinoma nodules from mice that were living under control or sEE conditions and received intravenous injection of 3LL cells. D, The numbers of nodules measured on the lung CT images on day 9 post cancer-cell injection; each dot represents a single mouse, while lines show mean values (n=5 per group; \* $P < 0.05$ ).

**The sEE increased *Ucp1* and *Adrb3* mRNA expression in iBAT at the point of transition from light to darkness**

Next, we analyzed whether an sEE could modulate the mRNA expression level of *Ucp1*. In sEE mice, the

expression of *Ucp1* mRNA was increased 3.5-fold in iBAT and 2.9-fold in rpWAT at the point of transition from light to darkness, whereas its expression in gWAT was downregulated to 0.1-fold at the point of transition from darkness to light compared with control mice (Fig.



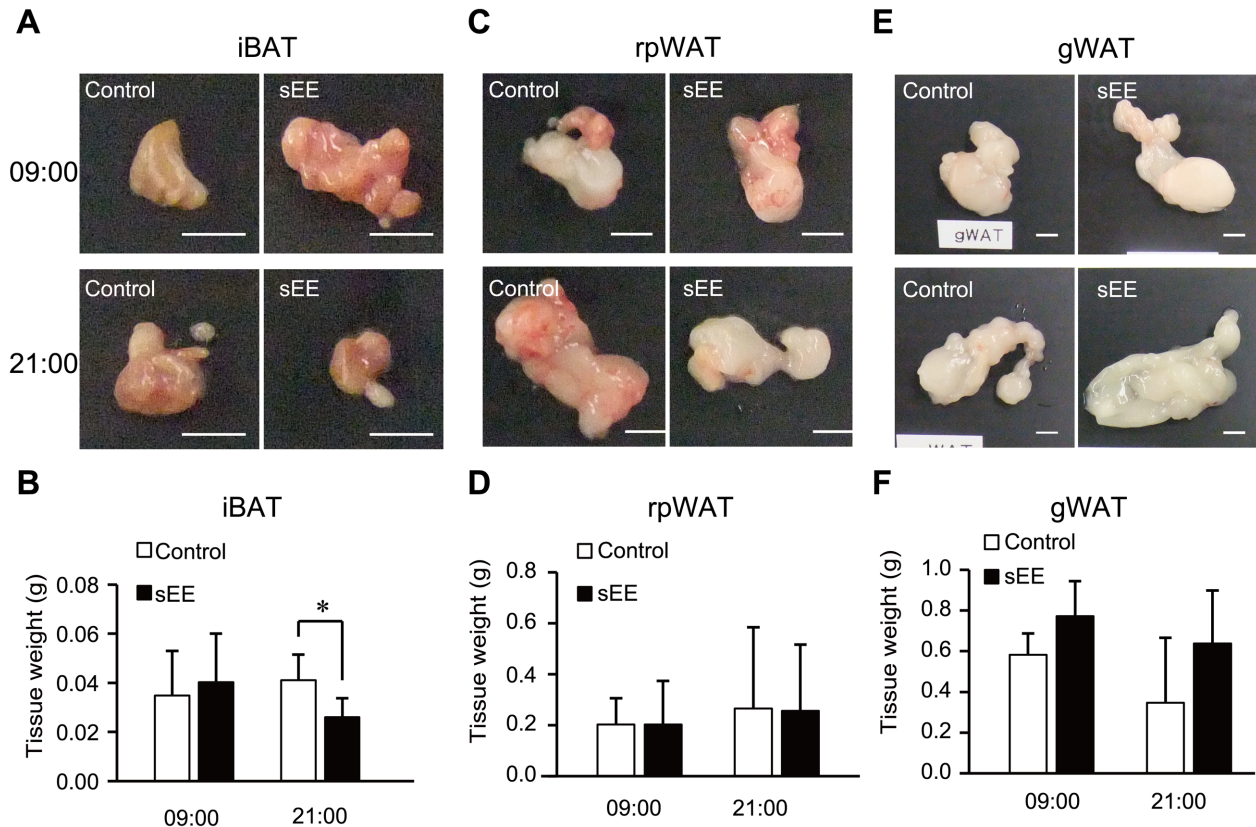
**Fig. 3.** The simplified enriched environment (sEE) is associated with a higher body temperature in mice. **A**, Average body temperatures of mice living under control or sEE conditions before tumor implantation. Significantly higher body temperatures are observed in sEE mice than in control mice. **B** and **C**, Average body temperature of carcinoma-bearing mice during the transition from light to darkness (08:00–08:30; **B**) and from darkness to light (20:00–20:30; **C**). The data are expressed as the mean  $\pm$  standard deviation ( $n=5$  per group;  $*P<0.05$ ).

5A). As  $\beta$ -adrenergic signaling plays an important role in the metabolism of BAT, we also assessed the expression of the *Adrb3* mRNA and found that it was increased by 2.4-fold in the iBAT of sEE mice at the point of transition from light to darkness compared with control mice (Fig. 5B) PGC-1 $\alpha$  is a BAT-specific marker encoded by the *Ppargc1a* gene, which mediates phenotypic transition from energy storage to energy expenditure [9], and we found that the expression of this gene was upregulated by 4.2-fold in the rpWAT of sEE mice at the point of transition from light to darkness (Fig. 5C). Overall, these data suggest that an sEE causes not only thermogenesis

in iBAT but may also affect the white-to-brown fat transformation in rpWAT.

### Immune checkpoint blockade and an sEE have additive effects on the survival of mice implanted 3LL cells

To test whether an sEE can potentiate cancer immunotherapy, mice in our spontaneous metastasis model were housed in an sEE with or without anti-PD-1 therapy. As shown in Fig. 6A, the administration of anti-PD-1 antibody significantly improved the survival rate when combined with an sEE. Micro-CT analysis indicated that



**Fig. 4.** The simplified enriched environment (sEE) reduces brown adipose tissue mass at the point of transition from light to darkness. Representative images of interscapular brown adipose tissue (iBAT; A), retroperitoneal white adipose tissue (rpWAT; C), and gonadal white adipose tissue (gWAT; E) from mice housed under control or sEE conditions. Mean weights of iBAT (B), rpWAT (D) and gWAT (F) at the point of transition from light to darkness (09:00) and from darkness to light (21:00). The data are expressed as the mean  $\pm$  standard deviation ( $n=4-5$  per group;  $*P<0.05$ ).

the anti-PD-1 therapy suppressed the development of lung tumor nodules (Fig. 6B). These data strongly suggest that the sEE enhanced immune system activity around tumors that are sensitive to PD-1 blockade.

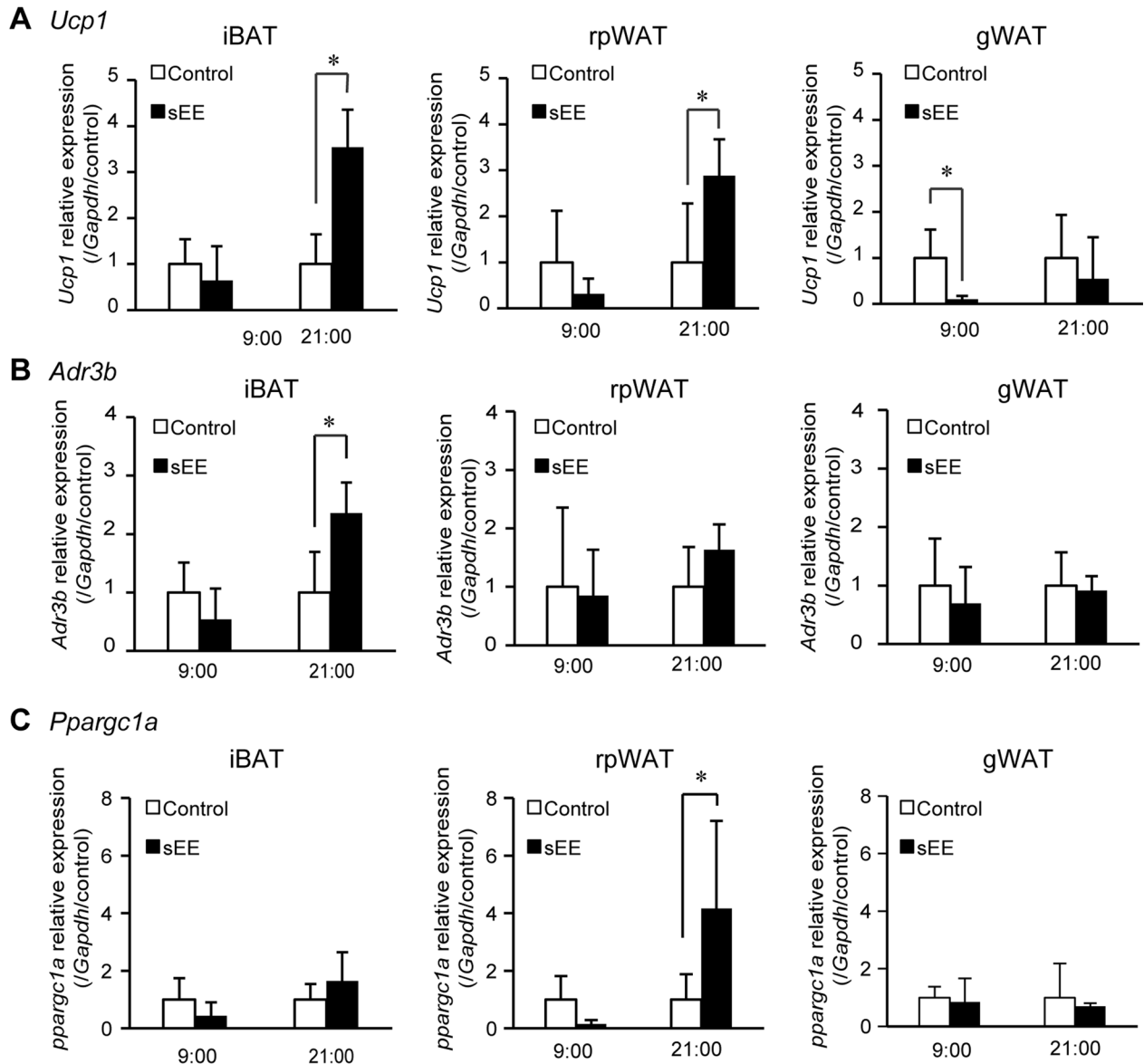
## Discussion

Ours is the first study showing that the sEE increased body temperature at the point of transition from darkness to light and that this could be associated with beneficial effects on cancer, thus suggesting a mechanism for cancer prevention via the sEE. Despite a long history of anecdotal and epidemiological reports showing that hyperthermia is associated with anticancer effects, systematic evidence has been poor. The results of our study indicate that, similar to the complex EE, the sEE increases body temperature even after tumor transplantation and that it is associated with the upregulation of metabolism-related genes as well as the inhibition of tumor growth and metastasis. This notion is consistent with earlier findings indicating that increased body temperature may influence the tumor microenvironment through temperature-dependent mechanisms, which in

turn affect tumor metabolism and blood supply as well as anticancer immune reactions including lymphocyte infiltration and expression of pro-inflammatory cytokines [20]. Our previous study showing that continuous intake of *I. obliquus* extract can potentially suppress cancer development through maintenance of body temperature [1] supports the notion of an association between oncogenesis and thermogenesis. Furthermore, it was found that maintaining mice at a higher temperature (30–31°C) slows tumor growth via the reduction of immunosuppressive T cells [11]. These data are in agreement with the previous finding showing that hypothermia activates adipocytes to stimulate tumor growth [7] and suggest that the upregulation of body temperature in an sEE could play a critical role in tumor suppression.

A previous study showed that a complex EE decreased adiposity, stimulated energy expenditure, and increased brown-like cells in white fat [4]; therefore, an sEE may produce similar effects. Interestingly, a reduction in iBAT mass was observed only at the point of transition from light to darkness, not at the point of transition from darkness to light; this may be attributed to specific circadian signals from the hypothalamus that shape adipose tissue

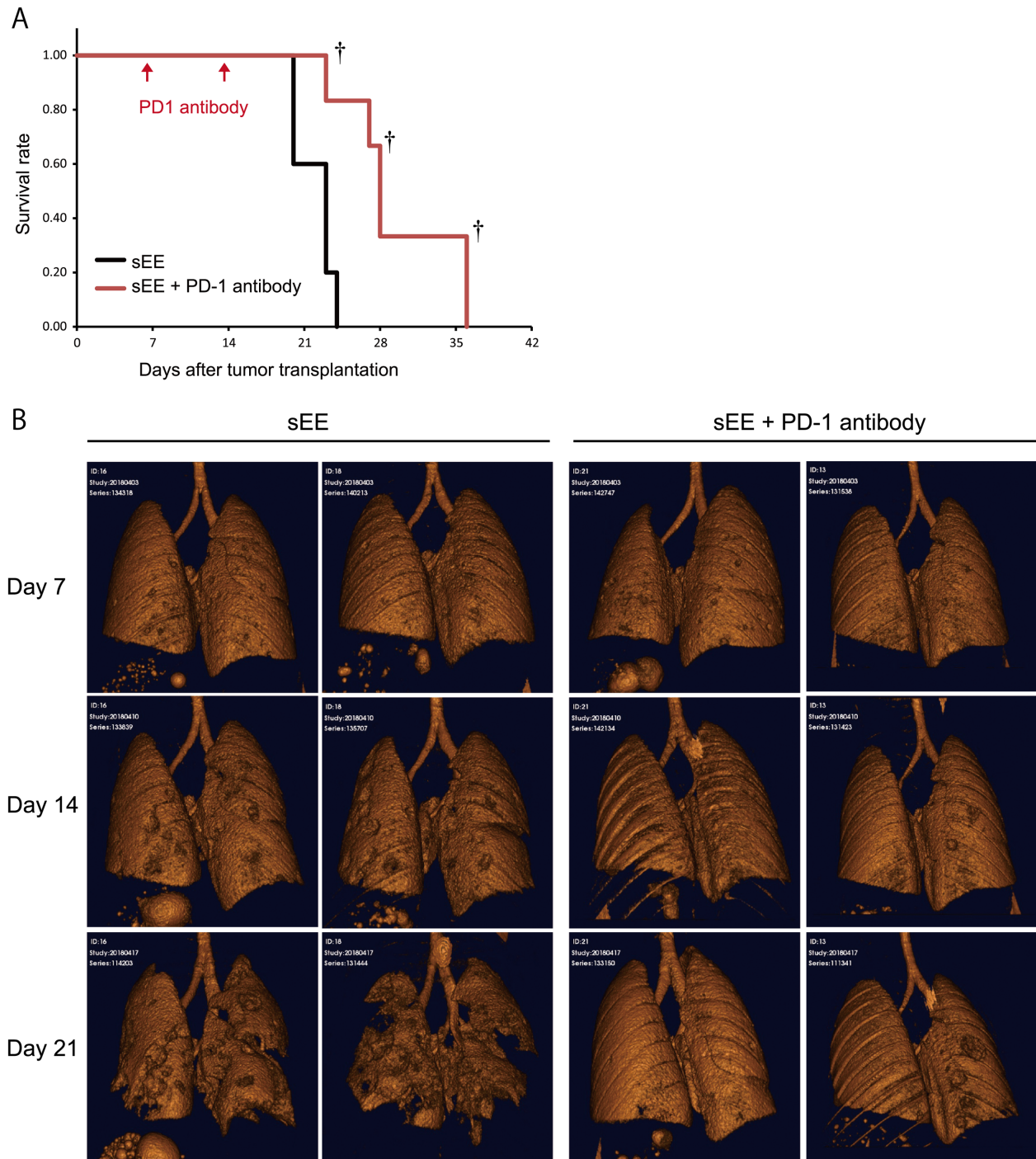




**Fig. 5.** The simplified enriched environment (sEE) increased *Ucp1* and *Adrb3* mRNA expression in interscapular brown adipose tissue (iBAT) at the point of transition from light to darkness. Mice living under control or sEE conditions were analyzed for gene expression in iBAT, retroperitoneal white adipose tissue (rpWAT), and gonadal white adipose tissue (gWAT) at the point of transition from light to darkness (09:00) and from darkness to light (21:00). A, *Ucp1*. B, *Adrb3*. C, *Ppargc1a*. The data are expressed as the mean  $\pm$  SD (n=4–5 per group; \* $P$ <0.05).

metabolism and can influence glucose uptake and energy expenditure in BAT [17]. BAT dissipates energy in the form of heat through thermogenesis in a process primarily mediated by the mitochondrial protein UCP1. In humans, mRNA expression of *UCP1* is highest just before waking up and gradually decreases while awake [12], indicating that BAT participates in glucose clearance. In this scenario, the sEE may activate the sympathetic nervous system to maintain glucose consumption and calorie burning in BAT at the start of the active period. In this study, the body temperature of control mice immediately increased after waking up (the point of transition from light to darkness) and gradually de-

creased during the waking period, reaching the lowest level at the point of transition from darkness to light (Fig. 3A). On the other hand, the body temperature of the mice in the sEE was maintained at a higher level while they were awake and gradually decreased after the point of transition from darkness to light but was still at a higher level than in the control mice. This delayed decrease of body temperature in sEE mice during the daily light cycle could be explained by rapid consumption of iBAT at the point of waking and upregulated consumption of iBAT while they were awake. The weight of iBAT in sEE mice was significantly decreased at 21:00 (the point of transition from light to darkness) but returned to the same



**Fig. 6.** Immune checkpoint blockade using an anti-programmed cell death 1 (PD-1) antibody has a synergistic effect with sEE on improving the survival of mice implanted with Lewis lung carcinoma cells. A, Kaplan-Meier curves showing survival;  $P < 0.05$ , sEE vs. sEE+anti-PD-1 antibody.  $n = 5-6$  per group. The † symbol indicates humane endpoint euthanasia. The other animals died before meeting criteria for euthanasia. B, Representative computed tomography images of mouse lungs containing carcinoma nodules on days 7, 14, and 21 post tumor transplantation under sEE conditions with or without the administration of 0.1 mg anti-PD-1 antibody 7 and 14 days post tumor transplantation.

level at 08:00 (the point of transition from darkness to light) (Fig. 4A and B). These data support the hypothesis that sEE stimulates energy metabolism.

Notably, the upregulation of UCP1, which is involved in the signaling pathway dissipating the mitochondrial proton gradient as well as in heat generation, was only

detected at the point of transition from light to darkness. This finding is consistent with the observation that BAT in humans and rodents exhibits rhythmicity in glucose consumption, which peaks before waking [17]. Moreover, the temporal oscillation of UCP1 levels in BAT explants from healthy adults is inversely correlated with

the expression pattern of the nuclear receptor REV-ERB $\alpha$ , which is known to participate in the circadian regulation in adipose tissue by directly suppressing *Ucp1* expression and thermogenic capacity [17]. Together with our results, these data collectively suggest that the anti-tumor effects of eustress modeled by the EE are mediated by the regulation of thermogenesis via pathways that are similar in both rodents and humans.

Although immunotherapy is a promising treatment, most patients do not show long-lasting remission; this underscores the need to better understand the variation in benefits of PD-1 pathway modulation among patients. Recent studies found that the gut microbiome influences the efficacy of PD-1-based immunotherapy against melanoma and other epithelial tumors [10, 14, 22]. Surprisingly, obesity is also reported to have a positive effect on the efficacy of PD-1 antibody therapy [30], while another study showed that  $\beta$ -adrenergic signaling in mice undermined checkpoint inhibitor efficacy [3]. In our study, we found that PD-1 antibody therapy improved the survival rate of sEE mice implanted with tumor cells. Although further experiments are required to clarify the mechanism of action, our model can be a useful tool because of its reproducibility and the fact that the mice are immunologically naïve.

In conclusion, our sEE model produces results that are consistent with those obtained using complex EEs regarding mouse behavior and anticancer effects. Thus, the sEE can potentially be used in further studies to standardize EE conditions. The sEE promoted suppression of tumor growth and lung metastasis, which could correspond to the increase in body temperature and iBAT mass, especially during the transition from darkness to light. Our findings indicate that the sEE model could be a useful tool for studying the antitumor effects of eustress, in combination with existing chemotherapeutic agents such as immune checkpoint inhibitors. The sEE provides motor and social stimulations to the animals, which mimics a social environment for an active lifestyle of humans with adequate physical exercises and supportive social interactions. Therefore, our data potentially open new perspectives in cancer prevention and treatment.

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