New cancer cachexia rat model generated by implantation of a peritoneal dissemination-derived human stomach cancer cell line

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Terawaki K, Sawada Y, Kashiwase Y, Hashimoto H, Yoshimura M, Suzuki M, Miyano K, Sudo Y, Shiraishi S, Higami Y, Yanagihara K, Kase Y, Ueta Y, Uezono Y. New cancer cachexia rat model generated by implantation of a peritoneal dissemination-derived human stomach cancer cell line. Am J Physiol Endocrinol Metab 306: E373–E387, 2014. First published December 17, 2013; doi:10.1152/ajpendo.00116.2013.—Cancer cachexia (CC), a syndrome characterized by anorexia and body weight loss due to low fat-free mass levels, including reduced muscle mass, markedly worsens patient quality of life. Although stomach cancer patients have the highest incidence of cachexia, few experimental models for the study of stomach CC have been established. Herein, we developed stomach CC animal models using nude rats subcutaneously implanted with two novel cell lines, i.e., MKN45c185, established from the human stomach cancer cell line MKN-45, and 85As2, derived from peritoneal dissemination of orthotopically implanted MKN45c185 cells in mice. Both CC models showed marked weight loss, anorexia, reduced muscle and muscle strength, increased inflammatory markers, and low plasma albumin levels; however, CC developed earlier and was more severe in rats implanted with 85As2 than in those implanted with MKN45c185. Moreover, human leukemia inhibitory factor (LIF), a known cachectic factor, and hypothalamic orexigenic peptide mRNA levels increased in the models, whereas hypothalamic anorexigenic peptide mRNA levels decreased. Surgical removal of the tumor not only abolished cachexia symptoms but also reduced plasma LIF levels to below detectable limits. Importantly, oral administration of rikkunshito, a traditional Japanese medicine, substantially ameliorated CC-related anorexia and body composition changes. In summary, our novel peritoneal dissemination-derived 85As2 rat model developed severe cachexia, possibly caused by LIF from cancer cells, that was ameliorated by rikkunshito. This model should provide a useful tool for further study into the mechanisms and treatment of stomach CC.

cachexia; leukemia inhibitory factor; rikkunshito; stomach cancer model; anorexia

CANCER CACHEXIA, A MULTIFACTORIAL SYNDROME characterized by anorexia and the loss of body weight, adipose tissue, and skeletal muscle, is observed in 80% of advanced cancer patients and accounts for at least 20% of cancer-related deaths (20, 35, 42). This syndrome causes not only poor quality of life (QOL) but also poor responses to chemotherapy, highlighting the need for improved cancer cachexia treatments. Weight loss, the most prominent clinical feature of cachexia, is observed in 30–80% of cancer patients, depending on tumor type. For example, weight loss occurs at a very high frequency (83%) in stomach and pancreatic cancer patients but is less prominent in patients with breast cancer, acute nonlymphocytic leukemia, and sarcomas (35). Although cachexia strongly impacts the success of therapeutic treatments, the mechanisms underlying this syndrome are not fully understood. Stomach cancer patients in particular have the highest incidence of cachexia; however, few experimental models for the study of stomach cancer cachexia have been established (4, 14, 66).

A useful cachexia model must meet three of the following five diagnostic criteria in addition to weight loss: anorexia, decreased muscle strength, fatigue, low fat-free mass (FFM) index, and abnormal biochemistry (anemia, increased inflammatory markers, and low serum albumin) (14). Moreover, Argilés et al. (1) reported that two specific indicators, anorexia and metabolic disturbances, should be identified before arriving at a diagnosis of cachexia-associated weight loss. Although body weight maintenance is the most important endpoint of any cachexia treatment, body composition and QOL should also be monitored (1). Accordingly, anorexia and body composition are very important in both the diagnosis and treatment of cachexia and should be present in any experimental model designed to study this syndrome.

To address the need for an experimental stomach cancer cachexia animal model, we previously screened 15 human stomach cancer cell lines for their ability to induce weight loss in mice after subcutaneous implantation (63). Among the cell lines that were screened, only the MKN-45 cell line induced body weight loss, with an incidence of 40% in tumor-bearing mice (63). On the basis of these findings, we established two novel cell lines from MKN-45 cells: MKN45c185 and 85As2 (63). Implantation of MKN45c185 cells induces body weight loss in mice with 100% efficiency. The 85As2 cell line, derived from peritoneal metastasis of orthotopically implanted MKN45c185 cells, has a strong capacity to induce peritoneal dissemination and body weight loss (100% efficiency) in mice. Anorexia is a key factor in both the diagnosis and treatment of cachexia. Appetite facilitation reinforces physical strength and improves QOL. Thus, anorexia is very important for the evaluation of cachexia. In our previous mouse model, evaluation of cachexia based on weight loss was possible, whereas anorexia could not be used to assess cachexia because of instability in the reduction of food consumption (63). There-
fore, our mouse cachexia model was not suitable to evaluate drug efficacy or mechanisms of cachexia-associated anorexia. To address this problem, we aimed to establish two novel stomach cancer cachexia models by implanting MKN45cI85 and 85As2 cell lines into nude rats. We determined the usefulness of these cancer cachexia models in evaluating anorexia, body composition changes (including low FFM), and weight loss. Moreover, body composition changes, including low FFM, are useful in elucidating the mechanisms of anorexia associated with stomach cancer cachexia. To investigate the underlying mechanisms of cachexia in these models, plasma levels of cytokines known to be involved in cancer cachexia development, such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)α, and leukemia inhibitory factor (LIF) (13, 19, 40, 58), were also evaluated. Because the hypothalamus is a key regulator of energy homeostasis and a major site for the integration of metabolic signals in the central nervous system, the expression of hypothalamic feeding-regulating peptides was determined. In addition, the expression of the muscle-specific E3 ubiquitin ligases atrogin-1/muscle atrophy F-box (MAFbx) and muscle RING finger 1 (MuRF-1), which are important mediators of skeletal muscle loss, was also evaluated (6, 25).

To assess the efficacy of our novel stomach cancer cachexia rat models in evaluating treatment outcomes, we examined the impact of rikkunshito therapy on cachexia-associated symptoms in these models. Rikkunshito has been approved by the Ministry of Health, Labor, and Welfare of Japan and is widely prescribed as a remedy for various gastrointestinal syndromes, such as anorexia, dyspepsia, and gastritis (29, 44). Rikkunshito was found to improve anorexia symptoms in a double-blind study of patients with functional dyspepsia (29). Additionally, increasing evidence from experimental animal models has shown that rikkunshito ameliorates several types of anorexia (23, 24, 57). It has been reported that with rikkushito there is an increase in the secretion of the orexigenic hormone ghrelin by the inhibition of 5-HT2B/2C receptors, an enhancement of ghrelin receptor (GHSR) signaling efficiency, and a facilitation by the inhibition of 5-HT2B/2C receptors, an enhancement of stomach emptying and gastric adaptive relaxation, all of which are important mediators of skeletal muscle loss, was also evaluated (6, 25).

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**MATERIALS AND METHODS**

**Animal experimentation.** Six-week-old male F344/NJcl-mu/mu rats (Cleaa-Japan, Tokyo, Japan) were housed individually under a 12:12-h light-dark cycle (lights on at 0800) at a constant temperature and humidity, with ad libitum access to food and water. Rats were allowed to acclimate to laboratory conditions for 2 wk prior to experimentation. All studies were performed according to the Guidelines for Animal Experiments drafted by the Committee for Ethics in Animal Experimentation of the National Cancer Center and approved by the committee (approval nos. T09-050-M02 and T09-050-C04), and they met the ethical standards required by the law and the guidelines concerning experimental animals in Japan.

**Cell lines and culture conditions.** MKN45cI85 and 85As2 cell lines were established from the human stomach MKN-45 cancer cell line, as described previously (63). Cells were maintained in RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 IU/ml penicillin G, and 100 µg/ml streptomycin sulfate (Nacalai Tesque) under a 5% CO₂ and 95% air atmosphere at 37°C.

**Tumor cell implantation.** MKN45cI85 and 85As2 cells were harvested from subconfluent cultures after brief exposure to 0.25% trypsin and 0.2% ethylenediaminetetraacetic acid. Cells were washed once in serum-free medium and resuspended in phosphate-buffered saline. Anesthetized rats were subcutaneously inoculated with either 1 × 10⁷ cells/site (tumor-bearing rats) or saline alone (non-tumor-bearing control rats) in the left and right flanks. The major and minor tumor axes were measured, and the tumor volume was estimated using the following equation: tumor volume (cm³) = major axis (cm) × minor axis (cm) × minor axis (cm) × 1/2, and tumor volume was converted to tumor weight (mg/mm³) (14, 17, 66).

**Rat model characterization.** Body weight and composition, organ tissue weight, food and water consumption, and grip strength were evaluated in each model. Body weight and food and water consumption were measured weekly. Body composition was determined using the Impedimed Bioimpedance Spectroscopy device (ImpediMed, Brisbane, Australia) to accurately measure FFM, fat mass (FM), and total body water (TBW) (52). Blood collected from the abdominal aorta was centrifuged (3,000 rpm, 10 min), and the plasma was stored at −80°C until analysis. Organ tissues were immediately dissected and weighed. Gastrocnemius muscle tissue was fixed with 10% formalin and embedded in paraffin for histological analysis. Cross sectional area, perimeter, Feret diameter, and minimum Feret diameter were calculated using Image J software (15). Grip strength was measured using a grip strength meter for rats and mice (MCF-380CM/F; Muromachi Kikai, Tokyo, Japan).

**Tumor removal experiment.** Anesthetized rats were subcutaneously inoculated with either 85As2 1 × 10⁷ cells/site (tumor-bearing rats) or saline alone (non-tumor-bearing control rats) in the left and right flanks. Tumor growth, body weight, and food and water intake were measured weekly. Cancer cachexia clearly developed in tumor-bearing rats 2 wk after implantation. After that, tumor-bearing cancer cachectic rats were divided into two groups; one group was not treated and the other group had their tumors removed. Tumors were removed with surgical scissors, and the incision was closed using a surgical needle and sutures under anesthesia. The body composition was measured at weeks 0 (before tumor implantation), 2 (before tumor removal), and 4 and 5 (after tumor removal). Grip strength was measured, and the plasma and gastrocnemius muscle were collected 5 wk after implantation.

**Real-time polymerase chain reaction.** Real-time polymerase chain reaction (PCR) was performed as described previously (56). Briefly, the hypothalamic area was dissected on an ice-cold metal plate, and total RNA was isolated using an Isogen kit (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. First-strand cDNA was reverse transcribed from 5 µg of total RNA using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA) in a final volume of 100 µl. Diluted cDNA (2 µl) was amplified in a rapid thermal cycler (LightCycler; Roche Diagnostics, Barcelona, Spain) using LightCycler 480 SYBR Green I Mastermix (Roche, Barcelona, Spain) and the following primers: atrogin-1/MAFbx 5'-GGA GAC CCG CTA CTT GGG AA-3' (forward) and 5'-ATC AAT CGC GAG CCT GTC CTT ATC CA-3' (reverse), MuRF-1 5'-AGG ACT CCT GCT GAG TTA C-3' (forward) and 5'-TTG TGG CTC ACG TCC TT-3' (reverse), forkhead box 01 (Foxo-1) 5'-TCA GGC TAG GAG TTA GTG AGC A-3' (forward) and 5'-AGG GAG GGG TGA AGG GCA TC-3' (reverse), Foxo-3 5'-CTC AGC CAG TGG ACA GTT AA-3' (forward) and 5'-GCT CTG TAG GAA TGC TG-3' (reverse), Foxo-4 5'-GCC GAC CCT GTC CTT ATC CA-3' (forward) and 5'-TTG AGT GTG TGA CCA CCT ATC CCT A-3' (reverse), IGF-I 5'-GCC GAC CCT GTC CTT ATC CA-3' (forward) and 5'-TCC GAA TGC AGC CAT A-3' (reverse), and glyceraldehyde-3-phosphate dehydrogenase 5'-CCC CCA ATG TAT CCG TTG TG-3' (forward) and 5'-TAG CCC AGG ATG CCC TTT AGT-3' (reverse). PCR products were quantified using Light-
Cycler 480 software to analyze the exponential phase of amplification and the melting curve as recommended by the manufacturer. The amount of target mRNA in the experimental group relative to that in the control group was determined from the resulting fluorescence and threshold values (CT) using the $2^{-\Delta\Delta CT}$ method (37).

**Cytokine measurements.** Plasma levels of human IL-1β, IL-6, IL-8, TNFα, and LIF were measured using the Luminex Multiplex Assay (Affymetrix, Billerica, MA) (18). Rat IL-1β, IL-6, TNFα, keratinocyte-derived chemokine (KC), and interferon (IFN)γ plasma levels were measured using the Procarta Cytokine Assay Kit (Affymetrix). Plasma α1-acid glycoprotein and albumin levels were measured using a rat α1-acid glycoprotein enzyme-linked immunosorbent assay (ELISA) kit (ImmunoLogic Consultants Laboratory, Newberg, OR) and a rat albumin ELISA kit (Shibayagi, Gunma, Japan), respectively. Human cytokine levels were also measured in MKN45c185 and 85As2 cell culture supernatants ($5 \times 10^5$ cells/well) at 24 and 48 h.

**In situ hybridization.** In situ hybridization was performed as described previously (55). Briefly, frozen 12-μm-thick coronal brain sections were prepared in a cryostat at −20°C, thawed, and mounted onto gelatin/chrome alum-coated slides. The paraventricular nucleus

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**Fig. 1.** Effects of MKN45c185 and 85As2 cell implantation on body weight (A), tumor size (B), fat-free mass (FFM; C), fat mass (FM; D), total body water (TBW; E), and muscle and adipose tissue weights in nude rats (F). Rats were inoculated subcutaneously (sc) with MKN45c185 or 85As2 cells in both flanks ($1 \times 10^7$ cells/each site) at week 0. Rats inoculated with saline served as a control group. Each data point or bar represents the mean ± SE of 4–5 rats. Differences between groups were evaluated using Student’s t-test or Welch’s t-test. *P < 0.05, **P < 0.01, ***P < 0.001 vs. the control group. Gre, greater pectoral muscle; Gas, gastrocnemius muscle; Sol, soleus muscle; Epi, epididymal fat; Ren, perirenal fat; Mes, mesentery fat.
Table 1. Body, tumor, muscle, fat, and organ weights in nude rats 4 wk after implantation with different 85As2 cell concentrations

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>1 × 10⁶</th>
<th>1 × 10⁷</th>
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<tbody>
<tr>
<td>TW</td>
<td>0.00 ± 0.00</td>
<td>5.19 ± 1.54*</td>
<td>11.00 ± 2.31***</td>
</tr>
<tr>
<td>TV</td>
<td>0.00 ± 0.00</td>
<td>3.77 ± 1.27**</td>
<td>8.75 ± 1.50***</td>
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<tr>
<td>BW</td>
<td>259.62 ± 5.84</td>
<td>219.78 ± 8.98***</td>
<td>192.96 ± 8.62***</td>
</tr>
<tr>
<td>%control</td>
<td>100.0 ± 2.3</td>
<td>84.7 ± 3.5***</td>
<td>74.3 ± 3.2***</td>
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<tr>
<td>BW CTW</td>
<td>259.62 ± 5.84</td>
<td>216.01 ± 10.18***</td>
<td>184.20 ± 9.25***</td>
</tr>
<tr>
<td>%control</td>
<td>100.0 ± 2.3</td>
<td>83.2 ± 3.9***</td>
<td>71.0 ± 3.6***</td>
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<tr>
<td>BW − TW</td>
<td>259.62 ± 5.84</td>
<td>214.59 ± 10.39***</td>
<td>181.96 ± 9.91***</td>
</tr>
<tr>
<td>%control</td>
<td>100.0 ± 2.3</td>
<td>82.7 ± 4.0***</td>
<td>70.1 ± 3.8***</td>
</tr>
<tr>
<td>Peak BW</td>
<td>226.89 ± 6.06</td>
<td>206.63 ± 7.15</td>
<td></td>
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<tr>
<td>%peak BW</td>
<td>94.4 ± 2.7</td>
<td>88.0 ± 3.5***</td>
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Muscle weights

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<th>1 × 10⁷</th>
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<tbody>
<tr>
<td>Greater pectoral</td>
<td>2.67 ± 0.11</td>
<td>1.88 ± 0.10***</td>
<td>1.42 ± 0.04***</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>1.37 ± 0.04</td>
<td>1.17 ± 0.04***</td>
<td>0.97 ± 0.06***</td>
</tr>
<tr>
<td>Tibialis</td>
<td>0.54 ± 0.02</td>
<td>0.42 ± 0.03***</td>
<td>0.41 ± 0.04***</td>
</tr>
<tr>
<td>Soleus</td>
<td>0.08 ± 0.01</td>
<td>0.07 ± 0.00*</td>
<td>0.07 ± 0.01*</td>
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</table>

Fat weights

<table>
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<th>Control</th>
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<th>1 × 10⁷</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epididymis</td>
<td>3.78 ± 0.19</td>
<td>2.73 ± 0.36*</td>
<td>1.79 ± 0.30***</td>
</tr>
<tr>
<td>Perirenal</td>
<td>2.58 ± 0.28</td>
<td>1.53 ± 0.45</td>
<td>0.64 ± 0.34***</td>
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<tr>
<td>Mesentery</td>
<td>1.41 ± 0.13</td>
<td>0.95 ± 0.29</td>
<td>0.40 ± 0.18***</td>
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</table>

Organ weights

<table>
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<th>1 × 10⁶</th>
<th>1 × 10⁷</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>10.36 ± 0.33</td>
<td>7.69 ± 0.56***</td>
<td>6.69 ± 0.31***</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.63 ± 0.03</td>
<td>0.48 ± 0.02**</td>
<td>0.49 ± 0.03***</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SE of 5 rats; all weight data are expressed in g. BW, tumor weight; TV, tumor volume; CTW, converted tumor weight. Rats were implanted subcutaneously with either 85As2 cells (1 × 10⁶ or 1 × 10⁷ cells/site) or saline alone in both flanks. TV was estimated using the following equation: TV (cm³) = major axis (cm) × minor axis (cm) × minor axis (cm) × 1/2, and the TV was converted to tumor weight (mg/mm³). BW and TV are expressed as the total for both sites. Values for bilateral tissues represent the mean of those for the 2 unilateral tissues. BW comparisons between the control group and 85As2 groups at 4 wk after implantation showed the following relationship: %control (%) = BW of each 85As2 group/BW of control group × 100. BW comparisons between peak BW and BW at 4 wk after implantation in each 85As2 group showed the following relationship: %peak body weight (%) = BW − TV) at 4 wk after implantation/peak BW (− CTW) × 100. Differences between groups were evaluated using Student’s t-test. Differences in BW and TV between the control group and 85As2 groups were evaluated using the Kruskal-Wallis test, followed by a post hoc Dunn’s multiple comparison test. *p < 0.05, **p < 0.01, and ***p < 0.001 vs. the control group; #p < 0.05 and ##p < 0.01 vs. the 85As2 cell 1 × 10⁶ cell group; †p < 0.05 vs. each peak BW.

Respiratory metabolism. Oxygen consumption was measured with an O₂/CO₂ metabolism-measuring system (MK-5000RQ; Muromachi Kikai, Tokyo, Japan) (33, 45). Each rat was kept unrestrained in a sealed chamber with an airflow of 0.5 l/min at 25°C for 20 h without food. Air was sampled every 3 min, and oxygen consumption (V˙O₂) and carbon dioxide production (V˙CO₂) were calculated (ml·min⁻¹/kg). Locomotor activity was measured simultaneously with an attached device. The respiratory quotient (RQ) was calculated by dividing V˙CO₂ by V˙O₂. Metabolic calories (E) were calculated using an MCID imaging analyzer (Imaging Research, St. Catherines, ON, Canada). The images were captured by a charge-coupled device camera (Dage-MTI, Michigan City, IN). Mean absorbance was measured and compared with simultaneously exposed ¹⁴C microscale samples (Amersham). The standard curve was fitted according to the absorbance of the ¹⁴C microscale on the same film.

Palliative therapeutic studies using rikkunshito. Rikkunshito was manufactured by Tsumura (Tokyo, Japan) by spray-drying a hot water
extract from the following eight crude drugs to form a powdered extract: Atractylodis lanceae rhizoma (4.0 g), Ginseng radix (4.0 g), Pinelliae tuber (4.0 g), Hoelen (4.0 g), Zizyphi fructus (2.0 g), Aurantii nobilis pericarpium (2.0 g), Glycyrrhizae radix (1.0 g), and Zingiberis rhizoma (0.5 g). The powdered rikkunshito extract was obtained from Tsumura. For oral administration into the stomach, rikkunshito was dissolved in distilled water (10 g/ml) over the same period. Non-tumor-bearing rats (control group) were administered DW (10 ml/kg) over the same period. Differences between groups were evaluated using the Mann-Whitney U-test. *P < 0.05 and ***P < 0.001 vs. the control group.

RESULTS

Implantation of MKN45cl85 and 85As2 cells induced cancer cachexia in rats. Subcutaneous implantation of either MKN45cl85 or 85As2 cells in rats induced progressive tumor growth beginning 1 wk after implantation and affected body weight and composition. Body weight was markedly reduced 2 wk after implantation of MKN45cl85 and 85As2 cells compared with controls, and thereafter, the differences gradually increased (Fig. 1, A and B). Additionally, all body composition parameters (FFM, FM, and TBW) were significantly lower in the MKN45cl85 and 85As2 groups than in the control group (Fig. 1, C–E). Moreover, all muscle and adipose tissue weights in cachectic rat (C) and adipose tissue weight in a control rat (B) and cachectic rat (C). D and E: expression of the E3 ubiquitin ligases atrogin-1 (D) and muscle RING finger 1 (MuRF-1; E) in gastrocnemius muscle tissue. Each bar represents the mean ± SE of 7–8 rats. Differences between groups were evaluated using the Mann-Whitney U-test. *P < 0.05 and ***P < 0.001 vs. the control group.

Fig. 3. Atrophy and expression of E3 ubiquitin ligases in the gastrocnemius muscle in 85As2-bearing cachectic rats 4 wk after implantation. Nude rats were inoculated subcutaneously with 85As2 cells (1 × 10⁷ cells/each site) or saline (control) in both flanks at week 0. A: macroscopic observation of the gastrocnemius muscle in a control rat (top muscle in the photo) and cachectic rat (bottom muscle in the photo). B and C: histological observation of the gastrocnemius muscle tissue in a control rat (B) and cachectic rat (C). D and E: expression of the E3 ubiquitin ligases atrogin-1 (D) and muscle RING finger 1 (MuRF-1; E) in gastrocnemius muscle tissue. Each bar represents the mean ± SE of 7–8 rats. Differences between groups were evaluated using the Mann-Whitney U-test. *P < 0.05 and ***P < 0.001 vs. the control group.
were significantly reduced in cachectic rats at 12 wk after implantation compared with control rats (Fig. 1F). Symptoms of cancer cachexia, including weight loss, low FM and FFM, and wasting of muscle and adipose tissues, were more pronounced in the 85As2 group than in the MKN45cl85 group.

85As2-induced cancer cachexia rat model characterization. Because the 85As2 model induced more severe cancer cachexia in rats than the MKN45cl85 model, the 85As2-induced cancer cachexia model was characterized further. Tumor volume grew progressively in a cell concentration-dependent manner, reaching 1.94 ± 0.57 and 4.38 ± 0.68 cm³ at 4 wk after implantation of 1 × 10⁶ and 1 × 10⁷ 85As2 cells, respectively (Fig. 2A). The body weight of the control group continued to increase during the experiment, whereas the body weight of the 85As2 groups did not. Body weight loss was higher in the 85As2 groups than in the control group beginning

Fig. 5. Effects of tumor removal on body weight (A), food intake (B), water intake (C), FFM (D), FM (E), TBW (F), atrogin-1 (G), MuRF-1 (H), forkhead box O (Foxo; J), insulin-like growth factor-1 (IGF-1; J), grip strength (K), and plasma leukemia inhibitory factor (LIF; L) levels in nude rats implanted with 85As2 cells (1 × 10⁷ cells/each site) or saline (control) in both flanks at week 0. Plasma and the gastrocnemius muscle of the rats were collected 5 wk after implantation. Each data point or bar represents the mean ± SE of 8–9 rats (A–C, K, and L). Each data point or bar represents the mean ± SE of 5 rats (D–J). Differences between groups were evaluated using Student’s t-test or the Mann-Whitney U-test. Differences in plasma LIF levels between groups were evaluated using the Kruskal-Wallis test, followed by post hoc Dunn’s multiple comparison test. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the control group; #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. the corresponding 85As2 group.
at 2 wk after implantation and became significant at 3 and 2 wk after implantation of $1 \times 10^6$ and $1 \times 10^7$ cells, respectively (Fig. 2A). The differences in body weight between the 85As2-implanted groups and corresponding control groups were greatest at 4 wk after implantation. The differences in body weight between the 85As2 groups and control group were 70.1–74.3 and 82.7–84.7% regardless of body weight with or without the tumor weight at 4 wk after implantation of $1 \times 10^7$

### Table 2. Plasma levels of human cytokines in the cancer cachexia rat models and cell culture supernatants

<table>
<thead>
<tr>
<th>Time after Inoculation</th>
<th>Cells</th>
<th>IL-1β</th>
<th>IL-6</th>
<th>IL-8</th>
<th>TNFα</th>
<th>LIF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasm (2 wk)</td>
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<td>10.36 ± 0.70***</td>
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<td>611.74 ± 3.84**</td>
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<tr>
<td>MKN45cl85</td>
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<td>321.47 ± 22.98</td>
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<tr>
<td>85As2</td>
<td>$5 \times 10^3$</td>
<td>ND</td>
<td>ND</td>
<td>19.02 ± 3.93***</td>
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<td>559.98 ± 25.16</td>
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<td>729.91 ± 22.50</td>
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Cytokine levels in cell culture supernatants are expressed as the mean ± SE of triplicate wells in pg/ml, and plasma cytokine levels are expressed as the mean ± SE (pg/ml) values for 4–5 rats. LIF, leukemia inhibitory factor; ND, not detectable (below the minimum detection limit of the assay). Rats were implanted subcutaneously with MKN45cl85 or 85As2 cells ($1 \times 10^5$ or $10^6$ cells/site) or saline alone in both flanks. Differences in plasma cytokine levels between groups were evaluated using the Kruskal-Wallis test, followed by a post hoc Dunn’s multiple comparison test (*$P < 0.05$ and **$P < 0.01$ vs. the corresponding control group). Supernatants were collected from 24- or 48-h incubation cultures. Differences in the cytokine levels in cell culture supernatants for the groups were evaluated using Student’s $t$-test ($**P < 0.01$ and ***$P < 0.001$ vs. the corresponding MKN45cl85 group).
and 1 $\times 10^6$ cells, respectively (Table 1). Comparison between peak body weight and body weight 4 wk after implantation showed body weight loss in each cachectic rat (1 $\times 10^7$ cells, 88.0 $\pm$ 3.5%; and 1 $\times 10^6$ cells, 94.4 $\pm$ 2.7%). These comparisons were made using the corresponding individual peak body weight. All body composition parameters (FFM, FM, and TBW) were also substantially lower in the 85As2 groups than in the control groups (Fig. 2, E–G). Reductions in food and water intake were observed beginning at 1 wk after implantation in the 85As2 groups compared with the corresponding control groups and became significant 2–4 wk later (food intake: 1 $\times 10^7$ cells, 73.5–78.7%; and 1 $\times 10^6$ cells, 89.0–90.7%; water intake: 1 $\times 10^7$ cells, 71.2–80.1%; and 1 $\times 10^6$ cells, 83.4–88.7%; Fig. 2, C and D). Similarly to the 85As2-induced cachexia model, MKN45cl85-implanted rats also exhibited marked decreases in food intake (Fig. 2H). However, the decrease in food intake was less pronounced in MKN45cl85-implanted rats than in 85As2-implanted rats (MKN45cl85, 1 $\times 10^7$ cells, 83.8–87.0% compared with the corresponding control groups).

Muscle (greater pectoral, gastrocnemius, tibialis, and soleus), adipose tissue (epididymal, perirenal, and mesentery fat), liver, and spleen weights decreased substantially in a cell concentration-dependent manner at 4 wk after implantation in cachectic rats compared with that in control rats (Table 1). Macroscopic and histological observations confirmed gastrocnemius muscle atrophy in the 85As2 group (1 $\times 10^7$ cells) at 4 wk after implantation (Fig. 3, A–C). Furthermore, all cells in Fig. 3, B and C (control, n = 51; 85As2, n = 75), were measured, and the cross-sectional area, perimeter, Feret diameter, and minimum Feret diameter were calculated. The cross-sectional area (1,460.4 $\pm$ 76.3 vs. 2,023.9 $\pm$ 85.2 $\mu m^2$, $P$ <

Fig. 7. Time course changes in oxygen consumption (VO$_2$, ml·min$^{-1}$·kg$^{-1}$; A), locomotor activity (B), respiratory quotient (RQ; C), average cumulative locomotor activity (D), VO$_2$ (ml·min$^{-1}$·kg$^{-1}$) (E), and metabolic calories (cal·min$^{-1}$·kg$^{-1}$; F) from 1000 to 1400 at 4 wk after 85As2 cell implantation in nude rats. Rats were inoculated sc with 85As2 cells (1 $\times 10^7$ cells/each site) or saline (control) in both flanks at week 0. Respiratory metabolism was measured 4 wk after implantation. Changes in body weight (G) and food intake (H) over time. Changes in body weight and food intake were evident at 4 wk after implantation. Each data point or bar represents the mean $\pm$ SE of 7 rats. Differences between groups were evaluated using Student’s t-test or the Mann-Whitney U-test. *$P$ < 0.05, **$P$ < 0.01, and ***$P$ < 0.001 vs. the control group.
NEW CANCER CACHEXIA MODEL

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0.0001), perimeter (151.1 ± 4.3 vs. 179.2 ± 4.1 μm, P <
0.0001), Feret diameter (57.5 ± 1.7 vs. 67.2 ± 1.8 μm, P <
0.001), and minimum Feret diameter (36.4 ± 1.1 vs. 44.1 ±
1.2 μm, P < 0.0001) were lower in the 85As2 group than in
the control group. A corresponding increase in the expression
of muscle-specific E3 ubiquitin ligases (atrogin-1 and MuRF-1;
Fig. 3, D–E) and reduction in grip strength were also observed
(5F). Three Foxo family members present in skeletal muscle
(Foxo1, Foxo3, and Foxo4) (49) were upregulated in the
gastrocnemius muscles of the 85As2-induced cachetic rats,
and the increase in Foxo1 was particularly prominent (Fig.
5, G–I). IGF-1 was upregulated in cachetic rats (nonsignific-
ant) (Fig. 5J). Plasma albumin level decreased in 85As2 (1 × 10^7
cells)-bearing cachetic rats, whereas the level of α1-acid
glycoprotein, the murine counterpart of human C-reactive
protein, was increased (Fig. 4, A and B). Importantly, tumor
removal restored body weight loss, food and water intake, body
composition (FFM, FM, and TBW), and grip strength (Fig. 5,
A–F and K). Furthermore, tumor removal reduced the increased
expression levels of not only atrogin-1 (85As2, 321.2% ± 123.2%;
tumor removal, 155.2% ± 40.8%) and MuRF-1 (85As2, 261.5%
± 71.3%; tumor removal, 150.7% ± 27.0%) but also
Foxo-1 (85As2, 683.7% ± 368.7%; tumor removal, 140.5% ±
29.6%) in the gastrocnemius muscle (Fig. 5, G–I).

Cytokine levels. To investigate the underlying causes of
cancer cachexia, the plasma concentrations of several proin-
flammatory cytokines were measured. Human LIF levels were
remarkably elevated in a cell concentration- and time-depen-
dent manner in rats implanted with 85As2 cells, whereas the
levels of human IL-1β, IL-6, and TNFα were not elevated in
this model at 12 wk (Table 2). Similar results were obtained in
MKN45cI85-implanted rats at 12 wk. Additionally, rat IL-1β,
IL-6, KC, and TNFα levels were below detection limits, and
IFNγ levels were unchanged in both rat models (data not shown).
Moreover, human LIF production was observed in cell
culture supernatants from both cell lines, although 85As2 cells
produced substantially higher amounts of LIF than
MKN45cI85 cells (Table 2). Human IL-8 production was also
observed in both cell lines. Furthermore, tumor removal re-
versed the increase in plasma LIF levels in 85As2-bearing
 cachetic rats (Fig. 5L).

Gene expression of hypothalamic orexigenic/anorexigenic
peptides in the 85As2-induced cachexia model. Cachexia
symptoms such as body weight loss and anorexia were induced in
rats implanted with 85As2 cells (1 × 10^7 cells; Fig. 6, B and C).
Hypothalamic feeding-regulating peptide levels were evaluated
4 wk after implantation of 85As2 cells. Orexigenic peptide
mRNA levels (NPY and AgRP in the ARC, ORX and MCH
in the LHA) were higher in 85As2 cachetic rats than in control
rats, whereas anorexigenic peptide mRNA levels (POMC and
CART in the ARC, CRH in the PVN) were lower in cachetic
rats than in control rats (Fig. 6A).

Respiratory metabolism in the 85As2-induced cachexia
model. Cachexia symptoms such as body weight loss and
anorexia were induced in rats implanted with 85As2 cells (1 × 10^7
cells; Fig. 7, G and H). V̇O_2 was higher in 85As2-induced
cachetic rats than in control rats 4 wk after implantation
(Fig. 7A). Locomotor activity was noticeably lower during
the “active” overnight period in cachetic rats than in
control rats (Fig. 7, B and D). RQ, V̇O_2, and metabolic
calorie levels were significantly higher in cachetic rats than in
control rats during the daytime period, although locomo-
tor activity was not different between the groups during this
time period (Fig. 7, C–F).

Rikkunshito ameliorates cachexia in the 85As2-induced
cahexia model. Rikkunshito increased food and water intake
rates (Fig. 8, A–F) and alleviated body weight loss, FFM,
TBW, and total muscle weight loss in 85As2-induced
 cachetic rats (Fig. 8, G–J).

DISCUSSION

Herein, we established novel stomach cancer cachexia models
by implanting nude rats with MKN45cI85 and 85As2 cells,
both of which were derived from the human stomach cancer
cell line MKN-45. These models enabled us to sequentially
evaluate anorexia and body composition changes (low FFM)
that correspond to poor QOL in human cancer patients. In
addition to anorexia and low FFM, the cachexia models
showed significant weight loss, reduced musculature and
muscle strength, and abnormal biochemistry (increased inflamma-
tory marker levels and low serum albumin levels), thereby
fulfilling the cachexia diagnostic criteria (20). Interestingly,
cancer cachexia developed earlier and was more severe in
the 85As2-bearing model than in the MKN45cI85-bearing model,
indicating that 85As2 cells derived from peritoneal dissemina-
tion possessed an enhanced ability to cause cachexia. Indeed,
the presence of peritoneal metastasis promotes cachexia and is
associated with a poor prognosis and low QOL in patients with
advanced-stage stomach cancer. Our 85As2-bearing model
may provide a useful tool for further study into the mechanisms
and potential treatment of cancer cachexia.

Characterization of the 85As2-induced cachexia rat model
showed marked weight loss and reductions in food and water

Fig. 8. Effects of rikkunshito on anorexia and body composition changes in the 85As2-induced cancer cachexia model. Changes in food intake (A), water intake (B), cumulative food intake (C), and cumulative water intake (D) over time. Comparison of food intake (E), water intake (F), body weight (G), FFM (H), TBW (I), and total muscle and fat weight (J) before and after rikkunshito administration. Rats were implanted sc with 85As2 cells in two flanks (1 × 10^7 cells/eat site) on day 0. Rikkunshito (1 g/kg “day”) or distilled water was administered orally twice a day for 7 days from day 0. Rats inculcated with saline served as a control group and were similarly administered distilled water. Each data point or bar represents the mean ± SE of 10–11 rats. Differences between saline-implanted and 85As2-implanted rats were evaluated using Student’s t-test; *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the corresponding control +
distilled water-treated group (A, B, and G–J). Differences between rikkunshito and distilled water treatments were evaluated using Student’s t-test; #P < 0.05 and
##P < 0.01 vs. the corresponding 85As2 + distilled water-treated group (A, B, and H–J). Differences between groups in the time course of cumulative food
and water intake were evaluated using 2-way repeated-measures ANOVA, followed by post hoc Bonferroni test; **P < 0.01 and ****P < 0.001 vs. the
corresponding control + distilled water-treated group; #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. the corresponding 85As2 + distilled water-treated group
(C and D). Differences between groups were evaluated using a 1-way ANOVA, followed by post hoc Dunnett’s multiple comparison test; **P < 0.01 and
***P < 0.001 vs. the 85As2 + distilled water-treated group (E and F). Differences before and after administration of either rikkunshito or distilled water were
evaluated using the paired t-test; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. the corresponding before-administration group (E and G).
atrogin-1 and animal models (2, 16). Moreover, increased expression of has been associated with muscle wasting in other cancer cachexia studies. In the present experimental models, tumor growth or cachexia developed very early. However, young rats (aged 8 wk at the time of implantation) were used; therefore, senescence, which is common in human cancer, may have been a limitation of the study. Although 85As2 cell implantation can cause peritoneal dissemination accompanied by ascites (63), the decreased food intake in our model was not associated with either condition. Reduced FFM in cachetic rats was thought to be caused primarily by wasting skeletal muscle and organ tissues, as evidenced by the reduction in all measured musculature weights, muscle atrophy (e.g., gastrocnemius muscle), and reduced spleen and liver weights. Because intracellular skeletal muscle proteins are degraded primarily by the ubiquitin-proteasome system (2, 16), increased expression of the E3 ubiquitin ligases atrogin-1 and MuRF-1 likely contributed to skeletal muscle loss by accelerating muscle protein breakdown during cancer cachexia development, as these enzymes have been associated with muscle wasting in other cancer cachexia animal models (2, 16). Moreover, increased expression of atrogin-1 and MuRF-1 in the gastrocnemius muscles of cachectic rats has been associated with increased expression of Foxo, the master regulators of muscle-specific E3 ligases, and increased Foxo expression has been shown in cancer cachexia models (49). In our study, Foxo1, Foxo3, and Foxo4 were upregulated in 85As2-induced cachetic rats, and their increased expression was thought to be associated with the increased expression of atrogin-1 and MuRF-1. Notably, the elevation of Foxo1 levels was prominent, and its blockade suppressed cachetic muscle atrophy (36). Expression of the protein synthetic factor IGF-1 has been reported to decrease in cancer cachexia models (16). However, IGF-1 expression did not decrease in cachetic rats; rather, it unexpectedly increased, although the increase was not significant. Taken together, our findings indicated that protein degradation pathways in skeletal muscle were activated in 85As2-induced cachetic rats. Importantly, tumor removal reversed the cachexia symptoms, including body weight loss, decreased food and water intake, body composition changes, and increased expression of genes that accelerate muscle protein breakdown such as atrogin-1, MuRF-1, and Foxo-1 in 85As2-bearing cachetic rats. These findings strongly indicated that the 85As2 cancer cell xenograft induced cachexia symptoms.

Increasing evidence suggests that proinflammatory cytokines, including TNFα, IL-1, IL-6, IL-10, and TGFβ, may be involved in the development of cancer cachexia (13, 19, 58). For example, high IL-6 levels have been associated with increased inflammation (20) and weight loss in patients with non-small-cell lung, pancreatic, and prostate cancers (19, 46, 51). However, other studies have suggested that cancer cachexia is not fully attributable to IL-6 levels (53). In the present study, human and rat IL-6 were not detected in the plasma of MKN45c185- or 85As2-tumor-bearing cachetic rats or in cell culture supernatants, making it unlikely that IL-6 was a causative factor for cancer cachexia in our experimental model. Moreover, human and rat IL-1β and TNFα were not detected in the plasma of tumor-bearing cachetic rats, and human IL-10 and TGFβ were not detected in the cell culture supernatants, which was similar to our previous results in a stomach cancer cachexia mouse model (63). In contrast, plasma levels of human LIF, a pleiotropic cytokine belonging to the IL-6 family, were markedly elevated in a cell concentration- and time-dependent manner in rats implanted with 85As2 cells. These findings are in agreement with a previous study showing higher LIF levels in a melanoma SEK1-induced cancer cachexia mouse model (39, 40). Furthermore, we found that tumor removal not only abolished the cachexia symptoms induced by 85As2 cells but also decreased plasma LIF levels to below detectable levels. Therefore, our findings strongly suggested that LIF is a cachectic factor in the 85As2-bearing cachexia model. To date, genetic polymorphisms of cytokines such as IL-1β, IL-8, and IL-10 have been implicated in cachexia pathogenesis in stomach cancer patients (5, 26, 54).

Our study is the first to associate LIF with stomach cancer cachexia. Although clinical evaluation of LIF is currently ongoing, LIF may be a biomarker of pathogenesis and a therapeutic target for peritoneal dissemination and cachexia in stomach cancer.

LIF and its receptor LIF-R, a heterodimeric receptor complex consisting of the ligand-specific LIF-R and signal-transducing gp130 subunit (3), are expressed in POMC neurons in the ARC and have been shown to impact signaling in the hypothalamus. LIF has been shown to inhibit food intake by directly activating POMC neurons in the ARC and stimulating the release of α-melanocyte-stimulating hormone, which in turn transduces anorexigenic signals (27). Importantly, the blood-brain barrier is relatively permissive in the ARC, allowing the neurons to access circulating macromolecules. In fact, Pan et al. (47) showed that peripherally administered LIF reached the brain and spinal cord by crossing the blood-brain barrier. Taken together, these findings suggested that LIF produced by 85As2 and MKN45c185 cell implantation induced cachexia symptoms, including anorexia, in the present study by affecting LIF receptor signaling pathways in POMC neurons in the ARC. Moreover, LIF may contribute to differences in the onset and severity of cachexia in the 85As2 and MKN45c185 cachexia models. However, further study is necessary to determine the contribution of other cachectic factors to the varying degrees of cancer cachexia in these models.

In the present study, hypothalamic levels of orexigenic peptide mRNAs (NPY and AgRP in the ARC, ORX and MCH in the LHA) were increased in the 85As2-induced cachexia model, whereas the levels of anorexigenic peptide mRNAs (POMC and CART in the ARC, CRH in the PVN) were decreased. Previous studies have shown that hypothalamic NPY release is reduced and that the feeding response to hypothalamic injection of NPY is attenuated in anorectic tumor-bearing rats despite increased hypothalamic NPY mRNA expression (9–12, 38). Moreover, proinflammatory signals (e.g., IL-1β) have been shown to decrease AgRP secretion by increase AgRP gene transcription (50). Thus, despite increases in hypothalamic NPY and AgRP mRNA expression, the anorexia induced in our model may involve impairment of NPY and AgRP release or feeding response to NPY. Interestingly, our previous study using a cisplatin-induced cachexia rat model yielded contrasting results to those of the 85As2 model, although both models exhibited decreased food intake. In the cisplatin-induced cachexia model, hypothalamic orexigenic peptide mRNA levels decreased and anorexigenic peptide mRNA levels increased (65). Cisplatin has been shown to reduce the secretion of ghrelin that activates NPY neurons,
whereas it inhibits POMC and CART expression in the ARC (34, 57). Reduced ghrelin secretion may decrease NPY mRNA levels and increase POMC and CART mRNA levels in the ARC. These findings indicated that distinct underlying mechanisms may induce cachexia-associated anorexia development in different cachexia models.

In addition to body weight loss and anorexia, patients with cancer cachexia also exhibit a reduction in physical activity corresponding to daytime activities (22, 61). Similarly, locomotor activity in the “active period” or dark phase was substantially lower in 85As2-induced cachetic rats than in control rats, whereas locomotor activity was not different between these groups during the daytime period. Reduced activity during the dark phase, but not the light phase, in cachetic rats has also been observed in other cachexia models (41, 59). Because $V_{O2}$ is thought to be affected by the amount of locomotor activity, we evaluated this parameter during the daytime period. Reduced activity was also observed in other cachexia models (41, 59). Because $V_{O2}$, RQ, and metabolic calorie levels were significantly higher in cachetic rats than in control rats during this time period. These findings suggested that enhanced energy expenditure, in addition to anorexia, may exacerbate body weight loss caused by the decrease in adipose and muscle tissues in cachetic rats. In fact, exacerbated resting energy expenditure in patients with cancer cachexia has frequently been observed (7, 8, 21) and is in contrast to the resting energy conservation associated with starvation-induced body weight loss.

Body weight maintenance is the most important end point of any treatment for cachexia-associated anorexia. Rikkunshito therapy has been shown to be an effective anorexia treatment in several animal models (50, 57); therefore, we evaluated the effect of rikkunshito on 85As2-induced cachexia symptoms. Rikkunshito substantially ameliorated cancer cachexia symptoms, including anorexia, weight loss, decreased water intake, and reductions in FFM, TBW, and musculature in the 85As2-induced cancer cachexia rat model; however, rikkunshito did not reduce tumor growth or plasma LIF levels. These findings indicated that the anticachectic effects of rikkunshito are not related to tumor regression or LIF levels. Rikkunshito has been shown to decrease the secretion of ghrelin, an orexigenic hormone (57), and also to increase ghrelin receptor (GHSR) signaling efficacy (24). GHSR is expressed in the ARC and PVN of the hypothalamus, and ligand binding stimulates NPY/AgRP neurons, thereby transducing orexigenic signals to increase food intake. Thus, rikkunshito may ameliorate anorexia by activating GHSR-NPY/AgRP orexigenic signaling in the ARC and PVN. In fact, our previous study demonstrated that rikkunshito ameliorated cisplatin-induced anorexia in rats and reversed the cisplatin-induced decrease in hypothalamic orexigenic peptide mRNA levels (NPY in the ARC) and increase in orexigenic peptide mRNA levels (POMC and CART in the ARC) (65). Because anorexia induced by cancer cachexia and cisplatin may involve different mechanisms, further study is required to clarify the mechanisms by which rikkunshito ameliorates cancer cachexia-induced anorexia.

In conclusion, we established novel stomach cancer cachexia rat models by implanting nude rats with MKN45cl85 and 85As2 cells, both of which were derived from the human stomach cancer cell line MKN-45. The 85As2-induced cancer cachexia model, which was generated using peritoneal dissemination-derived 85As2 cells, induced earlier and more severe cachexia than the MKN45cl85 model, which may have been caused by differences in LIF production. The 85As2 model allowed for the early evaluation of cancer cachexia parameters associated with poor patient QOL and metabolic disturbances, such as anorexia and body weight loss (including low FFM). Our findings also indicate that rikkunshito may improve QOL in patients with stomach cancer cachexia. The 85As2 model should provide a useful tool for further study of cancer cachexia pathogenesis and treatment.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


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