A promising cell line for research on the control of locust plagues

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Article

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A promising cell line for research on the control of locust plagues

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Abstract

The desert locust (*Schistocerca gregaria*), which forms a huge swarm, consuming large amounts of wild and agricultural plants, is the most destructive migratory pest in the world. Recently, a large-scale locust plague occurred from Africa to South Asia. The main methods used to control these pests, which involve the use of inexpensive and highly residual insecticides, are raising concerns on their impact on humans, domestic animals, and the environment. Cell lines are a useful research tool in molecular biological analysis and drug discovery; however, no cell line is yet available for the research of *S. gregaria*. Here, we succeeded in establishing a cell line (Sg-155 cells) from *S. gregaria* embryos and validated its utility. Soaking with low concentrations of dsRNA induced high and long-lasting RNAi efficiency in Sg-155 cells. Furthermore, response to an insect hormone, a candidate target as an anti-locust agent, was observed at the gene expression level. Thus, the Sg-155 cell line is useful in exploring and evaluating target genes and could therefore be applied as a high-throughput screening tool in the development of anti-locust agents.

Introduction

The desert locust (*Schistocerca gregaria*) typically exists in a solitary state at low density. However, under unusual climate conditions, including widespread and heavy rains, rapid plant growth triggers an increase in *S. gregaria* populations, leading to their transformation to the gregarious phase that is accompanied by various behavioral, morphological, and physiological changes. In some cases, gregarious locusts form a huge swarm covering more than 2000 km^2, and each locust can fly up to 150 km per day. They consume large amounts of wild and agricultural plants in events described as “locust plagues”[1, 2]. Such plagues have been documented in texts such as the Bible and Quran and have threatened human life since ancient times. To combat locust plagues, here, we developed and validated an *S. gregaria* cell line as a potentially viable research tool.

Recently, a large-scale locust plague occurred from Africa to South Asia and was widely reported by the media. The Food and Agriculture Organization of the United Nations warned that this plague could cause approximately 42 million people from Africa to Southwest Asia to face a food crisis. This plague is believed to have been initiated after two unusual cyclones in the Arabian Peninsula, and the COVID-19 pandemic made locust control difficult in some regions[3]. Since the main control methods of exterminating locusts depend on inexpensive and highly residual insecticides, their impact on humans, domestic animals, and the environment has led to serious concerns[1]. Hence, the development of a novel insecticide that specifically acts on locusts and/or can control the phase transition is desirable for alleviating these problems. Although vast ecological and

physiological knowledge has accumulated on *S. gregaria*, molecular biological tools frequently used for studying other model insects have not been widely reported for *S. gregaria* except for the recent release of genomic information. Cell lines are among the most useful research tools for molecular biological analysis and drug discovery; however, no cell line is available for *S. gregaria*. Therefore, we aimed to establish a cell line of *S. gregaria* and validated its properties. Our finding demonstrated this cell line is useful as a research tool for solving locust plagues.

Results and Discussions

A previous study successfully maintained a primary *S. gregaria* culture for a few years; however, the line was eventually lost due to its poor adaptation to the culture medium[4]. We performed primary culture using *S. gregaria* embryos under high-temperature (30°C) and nutrient-rich conditions to replicate the locust growth environment (Fig. 1a). After cultures were passaged over 50 times (1,635 days after initiation), a cell line of S. gregaria was established and named NARO-Sg-155 cells (hereafter, Sg-155 cells). The identity of Sg-155 cells was confirmed using DNA barcoding with the mitochondrial cytochrome c oxidase 1 (*COI*) gene and BLAST in NCBI (Supplementary Fig. 1). Sg-155 cells were adherent for the majority of the culture duration. Each Sg-155 cell was approximately 20 μm in diameter (Fig. 1b), spindle-shaped soon after passaging, and then stretched to form a few cellular protrusions (Fig. 1b). The cell growth curve (population doubling time = 3.35 days) showed that cell numbers plateaued in 14 days (Fig.

1b), indicating that Sg-155 cells should be passaged once every two weeks. This cell line can be stored in liquid nitrogen using 10% dimethyl sulfoxide as described in the Supplemental Experimental Procedures.

To characterize the Sg-155 properties, we first investigated the efficiency of RNA interference (RNAi), an important tool for analyzing gene functions and gene regulation processes, such as complicated signaling pathways *in vitro*[5]. We selected the juvenile hormone receptor gene (*methoprene tolerant*, *Met*)[6] and *MalE* of *Escherichia coli* as the target and control genes, respectively. Soaking double-stranded RNA (dsRNA) without the transfection reagent significantly decreased transcription to approximately 20% of baseline levels by day 2; this reduced expression level was maintained until day 8 (Fig.1c). Moreover, a near-maximal RNAi effect occurred at 0.05 ng/μL dsRNA (Fig. 1c'). In contrast, *Met* transcript expression in cells treated with *MalE* dsRNA showed no change throughout the experiment (Fig. 1c, c'), suggesting that the lower *Met* transcript expression levels in cells incubated with *Met* dsRNA was a target-specific effect. Therefore, this cell line is useful for exploring and evaluating target genes for developing insecticides against *S. gregaria*.

The pharmaceutical field now frequently employs cell-based screening for drug discovery and development[7]. The insect-specific juvenile hormone (JH) suppresses precocious metamorphosis during the larval stage and has different chemical structures across insects. Hence, the JH signaling pathway may be a suitable target for developing novel insect growth regulators (IGRs) that selectively target pests and are safe in the ambient environment[8]. Recently, cell-

based screening targeting the JH receptor was reported in mosquitoes and silkworms[9, 10]. As a proof-of-concept, we evaluated whether Sg-155 cells can be used to explore JH agonists and antagonists for developing an anti-locust agent. The Krüppel homolog 1 gene (*Kr-h1*), a JH early-inducible gene that represses insect metamorphosis[6], was induced significantly within 2 h of treatment with a JH analog (JHA, 10 μM methoprene) (Fig. 1d). Dose-response analysis demonstrated that the *Kr-h1* transcript was responsive to sub-nanomolar levels of JH III [the median effective concentration (EC_{50}), 3.3×10^{-11} M], a natural JH of the desert locust, whereas JHA (EC_{50}, 6.3×10^{-8} M) and JH precursors (farnesoic acid, FA) induced transcript concentrations that were approximately 500-fold higher than those induced by JH III (Fig. 1d'). These results suggest that this cell line can be applied as a high-throughput screening tool for developing IGRs targeting JH and for producing a novel anti-locust compound targeting other molecules.

In conclusion, we established a promising cell line of *S. gregaria* and demonstrated its usefulness in molecular biological analysis and insecticide development. Currently, universal protocols for developing insect cell lines are not available. Moreover, existing processes are time-intensive and difficult to control. Our cell line thus contributes toward achieving the efficacious control of locust populations, as researchers at public research institutes and agrochemical companies can use it for screening and identifying effective anti-locust agents with selectively to the locusts and safety to the ambient environment.

Methods

Insects. *Schistocerca gregaria* was obtained from Professor H. J. Ferenz (Germany) and reared at 31°C under 16L-8D, as described previously[11]. *S. gregaria* eggs were laid into a plastic cup containing moist sand. At 10 days after oviposition, the eggs were used for experiments. The methods were performed in accordance with relevant guidelines and regulations approved by the Animal Care Committee of the Institute of Agrobiological Sciences, National Agriculture and Food Research Organization (NARO).

Primary culture. Egg masses were disassembled with fine forceps and sterilized via immersion in 70% ethanol for 10 min. Sterilized eggs were rinsed with sterile distilled water and dried briefly on a clean bench. Rinsed eggs were dissected with fine forceps in phosphate-buffered saline (PBS), and yolk in the midgut was excised from embryos. To thoroughly remove the yolk, embryos were soaked in PBS, centrifuged (300 ×g for 3 min), and the resultant supernatant was discarded. Cleaned individual embryos were gently homogenized with a manual pestle and rinsed twice with PBS, followed by centrifugation at 300 ×g for 3 min. Homogenized cells were cultured at 30°C with MGM-464 medium[12] containing 30% fetal bovine serum (FBS; Corning, Inc., Corning, NY, USA) and 5% antibiotic-antimycotic (Gibco, Grand Island, NY, USA) in a 12.5 cm^2 flask (BD Falcon, Franklin Lakes, NJ, USA) coated with poly-L-lysine (SIGMA, St. Louis, MO, USA)[13]. The medium was partially replaced (one-third) with fresh medium every 7–14 days. After 1 month, medium without antibiotic-antimycotic was used,

and the culture was maintained for 1 year. The medium was changed to MGM-450[14] containing 30% FBS, with the amount of FBS gradually reduced to 10% with nine passages.

DNA barcoding. The identity of Sg-155 cells was confirmed using DNA barcoding[15]. Genomic DNA was extracted from 1×10^6 cells using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). A fragment of the mitochondrial cytochrome c oxidase 1 (*COI*) gene was amplified from genomic DNA using *Ex taq* DNA Polymerase (Takara Bio, Shiga, Japan) and a primer set (Supplementary Table 1). Amplicons were purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and directly sequenced using a BigDye Terminator v3.1 Cycle Sequencing kit and DNA sequencer (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were analyzed using BLAST in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Growth curve and population doubling time. To obtain the growth curve, Sg-155 cells were seeded upon the 67th passage at a density of 2×10^5 cells/well in 24-well plates (Sumilon, Sumitomo Bakelite, Shinagawa-ku, Japan) containing 500 µL medium per well. The plate was incubated at 30°C, and cells were counted with a cell counter (Watson Biolab, Kobe, Japan) for 17 days. The population doubling time was calculated as follows: population doubling time = $(t - t_0) \log 2/(\log N_t - \log N_0)$, where t and N are duration of cell culture and cell number after culturing for t days, respectively[16].

Cryopreservation. A sterilized mixture of 70% MGM-450 medium, 20% FBS, and 10% dimethyl sulfoxide was prepared for cryopreservation of the Sg-155 cells. Cells were cultured until approximately 70% confluency in a 12.5 cm^2 flask and then separated with gentle pipetting. The cell suspension was transferred into a 15-mL tube and centrifuged at 300 ×g for 2 min. After removing the supernatant, 1.6 mL of the sterilized mixture was added to cells, and 800 µL of the mixture was dispensed into individual System 100 Cryogenic Vials (Thermo Fisher Scientific, Waltham, MA, USA). Vials were placed in a BICELL (Nihon Freezer, Osaka, Japan) and then transferred to a –80°C deep freezer. The next day, vials were placed in liquid nitrogen. To reactivate cells after cryopreservation, vials were warmed quickly to 40°C in a water bath and centrifuged at 300 ×g for 3 min. After removing the supernatant, cells were resuspended via gentle pipetting in 1 mL fresh medium and transferred into a 12.5 cm^2 flask containing 1 mL of fresh medium.

RNAi experiments. Template DNA fragments of *Met* and *MalE* were PCR-amplified using primers and templates listed in Supplementary Table 1 and purified with a Wizard SV Gel and PCR Clean-Up System (Promega). dsRNAs were synthesized from the amplified DNA using a RiboMAX T7 Large Scale RNA Production System, following manufacturer protocol (Promega).

To examine the temporal effect of RNAi, Sg-155 cells (5.0×10^5 cells/well) were first seeded in a 96-well plate containing 100 µL medium, and *Met* or *MalE*

196 dsRNA was added to each well (final concentration 5 ng/μL). Cells were cultured
197 for 1–8 days after the first dsRNA treatment at 30°C. To examine concentration-
198 response relationships, cells (5.0×10^5 cells/well) were seeded in 100 μL
199 medium containing various concentrations of *Met* or *MalE* dsRNAs (0.0005–500
200 ng/μL) in 96-well plates. Cells were incubated at 30°C for 66 h and collected for
201 RNA extraction.
202
203 **Chemicals.** JH III and farnesoic acid (FA) were purchased from Sigma-Aldrich
204 and Echelon Biosciences (Salt Lake City, UT, USA), respectively. Methoprene
205 (SDS Biotech, Tokyo, Japan) was supplied by Dr. Syo Sakurai (Kanazawa
206 University). Appropriate amounts of compounds dissolved in methanol were
207 transferred into glass vials coated with polyethylene glycol 20,000 (PEG20,000;
208 Wako, Osaka, Japan). Methanol was evaporated under a stream of nitrogen,
209 appropriate amounts of medium were added to each vial, and compounds were
210 dissolved via sonication using a Biorupter (Cosmo Bio, Carlsbad, CA, USA).
211
212 **JH treatment in Sg-155 cells.** To examine the concentration-response
213 relationship, cells were seeded at 5.0×10^5 cells/well in 100 μL medium in 96-
214 well plates coated with PEG20,000 and incubated for 1 day before JH treatment.
215 Fresh medium containing JH III, FA, or JHA was added to the precultured cells,
216 which were incubated at 30°C and collected for RNA extraction.
217
218 **qPCR.** Total RNA was extracted from cells using an RNeasy Plus mini kit

(Qiagen) and used to synthesize cDNA with a PrimeScript RT reagent kit (Takara Bio). Primer sequences used to quantify *Met*, *Kr-h1*, and *RPL32* are listed in Supplementary Table 1. The internal reference was *RPL32*. Reactions were performed in a 10-μL volume containing template cDNA derived from 1 ng total RNA, 5 μL SYBR Premix Ex Taq (Takara Bio), and forward/reverse primers (0.2 μM each) on a LightCycler 480 real-time thermal cycler (Roche, Basel, Switzerland). The PCR conditions were as follows: 95°C for 5 min, followed by 55 cycles of 95°C for 5 s, and 60°C for 20 s. Relative gene expression was determined with the $2^{-\Delta\Delta Ct}$ method[17].

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Author Contributions

K.W., S.T., and T.K. designed the study; K.W., R.S., S.T., and T.K. performed the experiments; K.W., S.T., and T.K. analyzed the data; and K.W., R.S., S.T., and T.K. wrote the manuscript.

Competing Interests

The authors declare no competing interests.

Figure

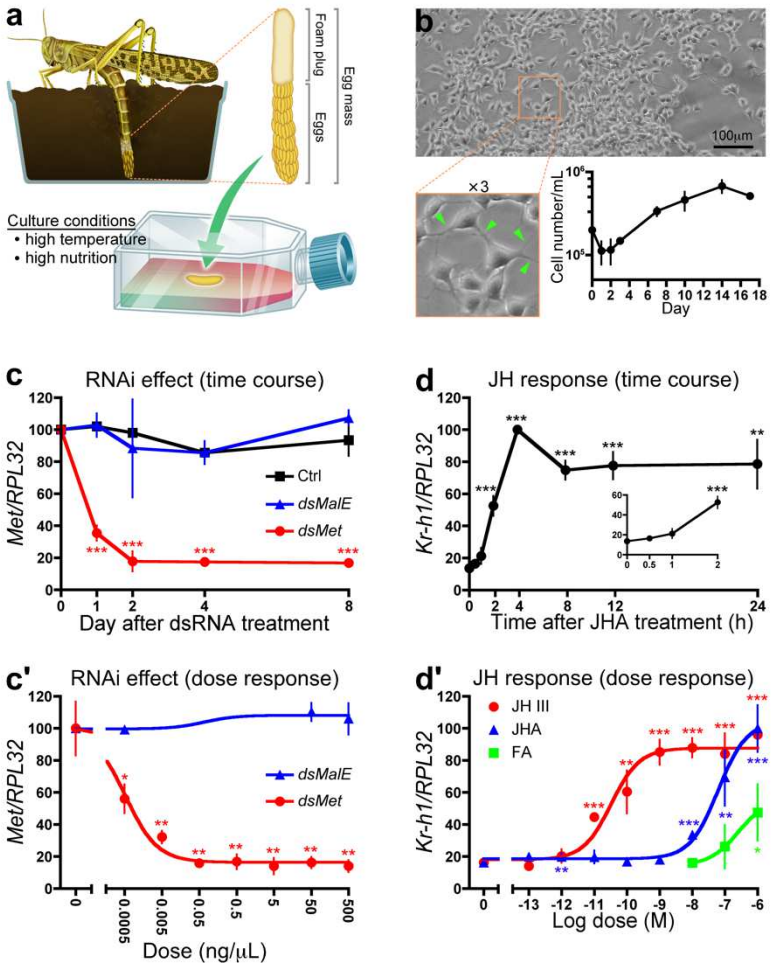

Fig. 1 Morphology and properties of Sg-155 cells. (a) Schematic overview of cell line establishment. Eggs of *S. gregaria* were separated individually and cultured under high-temperature (30°C) and eutrophic conditions (MGM-464 + 30% fetal bovine serum). (b) Phase-contrast micrograph and growth curves of Sg-155 cells. Green arrowheads indicate cellular protrusion. (c, c′) Effects of RNAi in Sg-155 cells. (c) Time course of RNAi. Cells were incubated with *Met* dsRNA (50 ng/μL *dsMet*, red), *MalE* dsRNA (50 ng/μL *dsMalE*, control, blue), or no dsRNA (water alone, control, black), and temporal changes in *Met* transcript

301 levels were measured using quantitative PCR (qPCR). (c′) Dose response of
302 RNAi. Cells were treated with different concentrations of dsRNA for *MalE* or *Met*,
303 and *Met* expression was determined using qPCR at 66 h after treatment. Data
304 were analyzed using Student's *t*-tests (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; not
305 indicated, $P > 0.05$). Each response was compared with that on day 0 (c) or that
306 for 0 ng/μL (c′). (d, d′) Responses to JHs in Sg-155 cells. (d) Time course of
307 response to JH analog (JHA, methoprene). Cells were treated with JHA (10 μM),
308 and temporal changes in *Kr-h1* transcript levels were measured using qPCR. (d′)
309 Dose-dependent response to JHs. Cells were treated with different
310 concentrations of JH III, JHA, and JH precursor (farnesoic acid, FA), and the
311 expression levels of *Kr-h1* were determined at 4 h after treatment. Data were
312 analyzed using Student's *t*-tests (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; not
313 indicated, $P > 0.05$). Each result was compared with that at 0 h (d) or that for 0
314 ng/μL (d′).

Figures

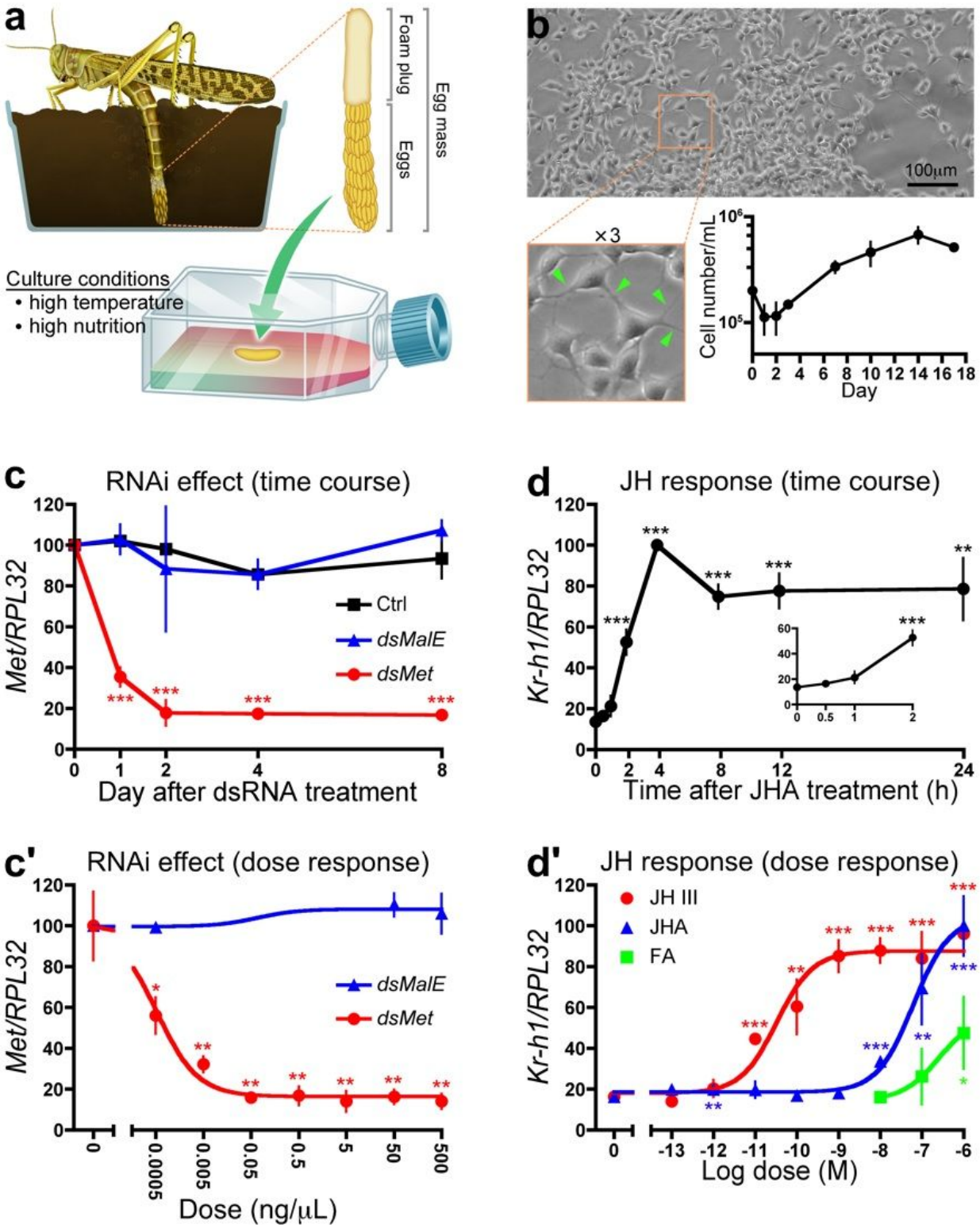

Figure 1

Morphology and properties of Sg-155 cells. (a) Schematic overview of cell line establishment. Eggs of S. gregaria were separated individually and cultured under high-temperature (30°C) and eutrophic conditions (MGM-464 + 30% fetal bovine serum). (b) Phase-contrast micrograph and growth curves of Sg-155 cells.

Green arrowheads indicate cellular protrusion. (c, c') Effects of RNAi in Sg-155 cells. (c) Time course of RNAi. Cells were incubated with Met dsRNA (50 ng/μL dsMet, red), MalE dsRNA (50 ng/μL dsMalE, control, blue), or no dsRNA (water alone, control, black), and temporal changes in Met transcript levels were measured using quantitative PCR (qPCR). 301 (c') Dose response of RNAi. Cells were treated with different concentrations of dsRNA for MalE or Met, and Met expression was determined using qPCR at 66 h after treatment. Data were analyzed using Student's t-tests (***P < 0.001; **P < 0.01; *P < 0.05; not indicated, P > 0.05). Each response was compared with that on day 0 (c) or that for 0 ng/μL (c'). (d, d') Responses to JHs in Sg-155 cells. (d) Time course of response to JH analog (JHA, methoprene). Cells were treated with JHA (10 μM), and temporal changes in Kr-h1 transcript levels were measured using qPCR. (d') Dose-dependent response to JHs. Cells were treated with different concentrations of JH III, JHA, and JH precursor (farnesoic acid, FA), and the expression levels of Kr-h1 were determined at 4 h after treatment. Data were analyzed using Student's t-tests (***P < 0.001; **P < 0.01; *P < 0.05; notindicated, P > 0.05). Each result was compared with that at 0 h (d) or that for 0 ng/μL (d').

Supplementary Files

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