Serum-free adapted Drosophila S2R+ line is amenable to RNA interference

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Abstract

We have previously adapted a select number of Drosophila cell lines to grow in serum-free media supplemented with fly extract. This condition is arguably more representative of a native growth environment. Here, we validated that the fly extract adapted line, S2R+ (FEx 2.5%) is amenable to RNAi. RNAi against Rho1 in both S2R+ and S2R+ (FEx 2.5%) produced phenotypes similar to ones previously described in Drosophila S2 cells.

![Image of cellular images and graphs showing growth ratio and Western blot analyses.]
Figure 1: A-D. Light micrographs of S2R+ and S2R+ (FEx 2.5 %) cells. S2R+ cells cultured in M3 + BPYE + 10% FBS (A,B) were treated with either dsRNA against cat (A) or dsRNA against Rho1 (B). S2R+ (FEx 2.5%) cells cultured in M3 + 2.5% FEx (C,D) were treated with either dsRNA against cat (C) or dsRNA against Rho1 (D). Scale bar = 10 micrometers. E-H Fluorescence confocal images of S2R+ cells and S2R+ (FEx 2.5%) cells. S2R+ cells cultured in M3 + BPYE + 10% FBS (E,F) were treated with either dsRNA against cat (E) or dsRNA against Rho1 (F). S2R+ (FEx 2.5%) cells cultured in M3 + 2.5% FEx (G,H) were treated with either dsRNA against cat (G) or dsRNA against Rho1 (H). * marks an enlarged and multi-nucleated cell. DAPI (purple) marks the DNA and phalloidin (green) marks cytoskeletal actin. Scale bar = 8 micrometers. I. A bar graph shows the ratio of cell numbers after seven days of RNAi treatment against Rho1 or cat, to the cell number on day 0. * denotes p < 0.05. J. Immunoblot of Rho1 and α-Tubulin from S2R+ and S2R+ (FEx 2.5%) cells, and treated with dsRNA against Rho1 or dsRNA against cat. α-tubulin serves as loading control.

Description

*Drosophila* Schneider S2 cell lines are susceptible to RNA interference (RNAi) and this attribute has cemented *Drosophila* cell lines as an important tool for high throughput functional genomics screening (Rogers and Rogers 2008; Zhou et al. 2013; Mohr 2014). RNAi against *Drosophila* Rho1 in S2 cells results in a block in mitosis, giving rise to enlarged and multinucleated cells (Rogers et al. 2004). Recently, we have adapted a select group of *Drosophila* embryonic cell lines to grow in media supplemented by adult fly extract (FEx), instead of fetal bovine serum (FBS) (Luhur et al. 2020). Here, we demonstrate that S2R+ (FEx 2.5%), the M3 + 2.5% FEx-adapted S2R+ line is also amenable to RNA interference (RNAi), similar to its parental S2R+ cells cultured in M3 BPYE + 10% FBS. We observed similar efficacious RNAi against Rho1 in S2R+ and S2R+ (FEx 2.5%) as the cells became enlarged (Figure 1A-D), multinucleated (Figure 1E-H) and failed to proliferate (Figure 1I). There was a comparable growth delay in S2R+ and S2R+ (FEx 2.5%) cells treated with Rho1 dsRNA, as the cell population doubled in 7 days (Figure 1I). In contrast, both S2R+ and S2R+ (FEx 2.5%) cells treated with double stranded RNA against a control target gene encoding the bacterial antibiotic resistance gene chloramphenicol acetyl transferase (cat) had significantly proliferated 5 fold more under similar conditions (Figure 1I). In addition, there were no significant differences in the growth ratio between S2R+ and S2R+ (FEx 2.5%) (Figure 1I) (Luhur et al. 2020). These results demonstrate that Rho1 RNAi was recapitulated robustly in S2R+ (FEx 2.5%), similar to its parental S2R+ cells. Lastly, to confirm the depletion of Rho1, we assayed for Rho1 protein levels in these cultures by Western blot. Our result indicated a strong reduction in the amount of Rho1 protein in both S2R+ (FEx 2.5%) and S2R+ cells after Rho1 knockdown (Figure 1J). In contrast, the control RNAi knockdown of cat did not affect Rho1 protein levels in either S2R+ or S2R+ (FEx 2.5%) (Figure 1J). In summary, this finding expands the utility of the fly extract-adapted cells for their use in functional genomics in a more physiologically relevant culture condition.

Methods

**Request a detailed protocol**

**Cell culture**

S2R+ (DGRC#150, FBtc0000150), S2R+ (FEx 2.5%) (DGRC#310, FBtc0000310) were cultured in M3 + BPYE + 10% FBS and M3 + 2.5% FEx, respectively, according to previously described protocol (Luhur et al. 2019).

**RNA interference (RNAi)**

Cells from the respective growth media were pelleted and then seeded at 1 million cells/ mL in serum free M3 media in a 24 well plate (1 mL per well). 10 mg/mL dsRNA was added slowly to the media and allowed to incubate at room temperature for 1 hour. After the incubation period, the M3 media was supplemented with equal volumes of either M3+BPYE+20% FBS or M3 + 5% FEx, to constitute the M3 + BPYE + 10% FBS and M3 + 2.5% FEx, respectively. Cells were allowed to grow for a week at 25°C before assaying for the loss of function phenotypes.

As a negative control, a 467-bp fragment of the chloramphenicol resistance cassette was amplified from pFastBacHT-CAT expression plasmid (Invitrogen) using the primers: T7-CAT-fwd: 5'-TAATACGACTCACTATAGGATCAAGAACAACCAGAACATCG-3′ and T7-CAT-rev: 5′-TAATACGACTCACTATAGGGGGCGAAGAAGTTGTCCATATTGGCCA-3′.

As a positive control, we amplified a 667-bp sequence for Rho1(FBgn0014020) using the primers: T7-Rho1-fwd: 5′-TAATACGACTCACTATAGGTTTGTTTTGTGTTTAGTCG-3′ and T7-Rho1-rev: 5′-TAATACGACTCACTATAGGATCAAGAACAACCAGAACATCG-3′, from a Rho1 expression construct, originally provided by Dr. Liguang Luo (Stanford University). The dsRNA synthesis protocols followed the protocol described (Rogers and Rogers 2008).

**Immunostaining and microscopy**

The cells were seeded on dishes coated with Concanavalin A. After one hour, the media was removed and the cells were fixed for 10 minutes in a solution containing 4% paraformaldehyde diluted in phosphate buffered saline (PBS). The cells...
were then rinsed in 0.1% PBS-Triton-X and incubated in 1:1000 phalloidin for two hours at room temperature. Subsequently, the cells were rinsed for three times in 0.1% PBS-Triton-X before being mounted on Vectashield mounting media containing DAPI (H-1300). Fluorescence imaging was carried out using the Leica SP8 confocal microscope.

**Protein extraction and Western Blotting**

Cell pellets from a single well of a 24-well plate cells subjected to RNAi against either Rho1 or cat (dsRNA control) were lysed with RIPA buffer. Proteins in the lysed samples were separated with a BioRad WGX 4-20% gel, transferred onto Nitrocellulose membrane (BioRad) and blotted with either mouse anti-Rho1 (p1D9, from Developmental Studies Hybridoma Bank deposited by Parkhurst, S) or mouse anti-a-tubulin (T9026, Sigma). The blots were treated with anti-mouse HRP and the signals were visualized using Pierce Enhanced Chemiluminescence Reagent (ThermoFisher). The experiment was conducted in duplicate.

**Cell counting and statistical analysis**

Live cells were counted using an automated cell counter (BIORAD) according to manufacturer’s instructions. Each condition had a total of three replicate counts. Statistical analysis of the differences in the growth ratio was carried out using Prism8 using ordinary-one way ANOVA test, with Sidak’s multiple comparison test.

**Acknowledgments:** The Rho1 antibody was procured from Developmental Studies Hybridoma Bank (University of Iowa, IA). We also acknowledge the Light Microscopy Imaging Center (LMIC) at Indiana University Bloomington.

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**Funding:** This work was supported by a NIH grant (2P40OD010949) awarded to the Drosophila Genomics Resource Center.

**Author Contributions:** Arthur Luhur: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review and editing, Formal analysis, Visualization. Daniel Mariyappa: Formal analysis, Investigation, Visualization, Writing - review and editing. Kristin M Klueg: Writing - review and editing. Stephen L Rogers: Resources, Writing - review and editing. Andrew C Zelhof: Resources, Supervision, Writing - review and editing, Funding acquisition.

**Reviewed By:** Anonymous

**History:** Received January 5, 2021  Revision received January 22, 2021  Accepted January 22, 2021  Published January 29, 2021

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**Citation:** Luhur, A; Mariyappa, D; Klueg, KM; Rogers, SL; Zelhof, AC (2021). Serum-free adapted Drosophila S2R+ line is amenable to RNA interference. microPublication Biology. https://doi.org/10.17912/micropub.biology.000362