RNA Interference

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Surface-Chemistry Control To Silence Gene Expression in *Drosophila* Schneider 2 Cells through RNA Interference**

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RNA interference (RNAi) is a mechanism in molecular biology in which double-stranded RNA (dsRNA) mediates the silencing of a specific gene expression in a variety of organisms and cell types.[1] Since its initial discovery in the early 1990's, RNAi has revolutionized several important areas of biomedical science and is regarded as a breakthrough technology platform for basic research and therapeutic applications.[2] In particular, RNAi technology has become an important tool in identifying disease-causing genes in medical fields ranging from cancer to human immunodeficiency virus (HIV) research.[3] By targeting and interfering with messenger RNA (mRNA), RNAi gene-silencing technology blocks the expression of the proteins implicated in disease progression.[4] This process not only enables researchers to identify the genes of interest but could also potentially treat the disease by inactivating the malignance of the disease-causing gene. Despite much research effort aimed at understanding how RNAi mediates specific gene silencing in cells, the specific mechanism of RNAi uptake and cellular processing remains elusive. A number of cells in organisms including *C. elegans* and *Drosophila* can naturally take up exogenous dsRNA through endocytosis.[5] However, the efficiency of RNAi uptake can vary significantly depending on several parameters. These factors include the organism, the length of the dsRNA, and the cell interaction with the underlying substrate.[6] For example, *Drosophila* Schneider 2 (S2) cells have been shown to take up dsRNA over a wide range of concentrations efficiently on tissue culture plastics but when the cells are induced to spread on surfaces coated with the lectin concanavalin A (con A), the cells round-up and detach from the surfaces upon dsRNA addition.[7] This observation clearly suggests that the interaction between the cells and the underlying substrate is critical for RNAi internalization and processing. A detailed surface-chemistry study that molecularly defines the cell–material interactions may provide important insights and control on the mechanism of RNAi entry and function. There is currently no model system developed to systematically study various surface effects, including surface-chemical properties, cell geometries, and cell confluency on RNAi function with cells. Herein, we report for the first time the combination of RNAi technology with surface chemistry to control RNAi-mediated gene silencing of *Drosophila* (S2) cells patterned on tailored self-assembled monolayers (SAMs) of alkanethiolates on gold.

Herein, we use a cultured cell line derived from *Drosophila* embryos (S2 cells) as the cell model system to investigate the influence of surface effects on RNAi. S2 cells have been shown to internalize and process dsRNA naturally and are exquisitely sensitive to RNAi-mediated gene silencing.[8,9] Furthermore, the genome of *Drosophila* is rarely redundant and therefore there is an increase in effectiveness of RNAi to reduce the expression of a specific targeted gene. Finally, S2 cells normally grow as round non-adherent cells, but can also be induced to flatten and spread on surfaces treated with con A. This allows for use in high-resolution microscopy techniques to visualize internal structures and in particular cytoskeletal proteins and framework.[10] S2 cells are able to take up and process RNAi when loosely attached but are unable when attached strongly and well-spread on a surface. The molecular mechanism of this particular phenomenon is not known. Therefore, there is an intricate balance between the S2-cell–material interaction and RNAi effectiveness.

Our first study was to determine the surface composition that is compatible with S2 cell adhesion and RNAi (Figure 1). We generated a variety of surfaces tailored with different chemical functional head groups and found that S2 cells attach to hydrophilic and hydrophobic surfaces but with different morphologies. However, only on hydrophobic methyl terminated surfaces (hexadecanethiol; C16) do the S2 cells attach and appear rounded. To determine if RNAi is possible on these surfaces, we used dsRNA that targets the silencing of Rho GTPase, an important protein involved in regulating the actin cytoskeleton during cell division and migration.[11] Cells with the Rho repression can undergo growth but not cell division. The phenotypic response is manifested by the development of multinucleated giant cells.[12] Interestingly, we found that most adherent S2 cells on hydrophobic SAMs surfaces became enormous after three days with only one round of RNAi treatment. Unlike silencing signaling proteins in S2 cells, which normally requires one round of RNAi treatment, genes that illicit a phenotypic response in S2 cells cultured on tissue plastics usually require two rounds of RNAi treatment for effective Rho silencing over a period of seven days.[8]

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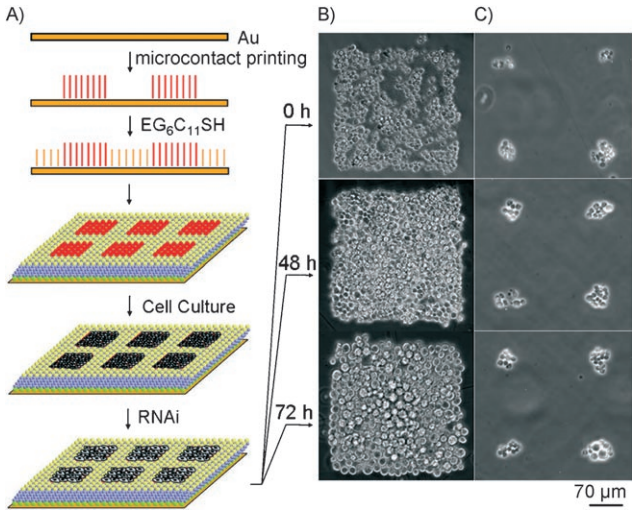

Figure 1. A) A schematic diagram for the RNAi-mediated silencing of Rho GTPase of S2 cells on patterned self-assembled monolayers. Hexadecanethiol (C16) was patterned into squares on gold-coated glass substrates by microcontact printing. The remaining bare gold was backfilled by hexa(ethylene glycol) alkanethiol to provide the cell-inert region. Addition of S2 cells to the SAM resulted in cell attachment only to the hydrophobic region of the surface. Patterned S2 cells were treated with dsRNA and then allowed to grow in serum media at room temperature. The extent of RNAi uptake for the S2 cells was characterized by determining the number of multinucleated giant cells on the patterned SAMs over time. B,C) Time-lapse microscopy for the RNAi-treated S2 cells on 280 μm and 50 μm square patterns. S2 cells on the larger patterns became enormous in cell size after 72 h, whereas the cells on the smaller patterns showed negligible phenotypic response over the same time period. EG = ethylene glycol.

To determine how cell population and confluency may affect RNAi uptake and processing, we generated high-throughput multiplexed patterned surfaces. We first microcontact printed hexadecanethiol (C16) to the surface in 280 μm square patterns and then filled the remaining bare gold regions with hexa(ethylene glycol) alkanethiol. This generated surfaces patterned with cell adhesive islands among an otherwise inert surface. When S2 cells were seeded onto the monolayer, they adhered exclusively to the hydrophobic patterns (Figure 1 A). We then treated the patterned S2 cells with RNAi and monitored the extent of RNAi-mediated Rho silencing by time-lapse microscopy (Figure 1 B). Time-lapse micrographs show that the average size of S2 cells on the pattern became enormous and multinucleated after 72 h. The observed phenotype is characteristics of Rho inhibition, suggesting that RNAi completely blocks the expression of Rho for S2 cells patterned on hydrophobic monolayer surfaces. It was determined that almost all ($\approx 90\%$) of the S2 cells on the patterns showed the Rho RNAi phenotype. We next repeated the same RNAi experiment for S2 cells adhered on patterns 50 μm in size to determine the efficiency of RNAi uptake by S2 cells confined on smaller patterns with fewer cells. When compared with the S2 cells on the 280-μm patterns, time-lapse micrographs show that the number of enlarged S2 cells on the 50-μm patterns is significantly reduced after 72 h (Figure 1 C).

To further confirm the extent of Rho inhibition for the adherent S2 cells, we next investigate the number of multinucleated S2 cells on each of the two different C16 pattern sizes by fluorescence microscopy (Figure 2). Although the

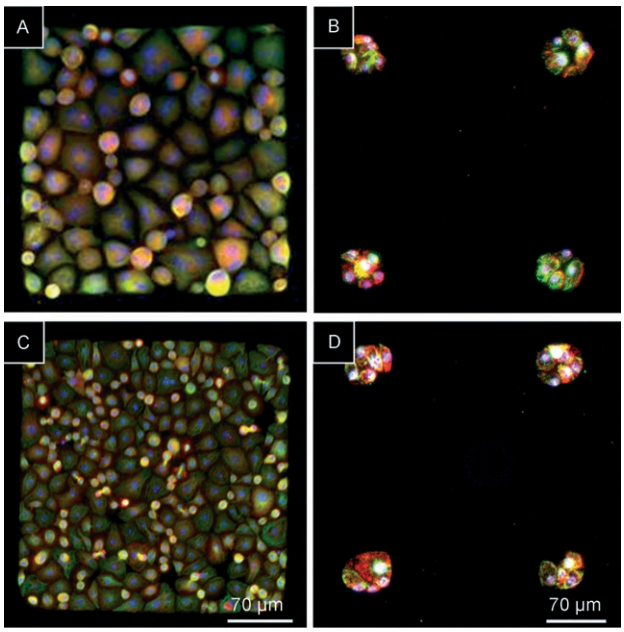

Figure 2. Fluorescent images of RNAi-treated S2 cells on patterns coated with con A. S2 cells obtained from the RNAi experiments shown in Figure 1 were transferred from microcontact-printed C16 patterns and reseeded onto patterns coated with con A to visualize the internal structures of the adherent cells. Cells were stained for F actin, microtubules, and nuclei. A) RNAi-treated S2 cells from the 280 μm pattern became enormous in size and multinucleated when compared with untreated S2 cells attached to patterns of the same size (C). When RNAi-treated S2 cells taken from the 50 μm patterns (B) are compared with the untreated S2 cells (D), both sets of cells appeared similar in size and were mononucleated, indicating RNAi was ineffective on these small patterns.

cells appear round, different phenotypes of Rho inhibition on the two different C16 pattern sizes are clearly shown by visualizing their change in size by optical microscopy (Figure 1). For high-resolution fluorescence microscopy of internal structures, the cells are required to spread and flatten. To compare the Rho phenotype images from the C16 surfaces with previous reports, the cells were removed from the two different C16 patterns and then reseeded separately onto patterned SAMs surfaces coated with con A.[8] It has been shown that S2 cells attach and spread on con A, which allows for detailed investigation of subcellular structures. Fluorescent images of the RNAi-treated S2 cells on con A show that most cells obtained from the 280-μm C16 patterns are in fact multinucleated (Figure 2 A), whereas the cells taken from the smaller C16 patterns are mononucleated (Figure 2 B). This surprising result suggests that the cell population and not confluency influences the endocytic efficiency of dsRNA of the adherent cells.

As further evidence that the size of the C16 patterns influence RNAi uptake and processing, immunoblots of S2

cells on the two, differently sized, C16 patterns were analyzed. The Rho immunoblots clearly show the role of pattern size and therefore cell population on the effectiveness of RNAi-mediated silencing of Rho (Figure 3). The Rho-protein

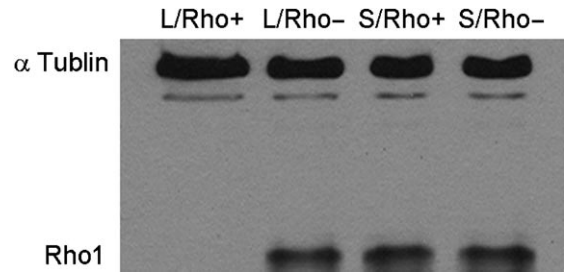

Figure 3. Immunoblots demonstrating the effectiveness of RNAi-mediated silencing of Rho in S2 cells on two different size C16 patterns. Immunoblotting revealed that RNAi completely reduced the Rho expression in S2 cells patterned on the large patterns (L/Rho+) when compared with the cells adhered to small-size patterns (S/Rho+) and the cells on both large (L/Rho−) and small patterns (S/Rho−) that express the protein at the endogenous level. The blot was probed with antibody against α-tubulin to show the relative protein concentration in each sample.

expression levels of S2 cells on the large C16 patterns are completely reduced upon dsRNA Rho treatment compared with nontreated cells. Interestingly, the cells on the small C16 patterns express similar Rho protein levels irrespective of dsRNA Rho treatment. Although the nature of the cell–material and cell–cell interactions are unclear and under investigation, this result demonstrates the importance of surface effects on the rate of RNAi uptake and processing in *Drosophila* S2 cells.

In conclusion, we have demonstrated RNAi-mediated gene silencing in *Drosophila* S2 cells patterned on self-assembled monolayers. We found that the RNAi uptake for S2 cells adhered on larger C16 sized patterns (280 μm wide) is significantly more efficient than the cells on smaller sized patterns (50 μm wide). This result suggests that cell–surface interactions play an important role in the mechanism of RNAi uptake through endocytosis. We are currently using other S2 cell lines (green fluorescent protein (GFP)-labeled and migratory cells) and a wide array of dsRNA in combination with molecularly defined tailored surfaces to systematically study the effect of cell–surface interactions on RNAi-mediated gene silencing. We believe that the use of microfabrication and microfluidic techniques on tailored surfaces in conjunction with RNAi functional genomic approaches will provide many new cell-screening opportunities and expand the scope of tools for fundamental understanding and utility of RNAi technology.

Experimental Section

Cell culture: *Drosophila* Schneider 2 (S2) cells (Invitrogen) were cultured in Schneider's *Drosophila* Medium (GIBCO BRL) containing 10% heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin (50 $\mu g\,mL^{-1}$). S2 cells were grown in 75-cm^2 flasks containing media (10 mL) for routine maintenance. For passage, cells are resuspended in the same 10 mL of medium that they were growing in, then 3 mL is transferred to 7 mL of fresh medium in a new flask.

Double-stranded RNAi: Individual DNA fragments approximately 650 bp in length containing coding sequences for the proteins to be "knock out" were amplified by using PCR according to protocol published by Rogers et al.[8] Briefly, each primer used in the PCR contained a 5′ T7 RNA polymerase binding site (GAATTAATACGACTCACTAGGGAGA) followed by sequences specific for the targeted genes. For Rho1, the forward primer sequence is ATCAAGAACAACCAGAACATCG and the reverse primer sequence is TTTGTTTTGTGTTTAGTTCGGC. The PCR products were purified by extraction with phenol/chloroform (50:50) followed by precipitation with ethanol/sodium acetate (50:50). The purified PCR products were used as templates by using a MEGASCRIPT T7 transcription kit (Ambion) to produce dsRNA. The dsRNA products were precipitated with ethanol/sodium acetate (50:50) and resuspended in water. The dsRNA molecules were annealed by incubation at 65 °C for 30 min followed by slow cooling to room temperature. dsRNA (6 μg) was analyzed by 1% agarose gel electrophoresis to ensure that the majority of the dsRNA existed as a single band of approximately 700 bp. The dsRNA was stored at −20 °C.

Preparation of monolayers: All gold substrates were prepared by electron-beam deposition of titanium (5 nm) followed by gold (15 nm) on glass cover slips ($75 \times 25\ mm^2$). All gold-coated glass substrates were cut into 1 cm^2 pieces and washed with absolute ethanol. A poly(dimethylsiloxane) stamp was used to microcontact print hexadecanethiol in patterns to the gold-coated substrates. The substrates were then immersed in an ethanolic solution containing hexa(ethylene glycol) alkanethiolates (1 mM) for 12 h. Furthermore, the slides were cleaned with ethanol prior to each experiment.

RNAi treatment of patterned S2 cells: RNAi treatment of adherent S2 cells on SAM surfaces was performed according to the following procedure: Suspended cells in serum media ($100\,000\ cells\,mL^{-1}$) were added onto the monolayer-coated gold substrates for 1 h to allow cell attachment to the surface. Substrates were then removed from the cell media and placed in a six-well plate containing serum free media(1 mL). dsRNA in water (20 μL; $1\ \mu g\,\mu L^{-1}$) was added to the well and mixed thoroughly. After 30 min, growth medium (1 mL) was added to the well to give a final volume of 2 mL. RNA-treated S2 cells were then allowed to grow at room temperature.

Fluorescent microscopy: RNAi-treated S2 cells on patterned monolayer surfaces were resuspended and plated onto microcontact printed monolayer substrates coated with Con A ($1\ mg\,mL^{-1}$ in water for 1 h) to allow attachment and spreading for 1 hour. The adherent cells were fixed in 3.7% paraformaldehyde in phosphate-buffered saline (PBS) buffer solution for ten minutes and then permeabilized with 0.1% Triton X in PBS (PBST) buffer solution for 10 min. Cells were then stained with anti-tubulin (1:1000) in PBS buffer solution containing 10% goat serum for 1 h followed by Alexa 488-conjugated goat anti-mouse IgG (1:100 in PBST buffer solution), phalloidin-tetramethylrhodamine B isothiocyanate (1:50 in PBST buffer solution), and 4,6-diamidino-2-phenylindole (DAPI; 1:300 in PBST buffer solution) for 1 h. Substrates were rinsed with deionized water before being mounted onto glass cover slips for microscopy. All optical and fluorescent micrographs were imaged by using a Nikon inverted microscope (model TE2000-E). All images were captured and processed by MetaMorph.

Assay of Rho Silencing (Western Blotting); Ten gold substrates with patterned S2 cells were used to generate each protein sample for the immunoblot. Patterned S2 cells were removed from the substrates in serum media. The cell media were centrifuged at 8000 rpm for 1 min. The pellet was resuspended in PBS-Tween (50 μL; 0.1% Tween) and was added to five times the concentration of the sample buffer solution (10 μL). Lysates were boiled for 3 min and then separated on a 12% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane. The membrane was blocked

with 5% nonfat milk in PBS-Tween for 15 min. The blot was incubated with anti-Rho1 (Developmental Studies Hybridoma Bank, the University of Iowa; 1:10) and anti-α tubulin (Sigma-Aldrich; 1:100) in 5% nonfat milk in PBS-Tween at room temperature for 1 h. The blot was washed three times for 5 min with PBS-Tween and then incubated with goat anti-mouse HRP (1:1000) in 5% nonfat milk in PBS-Tween for 30 min. The blot was washed three times for 5 min with PBS-Tween and visualized by using chemiluminescence (Amersham ECL Detection Reagents, GE Healthcare Life Sciences, Piscataway, NJ) on HyBlot CL film (Denville Scientific, Metuchen, NJ).

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