Solid-phase transfection; siRNA screens for DNA repair pathways as examples

Tomohiro Yoshikawa[1], Hiroyuki Hosokawa[1], Alun McCarthy[1,2], Cecilia Lundin[3], Bastiaan Evers[3], Daniel Ebner[4], Thomas Helleday[3]

[1]CytoPathfinder, Inc. [2]PGXIS Ltd. [3]Science for Life Laboratory, Stockholm University
[4]Target Discovery Institute, University of Oxford

INTRODUCTION

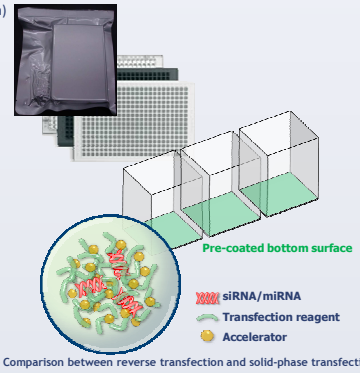
A solid-phase transfection technology makes siRNA screens more simple and easier. Here we show siRNA screens targeted for DNA repair pathways using our solid-phase transfection technique.

Solid-phase transfection

Gene knockdown using exogenous siRNA is a powerful tool for analyzing genetic network. We have developed a solid-phase transfection method in which the complexes of siRNA (miRNA) and transfection reagents are fixed on the bottom surface of the culture dish (plate) (Figure 1a). Our solid-phase transfection is a robust and simple-to-use technique for siRNA / miRNA transfection with excellent reproducibility and reliability.

We have shown that our solid-phase transfection is also effective for hard-to-transfect cells including primary cells (Yoshikawa T, et al., J Control Release. 2004, 28;96(2):227-32) and suspension cells (Figure 1e, f). Our solid-phase transfection technique allows us to perform assays using 384 /1536-well plates and to store the plates at least 12 months in a freezer. These characteristics of solid-phase transfection technique enable to perform siRNA screen using primary cells even with very limited amounts of samples.

a)



b) Cell type tested

Normal cells	rat brain cortex neuronal cells rat primary neuronal cells rat primary astrocyte primary mouse embryonic fibroblast (MEF) human endothelial cells (HUVEC) human epitherial cells (PrEC, NHBE, HMEC) human smooth muscle cells (UASMC) primary human hepatocytes
Human breast cancer cells	mice bearing human tumor xenografts
Stem cells	human mesenchymal stem cell (hMSCs) mouse neuronal stem cell
Suspension cells	Jurkat cells K562 cells

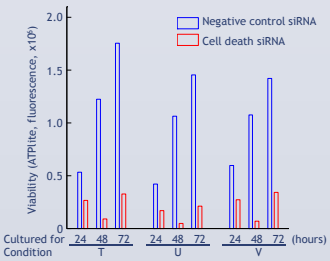
c) Comparison between reverse transfection and solid-phase transfection

	Reverse transfection	Solid-phase transfection
Applicable cell source	relatively narrow	wide
Cell type	suspension, adherent	suspension, adherent
Transfection efficiency	reliable	same as reverse transfection or better
Transfection dead volume	~50% of culture volume	0%
Storage	impossible	> 12 months
Maximum well density	384-well plate format	3456-well plate & glass array
Automation	yes	yes

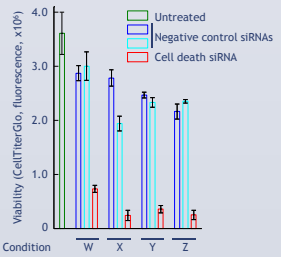
d) mRNA knockdown efficiencies in mouse primary neuronal cells

Targtet gene	Reverse transfection	Solid-phase transfection	
		Condition 1	Condition 2
A	43.4	68.3	65.7
B	73.3	88.0	87.6
C	24.7	9.1	48.2
D	70.9	97.4	91.7
E	36.0	94.3	85.2

e) siRNA transfection into Jurkat cells



f) siRNA transfection into K562 cells



Relative GAPDH mRNA level (%) at 48 h when transfected with GAPDH siRNA	7.5	4.8	6.6

Figure 1. Solid-phase transfection

DNA damage repair

DNA repair pathway has redundancies since DNA repair is important for maintaining genetic stability. Radiotherapy and cytostatic drugs such as camptothecin for cancer treatment cause DNA double strand breaks in cancer cells. DNA double strand breaks are repaired by two major pathways, non-homologous end joining and homologous recombination. The redundancies often enable cancer cells to survive using complementary DNA repair pathways. We have hypothesized that DNA repair pathways are rich sources of anti-cancer targets. Here, we performed two siRNA screens using solid-phase transfection.

1) A genome wide siRNA screen using RAD51 foci formation assay after irradiation in order to find novel target genes involved in homologous recombination
2) A double knockdown siRNA screen in order to find the synthetic lethal relationship between genes involved in DNA repair pathway

MATERIALS AND METHODS

Genes involved in homologous recombination (Figure 2a)

- Prepare solid-phase transfection plates (384-well plate x 58 , 18,262 target genes)
- Seed U2OS cells (time 0)
- Induce DNA double strand break by X-ray irradiation (4 Gy) at 68 hours
- Allow cells to repair DNA damage and fix cell at 72 hours
- Immunostaining with anti-RAD51 antibody and staining nuclear with DAPI
- Analyze the number of RAD51 foci (an index of homologous recombination) using IN Cell Analyzer

Gene combinations which cause synthetic lethality

- Prepare solid-phase transfection plates (384-well plate x 20 , 80 x 80 target genes)
- Seed MRC5 cells (time 0)
- Analyze the number of viable cells by resazurin assay at day 5

RESULTS

Genes involved in formation of RAD51 foci after X-ray irradiation (Genes involved in homologous recombination)

Figure 2b is a heat map showing the result of analyzing the numbers of foci formation after X-ray irradiation. There was a significant difference between positive control (siRNA for RAD51) and negative control (scramble) (Figure 2c). Z-factors between plates were in the range of 0.3-0.7. The correlation coefficient between first run (GW1) and second run (GW2) was 0.6889 (Figure 2d). The data was filtered depending on toxicity data and the number of RAD51-positive cells and hit selection was then performed by taking the top 250 hits both from first run(GW1) and from second run(GW2). Out of the 250 siRNA lists, 67 genes appeared in the both list. In those genes, there were several known homologous recombination proteins (e.g. BRCA1, BRCA2, and RPA1), indicating that this screen is reliable. Using gene enrichment analysis, we will evaluate if hits can be assigned to specific biological characteristics. We will perform secondary screen on those 67 candidate genes.

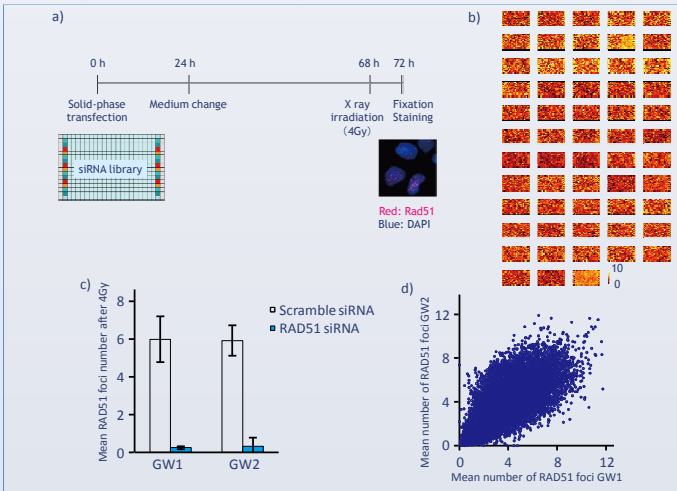


Figure 2. Screen novel factors involved in homologous recombination

Gene combinations which cause synthetic lethality

We carried out a double knockdown screen using 80 x 80 siRNAs on a normal diploid human cell line (MRC5 cells). Figure 3a is the heat map showing the results of resazurin assay. To find synthetic lethal interaction, genetic interaction score (ε) was defined as below.

$$K_{i,j} = \kappa \cdot R_i \cdot C_j \cdot \varepsilon_{i,j}$$

$K_{i,j}$ Viability of cells with siRNAs i and j
κ Baseline viability throughout screen
R_i Systematic row effect (mostly siRNAi)
C_j Systematic column effect (siRNAj and positional effect)
$\varepsilon_{i,j}$ Genetic interaction score between siRNAi and j

Data was fitted with linear model below to calculate genetic interaction scores(ε).

$$\log K_{i,j} = \log \kappa + \log R_i + \log C_j + \log \varepsilon_{i,j}$$

Genetic interaction scores(ε) are shown in Figure 3b with red for high synthetic lethal interaction. Successful double knockdown was confirmed by western blotting analysis (Figure 3c). We will further analyze some of the potential synthetic lethal interaction. Immunostaining with anti-γH2A.X, anti-53BP1 antibodies will be performed to clarify whether these synthetic lethal interactions are related to DNA repair pathway.

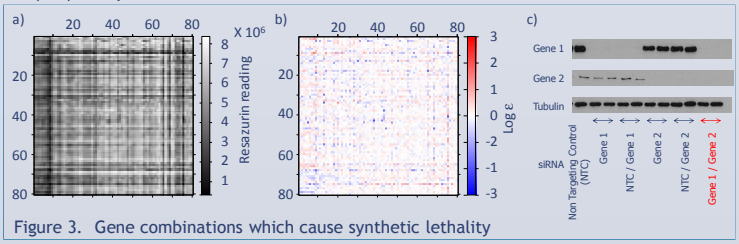


Figure 3. Gene combinations which cause synthetic lethality

SUMMARY

Using solid-phase transfection technology, we could perform

- a genome-wide siRNA screen (384-well plate x 58 x twice) with good reproducibility
- a double knock-down screen (384-well plate x 20 x 3 times) with good efficiency on both genes

As candidate genes for secondary screen, we selected

- 67 genes involved in RAD51 foci formation (homologous recombination)
- 1 strong and interesting synthetic lethal interaction

CONTACT

CytoPathfinder, Inc
Room 06106, Bio-IT Research Building, AIST Tokyo Waterfront,
2-4-7 Aomi, Koto-ku, Tokyo, 135-0064, JAPAN
TEL: +81-3-5530-8086
FAX: +81-3-5530-8087
http://www.cytopathfinder.com

www.PosterPresentations.com