QUANTITATIVE ANALYSIS OF SINGLE-CELL CLONAL EXPANSION AND CELL SENESCENCE BY USING MICROWELL CELL ARRAY

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ABSTRACT

This paper reports a quantitative method to study cell senescence by using a poly(dimethylsiloxane) (PDMS) based microwell cell array. GM639 human fibroblasts which have undergone shRNA-mediated depletion of the Werner Syndrome protein (*WRN*) were used in this experiment. Clonal expansion and cell senescence were quantified through the integration of a microwell cell array and an automated imaging system. Our results show that *WRN* depleted cells have a lower proliferative rate and a higher probability of becoming senescent which supports the role of WRN in determining cell proliferation, cell survival and maintenance of genetic integrity.

KEYWORDS: Microwell, Clonal Expansion, *WRN*, Cell Senescence

INTRODUCTION

Quantitatively measuring clonal expansion and detecting cell senescence are difficult and time consuming with the traditional pathological method. It usually requires one scientist to dedicate hours per day to screen and capture single cell activities by accurately locating the position of cells and manually taking images. Mistakes are usually made because of the manual control of device position resulting in inconsistent time intervals for the measurement of single cell activities. More and more newly developed platforms for single cell studies have shown the importance of tracking clonal expansion. However, the diverse and unique platforms which are used by groups usually require complicated techniques to fabricate and only fit their specific needs. Additionally, different platforms need different operations and sometimes the platforms only fit custom-made microscope stages. These customized platforms become a limitation and cause these platforms to be unable to be used widely in the pathological field. Furthermore, the process of tracking clonal expansion is monotonous but it needs precise spatiotemporal control in order to acquire the events of a large number of single cells. As a result of the inconsistent experiments in clonal expansion study, we developed a user-friendly platform by bonding a PDMS-based microarray device on a petri dish and integrated this platform with an automatic microscope [1]. Therefore, cell culture in this platform requires no training as the culturing technique is the same as the ones pathologists use daily.

THEORY

WRN-depleted or mutant cells have a much higher probability of becoming senescent. However, the lack of a quantitative method makes it difficult to confirm or better characterize this association. In this paper, we developed a platform to track clonal expansion and analyze cell senescence during clonal growth from the single cell level. Single cells were physically isolated and grown in their own islands. However, because the 900 microwell cell array was submerged in the medium. This ensures that cells see the same cell culture, nutrients, like in conventional cell culture methods. In order to minimize cell damage while transferring cells to the device, we use statistics to calculate the cell seeding density by Poisson distribution. The imaging process becomes relatively simple in this experiment by integrating this 900 microwell cell array and the automated microscope. The array of microwells can be easily positioned on the microscope stage and the single cells can be tracked by acquiring images automatically. An accurate spatiotemporal control in this system allows us to quantify single cell studies. In this experiment, we attempt to investigate the influence of the WRN gene to the induction of cell senescence by using this system. Theoretically, the setting of this system can be used widely in all kinds of clonal expansion experiments with the advantages of simple fabrication, user-friendly format (petri-dish), automated imaging acquisition and quantifiable single cell experiments.

EXPERIMENTAL

Device Fabrication. Photolithography was first used to fabricate the master of the 900 microwell cell array with the dimensions of 500 μm square or round wells and 400 μm in height. The spacing between each microwells is 500 μm. The 900 microwell cell array was then molded in PDMS using exclusion molding to make open wells on the PDMS membrane. Then the PDMS device was bonded onto a polystyrene petri dish after modification with aminopropyltriethoxysilane [2] (Fig. 1(a-d)).

Cell Preparation. The WRN protein in GM639 human fibroblasts was depleted by transducing cells with a *WRN* specific shRNA-expressing lentivirus for 24 hours, followed by an additional 72 hours of puromycin selection (1μg/ml) prior to use [3]. Depletion results were quantified by Western blot analysis (Fig. 2a).

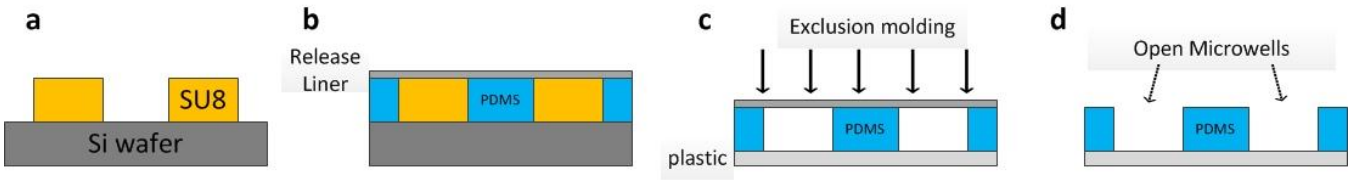
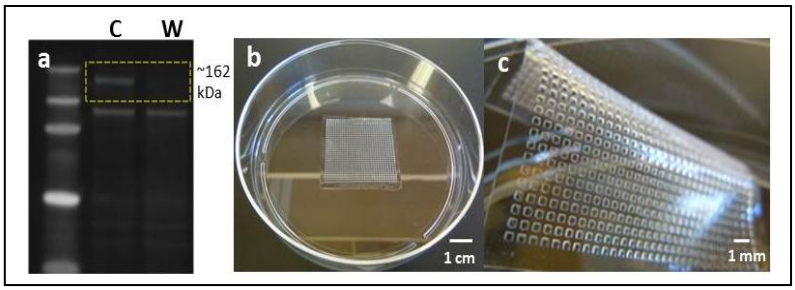


Figure 1: a) SU8 master. SU8 photoresist was spin-coated on a silicon wafer to pattern an array of microwells. b) PDMS replica molding. Uncured PDMS was poured over the features and exclusion molded with a 3M release liner (Mylar), before being baked at 70 ºC for at least 2 hrs. c) Cured PDMS replica was transferred with the release liner then plasma-bonded onto a plastic petri dish (polystylene). d) After bonding, the release liner was peeled off from the PDMS surface and the open microwells were formed.

Surface Coating. After the PDMS device was bonded onto a petri dish (Fig. 2(b-c)), we first treated the device with oxygen plasma for two purposes. First, to expose the surface to oxygen plasma to ensure the aminopropyltriethoxysilane was etched off from the polystyrene surface. Second, to modify the surface to make the polystyrene surface hydrophilic. Once the plasma treatment was done, then the 20 mM poly-d-lysine was used to coat the surface for 2 hours followed by 3 PBS washes.
Single Cell Seeding. The number of cells per dish was calculated by Poisson distribution and confirmed experimentally. We seeded 20,000 cells with 20 ml medium per dish, which resulted in ~36-40% of the wells containing single cells.
Imaging Operation. Nikon inverted microscope with Perfect Focus^{TM} system, motorized x-y control and imaging software were used to automate the process of tracking clonal expansion for each of the single cells. The cells were imaged every 24 hours for 7 days.



Fugure 2: a) Western blot analysis of GM639 cells after WRN- shRNA treatment, C: control, W: WRN. The predicted molecular weight of WRN is 162 kDa. b) 900 microwell cell array: The rounded square PDMS microarray is bonded to the polystyrene petri dish as a friendly pathological tool. c) PDMS membrane: The simple PDMS microarray membrane can be bonded or conformed onto pre-coated or pre-patterned substrates.

RESULTS AND DISCUSSION

Tracking Single Cell Proliferation. By using this device, each single cell has its own island. Thus, by following the images which are taken daily from each microwell, we can systemically track the condition of the proliferation from each single cell (Fig. 3a). We compare GM639 human fibroblasts between the WRN depleted group and the control group and track the proliferative rate for 7 days. The results show that the average proliferative rate of *WRN* depleted cells is about 40% slower than the control cells [4] (Fig. 3(b-c)), which is a significant difference. As we see the column charts shown in Fig. 2b and 2c, the tendency of proliferative rate is also faster in control group than in WRN depleted group.

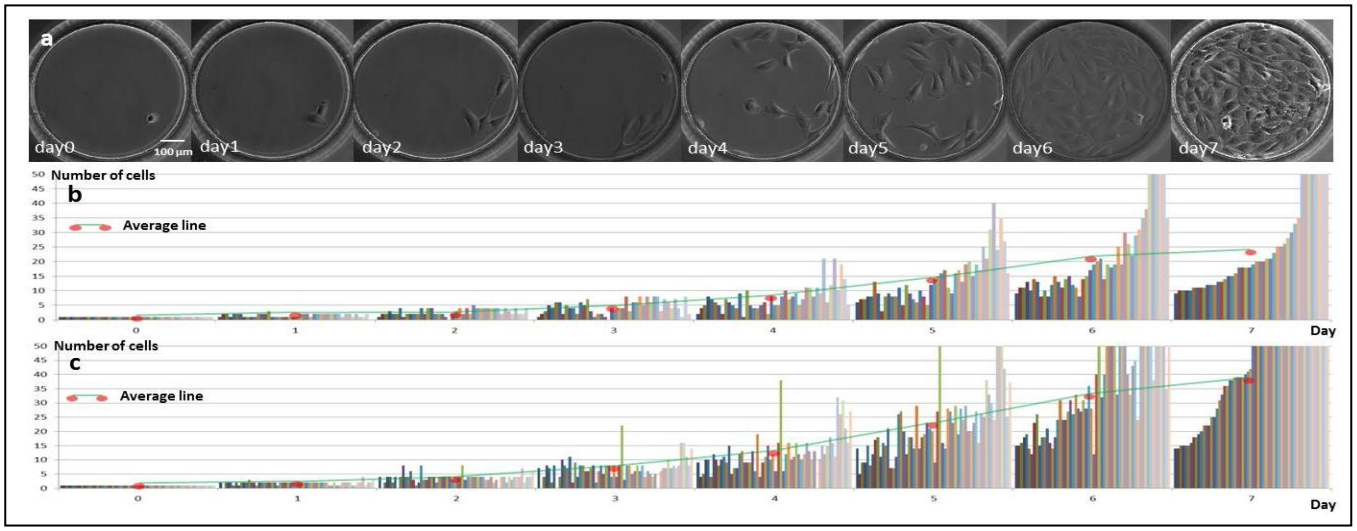


Figure 3: a) Representative images of single cell proliferation. The single cell was physically isolated in a 500 µm diameter, 400 µm height microwell with images acquired every 24 hours for 7 days. b,c) Proliferation of WRN depleted single cells (b) and control cells (c). Each bar in the plot represents a single cell in a microwell.

***WRN* Gene Depletion Induces Cell Senescence.** On the final day, the cells were stained with live/dead cell assay (Invitrogen, Carlsbad, CA) prior to the first imaging acquisition for phase and live/dead cells. Then 4% paraformaldehyde fixation was applied, followed by SA-βgal staining in order to identify senescent cells [5] (Figure 4a). The results confirm a higher fraction of senescent cells in cultures depleted of WRN (Fig. 4(b-c)).

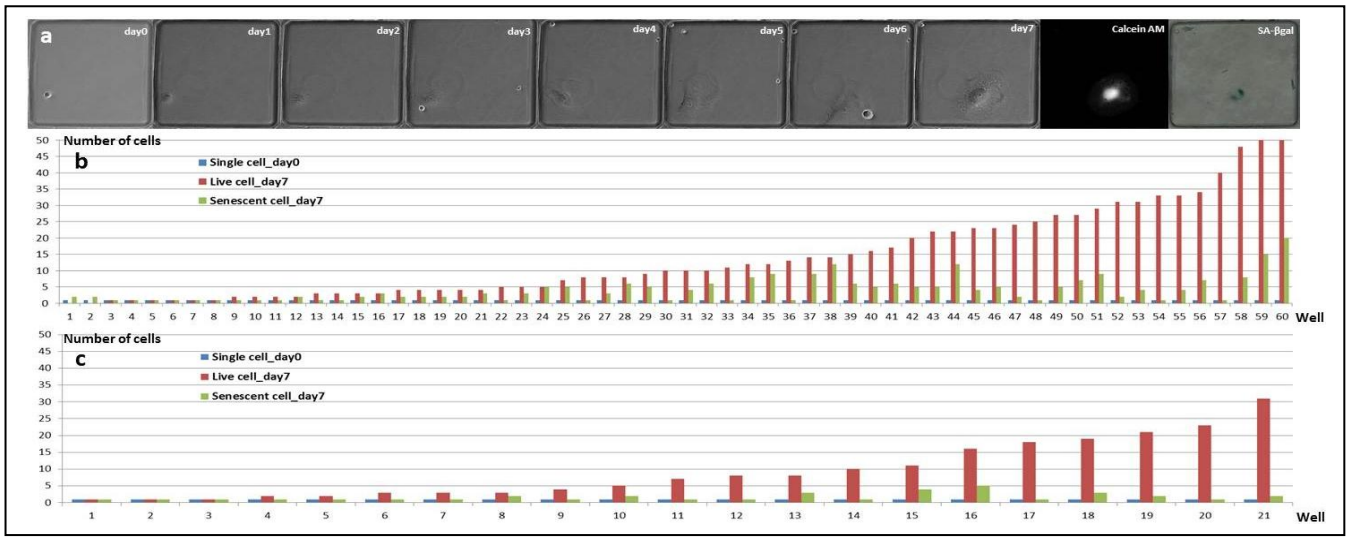


Figure 4: a) The trajectory of a senescent cell: Senescent cells could be traced back to the first day. SA-βgal was used to detect senescent cells and Calcein AM was used to confirm cell viability. b,c) Cell senescence plots: Senescent cells were detected in 60 out of 340 wells with WRN depleted cells (b) and 21 out of 320 wells in the control cells (c). The control has less senescent cells, indicating that the cells were already senescent cells or had started the senescence process before seeding. In contrast, WRN depletion leads to a higher probability of cellular senescence in individual cells.

CONCLUSION

The miniaturized format of the microarray facilitates high-speed data acquisition of clonal expansion and the detection of cellular senescence. The format also has the advantage of having a high capacity for parallel assays, low cost, user-friendliness and the speed of being automated. Our design could have in excess of 2500 wells within a 100 mm petri dish and the PDMS-based microarray device can be integrated with pre-coated or nano-patterned substrates. In conclusion, we demonstrate that important cellular phenotypes such as clonal expansion and cell senescence can be quantified in a microwell cell array system to provide data to understand cell physiology and function and the consequences of genetic or chemical perturbations.

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