Effect of “Phitenized” environment on cells

2020 Research Report

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NextGeM Inc.

【Purpose】

There have been various reports on the effects of "Phitenized" environments on living organisms, and it has been shown to improve various physical symptoms, but the detailed mechanism is still unknown. NextGeM Inc. has been studying the cellular level effects of "Phitenized" environments, especially cell proliferation and migration, and has identified that cell proliferation and migration tend to be enhanced in "Phitenized" environments. However, the changes are minute and it is important to capture the changes at the single cell level for more detailed analysis.

In 2020, in order to analyze the effects of "Phitenized" environments on cells in more detail, we aimed to analyze the cell proliferative capacity at the single cell level instead of the conventional analysis of cell populations.

For the analysis of cell proliferation, we used CellTrace™ Violet, a fluorescent dye that can track cell division for a long time without affecting morphology or physiological functions. We also studied the efficacy of the anticancer drug, etoposide in the "Phitenized" environments, because the enhanced cell division in the "Phitenized" environments may promote the efficacy of anticancer drugs.

【Materials and Methods】

(Materials)

- Ba/F3 Cells (murine pro B cell line) (Cat# RCB4476, RIKEN BioResource Center)
- CellTrace™ Violet Cell Proliferation Kit, for flow cytometry (Cat# C34571, Thermo Fisher Scientific, MA., U.S.A.)
- SYTOX™ Blue Dead Cell Stain, for flow cytometry (Cat# S34857 Thermo Fisher Scientific, MA., U.S.A.)
- Etoposide (Cat#　055-08431, Wako)
- 96-well plate (Control plate・"Phitenized" plate) (Cat# 353072 Corning, NY, U.S.A)
- BD FACS Aria™ III Cell Sorter (Becton, Dickinson and Company, NJ, U.S.A.)
- BD FACS Canto™ Flow Cytometer (Becton, Dickinson and Company, NJ, U.S.A.)
- BD FlowJo™ Software (Becton, Dickinson and Company, NJ, U.S.A.)

(Single-cell analysis of cell proliferative potential)

1. Ba/F3 Cells were labeled with CellTrace™ Violet following manufacturer's protocol.
2. Dispensed 200 uL of medium onto "Phitenized" and control 96-well plates.
3. Ba/F3 cells were sorted at 1500 cells per well using BD FACS Aria™ III. (n=16)
4. Cells were incubated at 5% CO_2 at 37°C for 72 hours. During incubation the "Phitenized" plate was covered with lead sheets (3 mm thick) to avoid the radiation effect of the "Phitenized" plate to the control plate (Fig. 1).
5. The fluorescence intensity of the cells was measured using BD FACS Canto™.
6. The number of live cells and cell divisions was analyzed by BD FlowJo™ software.

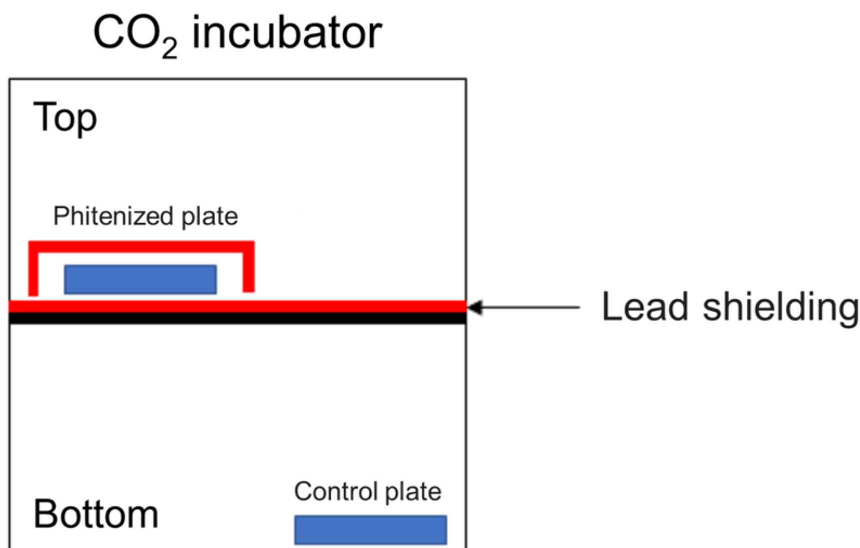

Fig. 1 Layout of the plate in the CO2 incubator.

(Evaluation of the efficacy of anticancer drugs)

1. Dispensed 200 uL of etoposide-containing medium onto control and "Phitenized" 96-well plates (0.001–100 uM, or none).
2. Ba/F3 cells were sorted at 5,000 cells per well using BD FACS Aria™ III. (n=8)
3. Cells were incubated at 5% CO_2 at 37°C for 24 hours.
4. Dead cells were stained with SYTOX™ Blue (final concentration 1/10,000 dilution).
5. Cells were measured by FACS Canto™.
6. The number of live cells was analyzed by BD FlowJo™ software.
7. The number of live cells was compared for each concentration of etoposide.

【Results】

Single-cell analysis of cell proliferative potential

Ba/F3 cells were cultured for 72 hours at 37°C using "Phitenized" and control plates, and the number of live cells and the number of cell divisions were measured (N=16). This experiment was repeated 5 times. As a result, a significant decrease in the number of cells in the "Phitenized" plates was observed in three experiments out of 5 experiments (Fig. 2, Exp. 1-3). On the other hand, there was no significant difference in the two experiments (Fig. 2, Exp. 4 and 5).

Next, we examined the number of cell divisions. Among the experiments 1-3 in which a significant decrease in the number of live cells was observed, a significant decrease in the number of cells with a high number of divisions (more than 5 times) was observed in the "Phitenized" plate in Exp. 3 (N=16 for each). On the other hand, in Exp. 4-5, where no significant difference was observed in the number of live cells, there was no significant difference in the number of cell divisions between "Phitenized" and control plates (Fig. 3).

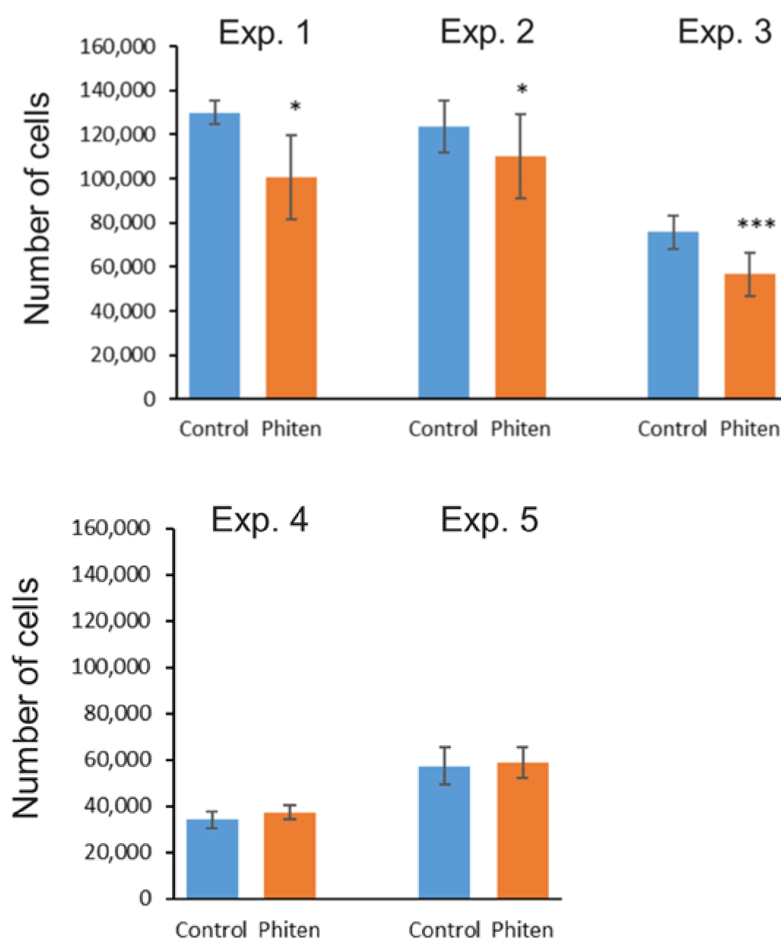

Fig. 2 Changes in the number of live cells in a "Phitenized" environment.

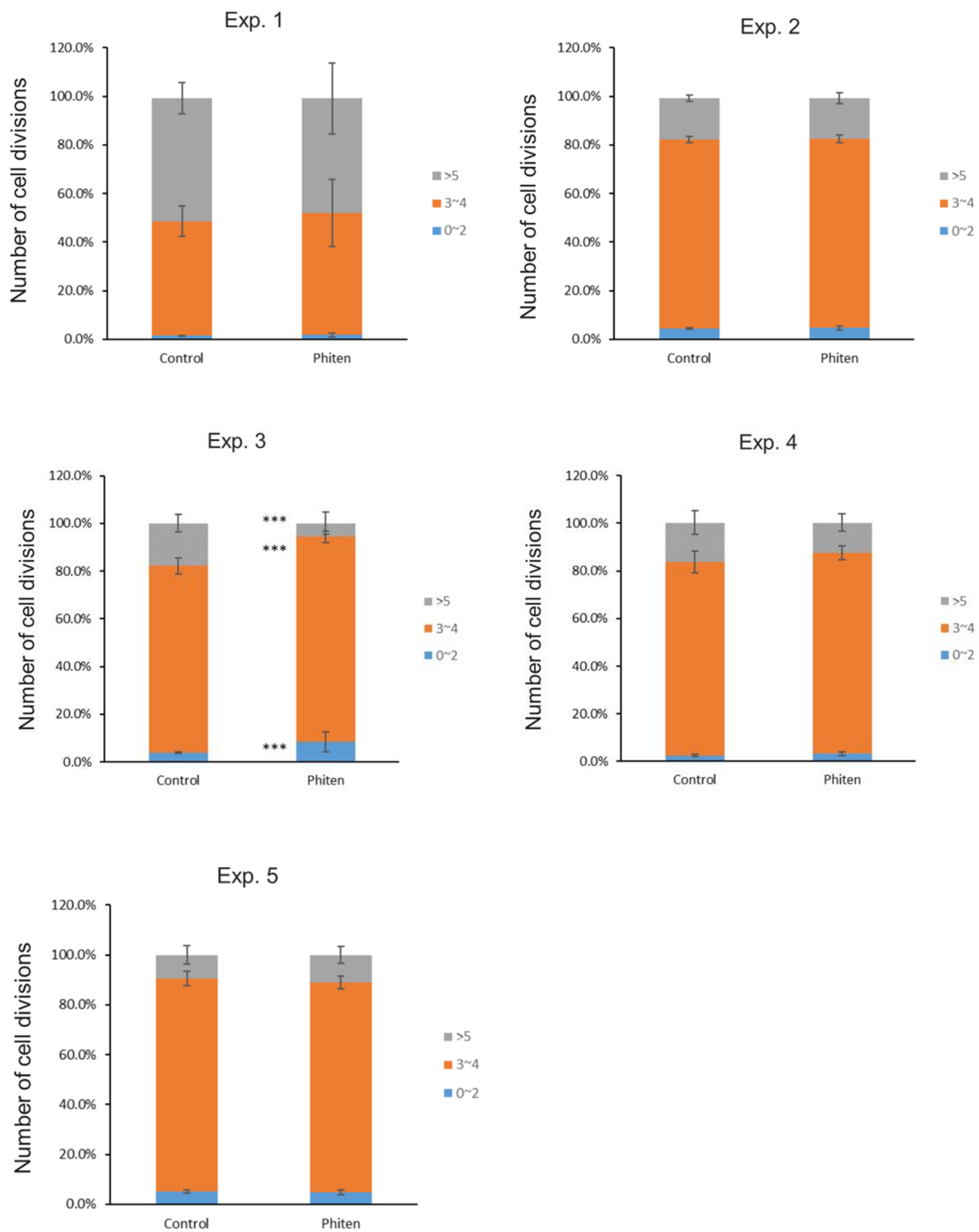

Fig. 3　Changes in the number of cell divisions in a "Phitenized" environment.

Evaluation of the efficacy of anticancer drugs

Ba/F3 cells were treated with etoposide for 24 hours. As a result, the live cell rate, which is an indicator of the cytotoxicity of etoposide, was decreased in the 10 and 100 uM groups. In particular, at the highest etoposide concentration (100 uM), the live cell rate was lower in the "Phitenized" plates compared to the control plates (N=8, p=0.08) (Fig. 4).

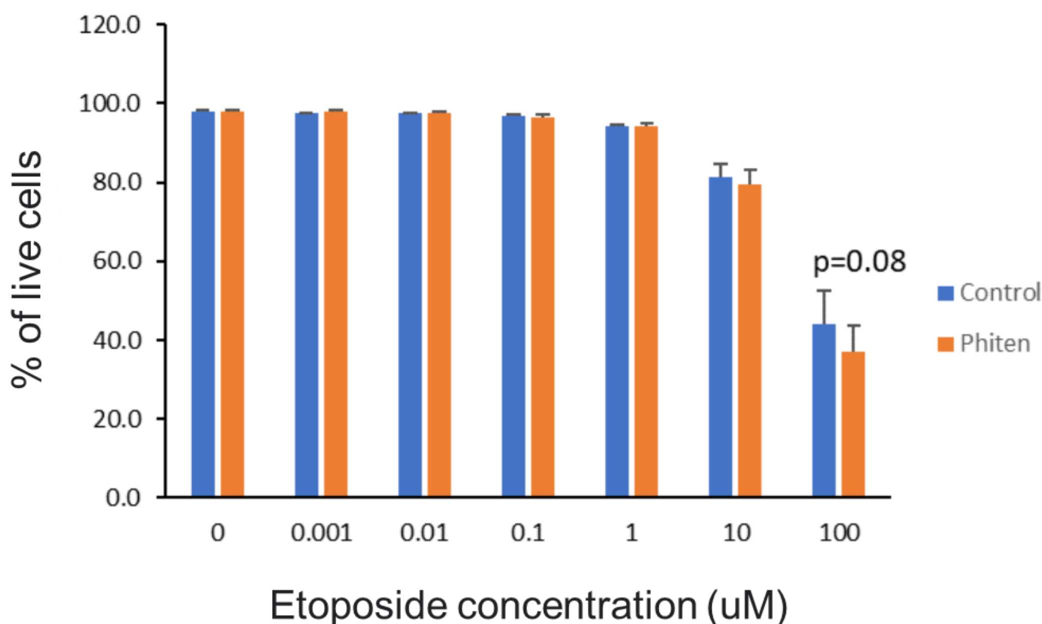

Fig. 4 Evaluation of the efficacy of the anticancer drug etoposide in a "Phitenized" environment.

【Discussions】

As for the effect of "Phitenized" on cell proliferation, cancer cells Ba/F3 were cultured in the "Phitenized" and control environments, and the results showed a significant decrease in the number of live cells in three out of five experiments, but no significant difference in two experiments (N=16 for each). In the report on July 27, 2020, a slight increase in cell number was observed in the "Phitenized" environment by the colorimetric method using water-soluble tetrazolium salt WST-8. However, in the report on November 11, 2020 using the single-cell analysis by flow cytometry there was no significant difference.

In this study, we considered comparing the "Phitenized" and the control plate in the same environment as much as possible in order to detect small changes. For example, the "Phitenized" and the control plate were incubated in the same incubator as shown in Figure 1.

The “Phitenized” plates were shielded by a 3 mm thick lead sheet. In the experiments shown in the previous reports, the “Phitenized” plate and the control plate were incubated in separate incubators. Although there seems to be no difference between incubators in normal cell biology experiments, the previous experimental results suggest that there may have been small differences in the environment between incubators.

As mentioned above, in this experiment, we protected the control plate by wrapping the “Phitenized” plate with a lead sheet. However, since the physicochemical mechanism of “Phitenization” is unknown, it is not clear whether the effect on the control plate was completely blocked by wrapping it with the lead sheet.

The fact that the physicochemical mechanism of “Phitenization” is unknown is a major issue in the design of the experiment. In parallel with investigating the cell biological effects, it may be necessary to observe and analyze the physicochemical phenomena. For example, it has been reported that in addition to the components of the culture medium, the charge on the culture plate also affects cell proliferation, and the zeta potential of the material surface has been shown to be significantly involved in promoting cell adhesion and cell proliferation[1]. Therefore, in order to evaluate the effect of the “Phitenized” plate on cells more precisely, it may be necessary to measure the charge generated on the culture plate by “Phitenization”.

In this experiment, we used a cancer cell line, as is common in basic cell biology experiments, but the cancer cell line is already in a proliferation-accelerated state. Assuming that the proliferation of cells is promoted by the environment, it may be difficult to detect the change in cancer cell lines that are already in a proliferation-enhanced state. In order to solve this problem, it may be necessary to evaluate the effect of the “Phitenized” environment using normal cells, although this will increase the difficulty of the experiment.

On the other hand, for the evaluation of the efficacy of the anticancer drug, the treatment of Ba/F3 cells with the anticancer drug etoposide for 24 hours resulted in a decrease in the live cell rate, indicating the cytotoxicity of etoposide at 10 and 100 uM concentrations. In particular, under the high etoposide concentration condition (100 uM), there was a significant decrease in the live cell rate in the “Phitenized” plate compared to the control plate, suggesting that the drug effect of the anticancer drug etoposide is enhanced by the “Phitenized” plate. Etoposide is an anticancer drug that was synthesized in 1966 from podophyllotoxin contained in the underground stems of American mandrake (*Podophyllum peltatum*) and approved by the U.S. Food and Drug Administration (FDA) in 1983. The mechanism of action is thought to be that it binds to DNA topoisomerase II, an enzyme that cleaves and recombines double-stranded

DNA, and inhibits DNA replication, resulting in inhibition of cell proliferation or cell death[2, 3]. It has been shown that the effect is more potent in cells with high levels of DNA topoisomerase II expression, such as cancer cells[4]. In this experiment, we found that the drug effect of etoposide tended to be enhanced in the “Phitenized” environment, suggesting that the “Phitenized” environment directly or indirectly enhanced the expression level of DNA topoisomerase II. Although the detailed mechanism of this effect is still unclear at this stage, it may be possible to clarify the effect of the “Phitenized” environment on cell division by using DNA topoisomerase II as a clue to comprehensively analyze the expression levels of cell cycle-related genes.

On the other hand, from a clinical point of view, the fact that a “Phitenized” environment enhances the level of DNA topoisomerase II expression and the drug efficacy of etoposide suggests that exposure to a “Phitenized” environment, either locally or systemically, is expected to enhance the anticancer drug effect in human cancer treatment. However, the negative effects of further increasing the expression of DNA topoisomerase II in cancer cells, which already have an elevated expression level, need to be fully considered.

In recent years, it has become clear that cancer cell lines are not a homogeneous population of cells but have subpopulations. These cells have been named "cancer stem cells" because they are capable of self-renewal, multi-differentiation, and tumorigenesis, and characteristic marker molecules such as aldehyde dehydrogenase 1 (ALDH1) have been discovered[5].

In this study, we performed single-cell analysis using a flow cytometer, which allows for more detailed analysis than the previous method of measuring the entire cell population, and conducted various studies assuming the existence of a population whose cell division is altered by culturing in a “Phitenized” environment. However, as mentioned above, although there was a tendency to enhance the evaluation of drug efficacy of high concentrations of anticancer drugs, there was no clear significant difference in cell proliferative capacity in the five experiments (N=16 each). This suggests that the effects of the “Phitenized” environment on the cells are not as large as those of anticancer drugs, but are very small. In order to detect such small changes, it would be ideal if the only difference between the control and the “Phitenized” environment is the “Phitenization”, and it would be necessary to consider the physicochemical mechanism in the experimental design as described above.

【Conclusions】

In this study, we analyzed the effects of the “Phitenized” environment on cells. As a result, we could not draw any conclusions about the effects of the “Phitenized” environment on cell proliferation. On the other hand, it was found that the “Phitenized” environment enhanced the efficacy of anticancer drugs at high concentrations, suggesting that the “Phitenized” environment has some effect on the expression of intracellular molecules. The results of this study will help to elucidate the effects of the “Phitenized” environment on cells by investigating the molecular mechanisms in more detail.

【References】

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