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Cell cycle dynamics analysis of drug-tolerant persister cells in non-small cell lung cancer

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ABSTRACT

Molecular-targeted therapy remains effective in cancer treatment; however, drug resistance inevitably arises after prolonged administration for several years. Drug-tolerant persister cells (DTPs), which are subpopulations of cells derived from drug-sensitive cells that develop tolerance after short-term exposure (lasting days or weeks) to drugs, have gained substantial attention. However, the specific characteristics of DTPs remain unclear as they encompass a diverse cell population, although they generally exhibit low proliferative potential. Therefore, analysis of cell cycle fluctuations during and after the formation of DTPs is expected to elucidate DTPs characteristics and contribute to future therapies. Here, we introduced the Fucci probe into the PC9 cell line to create PC9-Fucci to observe dynamic cell cycle variations during and after the DTPs formation process. The results showed that during the formation of DTPs, the majority of cells showed red fluorescence and were arrested in the G1 phase, whereas a small number of S-phase cells showed green fluorescence. Furthermore, some cells proliferated after the DTPs phase under drug-free medium and drug-containing medium culture conditions. These results indicate cell cycle diversity, with the majority of cells exhibiting arrested cell cycle during the formation of DTPs, whereas others existed in the S phase. In addition, cell cycle diversity occurs not only in the process of DTPs formation but also in subsequent processes.

1. Introduction

Lung cancer has the highest mortality rate among both men and women, and is the primary cause of cancer-related deaths globally [1]. Non-small cell lung cancer (NSCLC) constitutes the majority of lung cancer cases, accounting for approximately 80 % of diagnoses [2,3]. Epidermal growth factor receptor (EGFR) mutations are among the most common oncogenic drivers of NSCLC [4–6]. EGFR tyrosine kinase inhibitors (TKIs), such as erlotinib and osimertinib have been introduced as the first-line treatment for NSCLC [7–11]. Targeted EGFR treatment offers superior outcomes compared to those with chemotherapy [5]. However, tumors inevitably acquire drug resistance after months or years of EGFR-TKI treatment due to acquired genetic mutation [12–15]. Mechanistically, the effectiveness of first- and second-generation TKIs is compromised by factors, such as the presence of mutations, including the EGFR T790M mutation and MET amplification [16]. To overcome EGFR-TKI-resistant tumors, osimertinib, a third-generation EGFR-TKI, was developed and approved for the treatment of EGFR T790M-positive NSCLCs [17]. However, the application of osimertinib is limited by acquired resistance, including C797S and L844V [18–22]. Therefore, it is imperative to understand the process of drug resistance and explore therapeutic options for overcoming acquired resistance.

Abbreviations: DTPs, Drug-tolerant persister cells; EGFR, Epidermal growth factor receptor; TKIs, Tyrosine-kinase inhibitors; EGFR-TKI, Epidermal growth factor receptor-tyrosine-kinase inhibitor; Fucci, Fluorescent ubiquitination-based cell cycle indicator; NSCLC, Non-small cell lung cancer; CO_2, Carbon dioxide.

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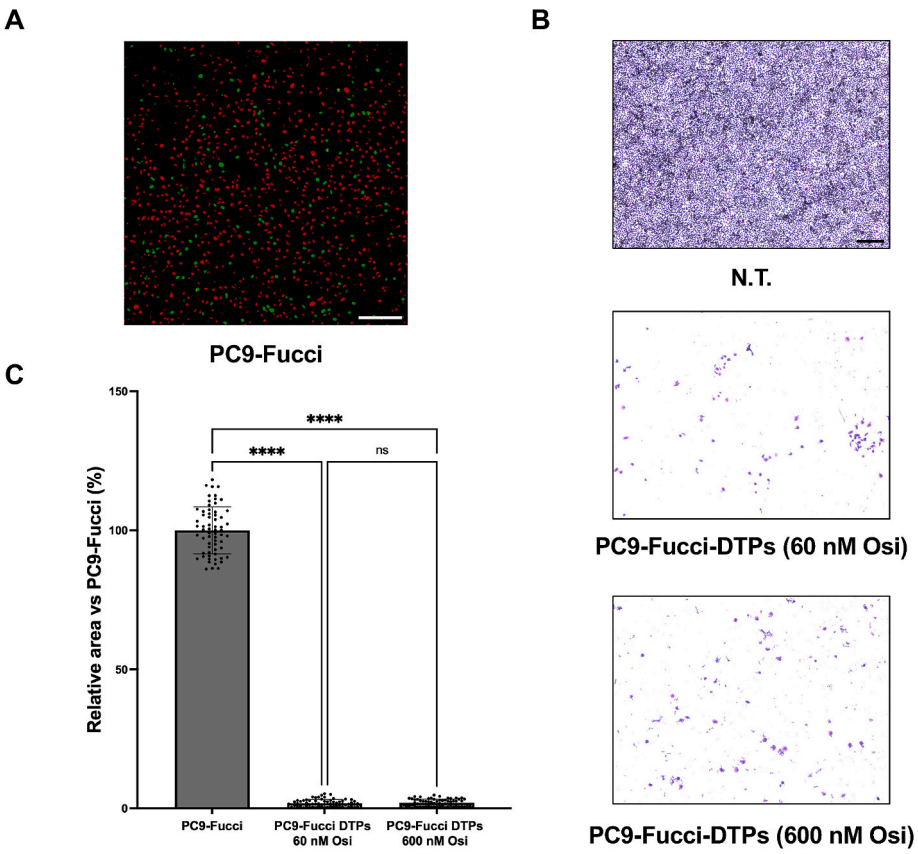

Fig. 1. Formation of PC9-Fucci DTPs. (A) PC9-Fucci cells were seeded on 12-well plates at high confluence and treated with 60 or 600 nM osimertinib for 9 days. Living cell were stained with crystal violet. Images were quantified corresponding to nine different fields. Representative images and histograms of relative area (B) were shown. Data are expressed as means ± SD ($n = 3$). This experiment was repeated third times with similar results. ****$P < 0.0001$, by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. The experiments were repeated with two times. Scale bar is 200 μm long. DTPs, drug-tolerant persister cells, Osi, osimertinib. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The recently identified drug-tolerant persister cells (DTPs) are the source of drug-resistant cells. DTPs have the potential to develop into drug-resistant cells through multiple resistance mechanisms [23]. Therefore, studying DTPs to prevent the emergence of drug resistance offers a novel perspective. Generally, DTPs are a small subpopulation of reversibly "drug-tolerant" cells that maintain viability in a mutation-independent manner after 9 days of drug exposure [24], when the majority of the cell population rapidly succumbs to treatment. Additionally, as a reversible subpopulation, the cell cycle is adaptable to drug exposure. During early treatment stages, most DTPs remain arrested in the presence of the drug, although a small subset of DTPs continues to proliferate and re-enter the cell cycle [25–27]. Oren et al. revealed proliferating DTPs have antioxidant gene upregulation programs across multiple cancer types [25]. However, alterations in cell cycle dynamics during both the formation and subsequent stages of DTPs remain poorly understood. In this study, we used Fucci probes for precise monitoring of cell-cycle progression, focusing on the variation in the cell cycle and number during and after the formation of DTPs to clarify the survival mechanisms of DTPs.

2. Material and methods

2.1. Cell lines and cell cultures

The lung adenocarcinoma cell line, PC9, (ATCC, Manassas, VA, USA) was used in this study. PC9 cells were cultured with 10 % fetal bovine serum (FBS, Biosera, Nuaille, France) and 1 % (v/v) penicillin-streptomycin-amphotericin B suspension (FUJIFILM Wako Pure Chemical, Osaka, Japan) in RPMI-1640 (Wako, Osaka, Japan), and maintained under standard cell culture conditions at 37 °C with 5 % CO_2 in air.

2.2. Establishment of PC9-Fucci cells

A ubiquitination-based cell cycle indicator (Fucci), which enables real-time monitoring of interphase and cell cycle progression, was used to establish PC9-Fucci [28] cells. Lentiviruses were packaged by co-transfection of HEK293T cells (ATCC) with Fucci (CA)2/pCSII-EF (RIKEN BRC, Tsukuba, Japan), psPAX2 (Addgene, Watertown, MA, USA #12260), and pMD2.G (Addgene #12259) using Fugene HD reagent (Promega). Viral solutions were prepared and used to transduce PC9 cells. Purified clones were selected using the serial dilution method in 96-well flat plates (Thermo Fisher Scientific, Waltham, MA, USA) and expanded for further analysis.

2.3. Generation of DTPs

PC9 and PC9-Fucci cells were treated with 60 or 600 nM of osimertinib (Selleckchem, Houston, TX, USA) for 9 days. The medium was replaced every 3 days. Viable cells that remained attached to the dish at the end of day 9 were considered DTPs.

2.4. DTP assay

Cells were seeded at 3×10^4 cells/well in 12-well plates (Nunc, Rochester, NY, USA) and treated with 60 or 600 nM of osimertinib for 9 days. Fresh medium containing drugs was replaced every 3 days. Any remaining cells were fixed with 4 % paraformaldehyde (Wako) and

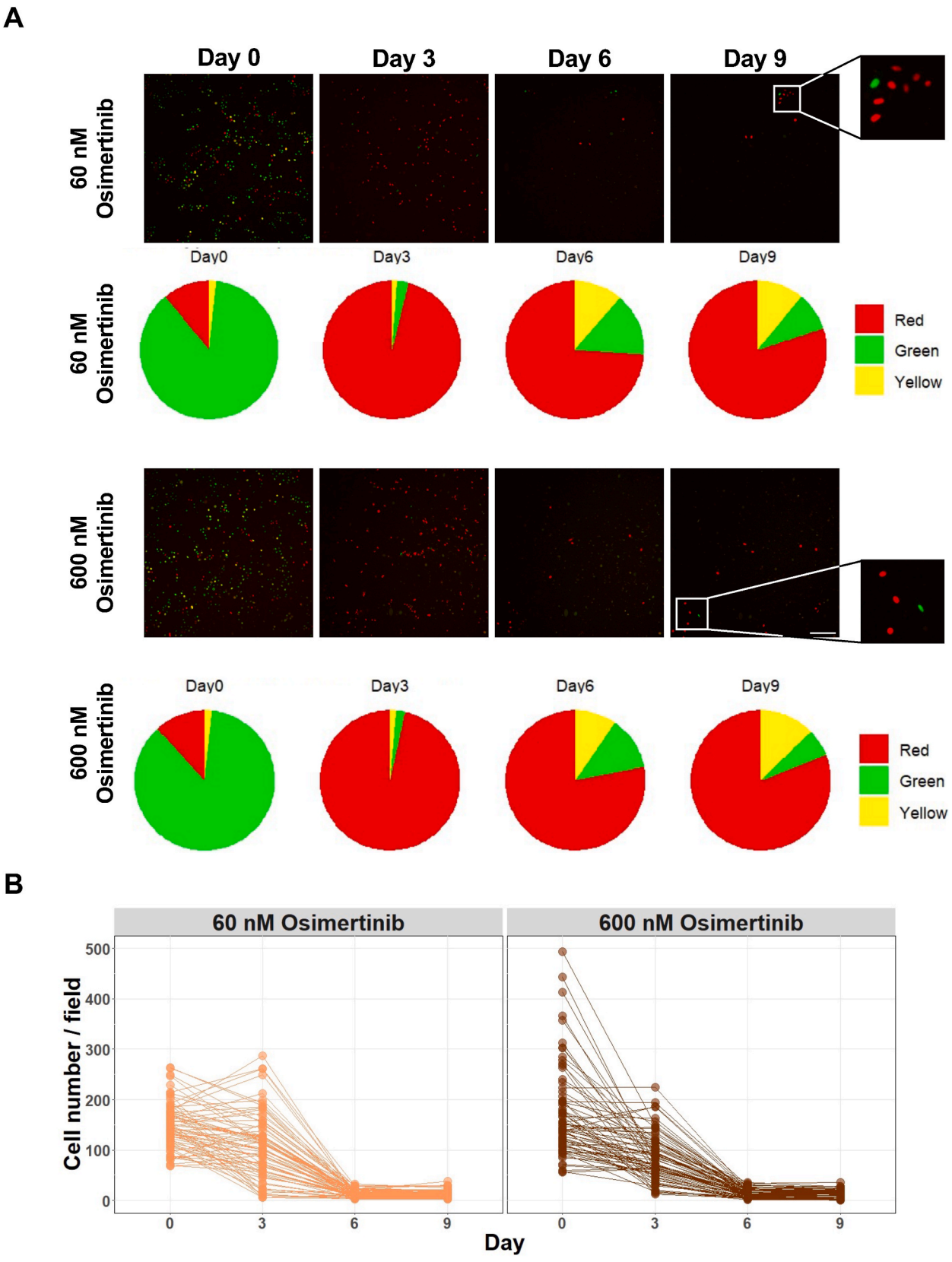

Fig. 2. Fluorescent observation during PC9-Fucci DTPs formation. After seeding on 96-well dishes and culturing overnight, PC9-Fucci cells were treated with 60 or 600 nM of osimertinib for 9 days. (A) Representative images of 60 or 600 nM of osimertinib-treated cells. Images were acquired with CellVoyager CV8000 High-Content Screening System (Yokogawa, Tokyo, Japan) every 3 days. Scale bars: 200 μm. The percentage of green, red, and yellow cells for each day is shown below each image. Image analysis was performed using ImageJ and CellProfiler. (B) Line chart shows the variation of cell counts during the DTPs formation. Each line represents cell counts per field of view for each group. The experiments were repeated with three times. DTPs, drug-tolerant persister cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

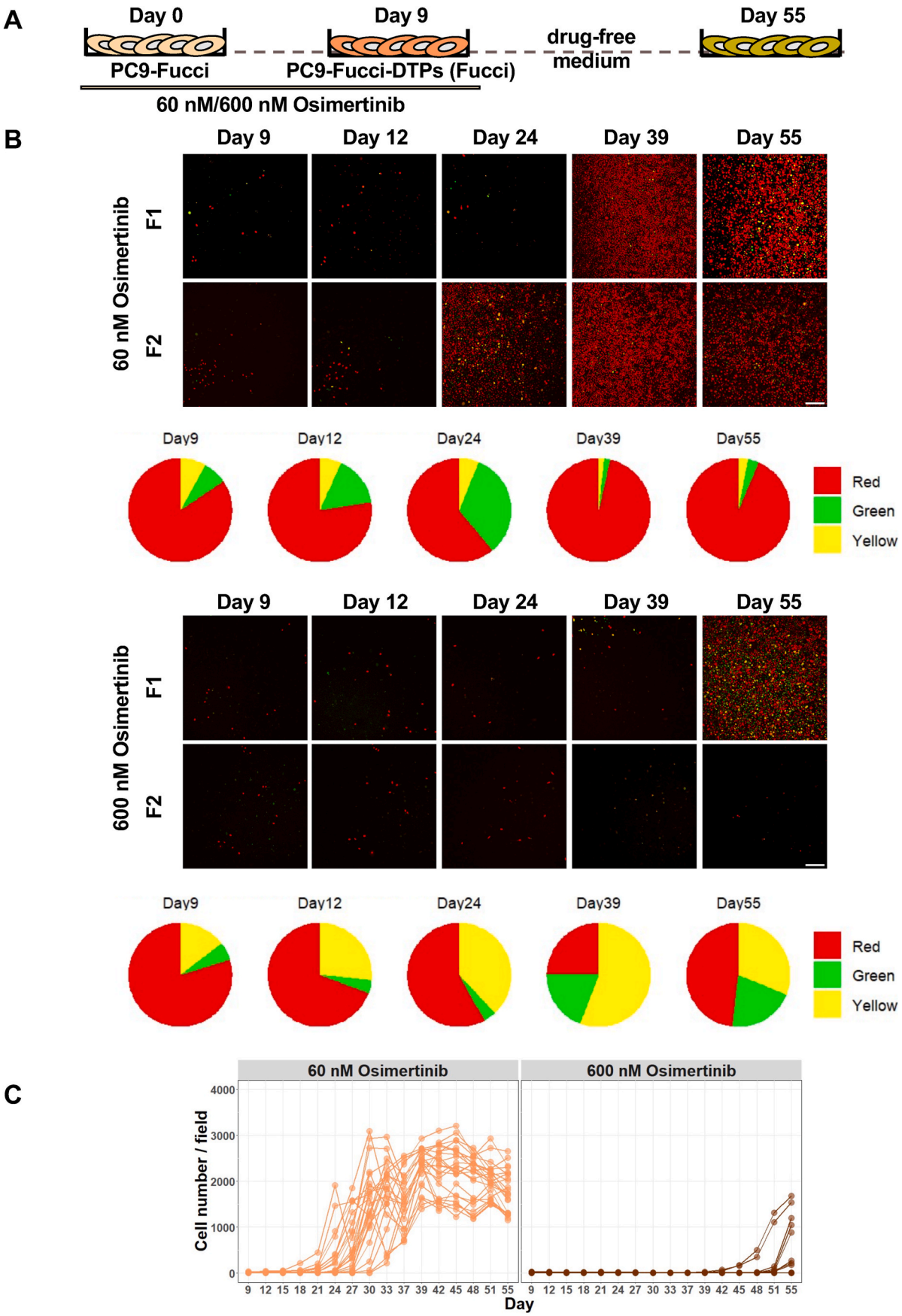

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Fig. 3. Variation in cell number in drug withdrawal state post DTPs formation.
(A) The process of drug treatment after DTP formation. After the formation of DTPs, cells were cultured in drug-free medium. Medium was changed every three days. (B) Representative images of cells cultured in drug-free medium in the field of view (F1, F2) after formation of DTPs. Images were acquired with CellVoyager CV8000 High-Content Screening. Scale bars: 200 μm. The percentage of green, red, and yellow cells for each day is shown below each image. Image analysis was performed using ImageJ and CellProfiler. (C) Line graph shows the change in the number of cells cultured in drug-free medium after DTPs formation. Each line represents the number of cells in each field of view for each group. The experiment was repeated with similar results. DTPs, drug-tolerant persister cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

stained with a crystal violet solution (Wako). Images of the stained cells were captured using a BZ-X800 fluorescence microscope (Keyence, Osaka, Japan) and the colony area was determined using ImageJ 1.53q (National Institutes of Health [NIH], MD, USA).

2.5. Cell cycle and cell number observation

PC9-Fucci cells were seeded at 8×10^3 cells/well in 96-well plates (PerkinElmer, Waltham, MA, USA) and treated with 60 or 600 nM of osimertinib. The cells were imaged using a CellVoyager CV8000 High-Content Screening System (Yokogawa, Tokyo, Japan). Images were analyzed using ImageJ (NIH) and Cellprofiler [29] (version 4.2.1). The R software (version 4.2.2) was used to visualize the data.

2.6. Statistical analysis

Microsoft Excel, Graph Pad Prism Mac version 9.0 (GraphPad Software, La Jolla, CA, USA; www.graphpad.com), and the R (version 4.2.2) software were used to analyze the data. Statistical analysis was performed using one-way ANOVA with the Tukey's test (Significance was set as $p < 0.05$).

3. Results

3.1. Establishment of PC9-Fucci and evaluation of PC9-Fucci-DTPs

The NSCLC PC9 cell line is often used as a cell model to establish DTPs [24], therefore, PC9 was selected as the primary focus of this study. A fluorescent ubiquitination-based cell cycle indicator (Fucci) probe, which was designed to dynamically visualize cell cycle progression by emitting green fluorescence during the S phase and red fluorescence during the G1 phase respectively [28], was introduced into PC9 cells by lentiviral transduction to establish PC9-Fucci (Fig. 1A). First, PC9-Fucci-DTPs was established by exposing two highly cytotoxic concentrations of osimertinib (60 nM/600 nM) to PC9-Fucci cells for 9 days. After 9 days of exposure, the remaining cells were stained with crystal violet to confirm the establishment of PC9-Fucci-DTPs (Fig. 1B and C).

3.2. Fluorescent observation during PC9-Fucci-DTPs formation

To further observe the variation of cell cycle and changes in cell number during the formation of DTPs, after seeding on 96-well plates and culturing overnight, PC9-Fucci cells were treated with osimertinib at a dose of 60 or 600 nM for 9 days. Images were acquired using a High-Content Screening System every 3 days and the percentage of the cells emitting green, red, and both fluorescence (yellow) were analyzed (Fig. 2A). On day 0, the majority of cells exhibited green fluorescence, indicating that the cell cycle was in the S phase (Fig. 2A). Only a small percentage of cells showed red fluorescence for the G1 phase or yellow fluorescence for the G2-M phase (Fig. 2A). Throughout DTPs formation, cell counts gradually diminished, with most cells emitting red fluorescence, indicating cell cycle arrest in the G1 phase (Fig. 2A). By the ninth day of drug exposure, when the DTPs were fully formed, most cells within each well exhibited red fluorescence, whereas a few percentages of the cells still displayed green and yellow fluorescence (Fig. 2A). This suggests that during the DTPs formation, most cells undergo drug-induced cell cycle arrest (red fluorescence), whereas a small subset of cells retains the capacity for increased cellular activity during DTPs formation. This underscores cell cycle diversity within the DTPs population. Next, the images were quantitatively analyzed to assess changes in cell numbers. The results revealed a rapid decrease in cell number from approximately 200 to <50 during DTPs formation under both 60 and 600 nM of osimertinib exposure conditions (Fig. 2B). Collectively, while the cell count decreased during DTPs formation, a subset of cells within the DTPs population exhibited green fluorescence (S phase).

3.3. Variation in cell number in drug-withdrawal state post DTPs formation

DTPs have a reversive phenotype [24]. To explore the reversibility of DTPs in the cell cycle after drug withdrawal, cells were cultured in a drug-free medium following the formation of DTPs, and fluctuations in cell number and cycle were monitored until day 55 (Fig. 3A). DTPs reproliferated in the absence of drug exposure. Upon cessation of drug addition, some cells showed green fluorescence and distinct cell clones appeared in the field of view (Fig. 3B). After quantifying the cell number, DTPs (with 60 nM of osimertinib) induced cell re-proliferation in all fields of view after drug withdrawal at various time points. Under 60 nM of osimertinib condition, as the cells resumed proliferation, an increased presence of green and yellow cells was observed (Fig. 3B). Once the cells reached confluence, the population predominantly returned to red, reflecting a G1-arrested state due to contact inhibition (Fig. 3B). On the other hand, by day 55, some cells post DTPs (with 600 nM of osimertinib) reproliferated, while other cells did not (Fig. 3C). When cells begun to proliferate in some regions, an increased presence of green and yellow cells was observed (Fig. 3B). Collectively, there was a temporal bias in the repopulation of these cells after drug removal. Additionally, after DTPs formation, these cells reproliferated after drug withdrawal, reflecting the reversibility of DTPs. In conclusion, an increased cell proliferation was observed in a drug-free medium after DTPs formation.

3.4. Variation in cell number in drug-continuance state post DTPs formation

To explore how DTPs develop and behave after 9 days of exposure to the drug, DTPs were exposed to either 60 or 600 nM of osimertinib, and fluctuations in cell number and cycle were monitored until day 55 (Fig. 4A). Some DTPs have reproliferating potential after continued exposure to 60 and 600 nM of osimertinib (Fig. 4B, F1). On day 46 of continuous drug exposure, distinct cell clones were observed. However, a subset of cells did not acquire reproliferative capacity and eventually disappeared (Fig. 4B, F2). While the majority of cells in the continuous drug treatment group remained in the red state, indicative of G1 arrest, a small proportion of green cells persisted, suggesting a retained proliferative potential. Moreover, as the cells resumed growth, an increased number of green cells appeared, consistent with cell cycle progression. Notably, in the 600 nM osimertinib group, only minimal regrowth was observed (Fig. 4B), and this was not comparable to the extent of proliferation seen in the drug-withdrawal group. This observation suggests that the capacity for recovery is influenced by the concentration of the drug. Similarly, cell numbers were quantified as in Fig. 3C, some proliferation was particularly observed in certain fields of view under the 60 nM condition and showed that cells began to proliferate again after day 36 (Fig. 4C). This indicated that a subset of DTPs has reproliferative potential under sustained exposure to the drug.

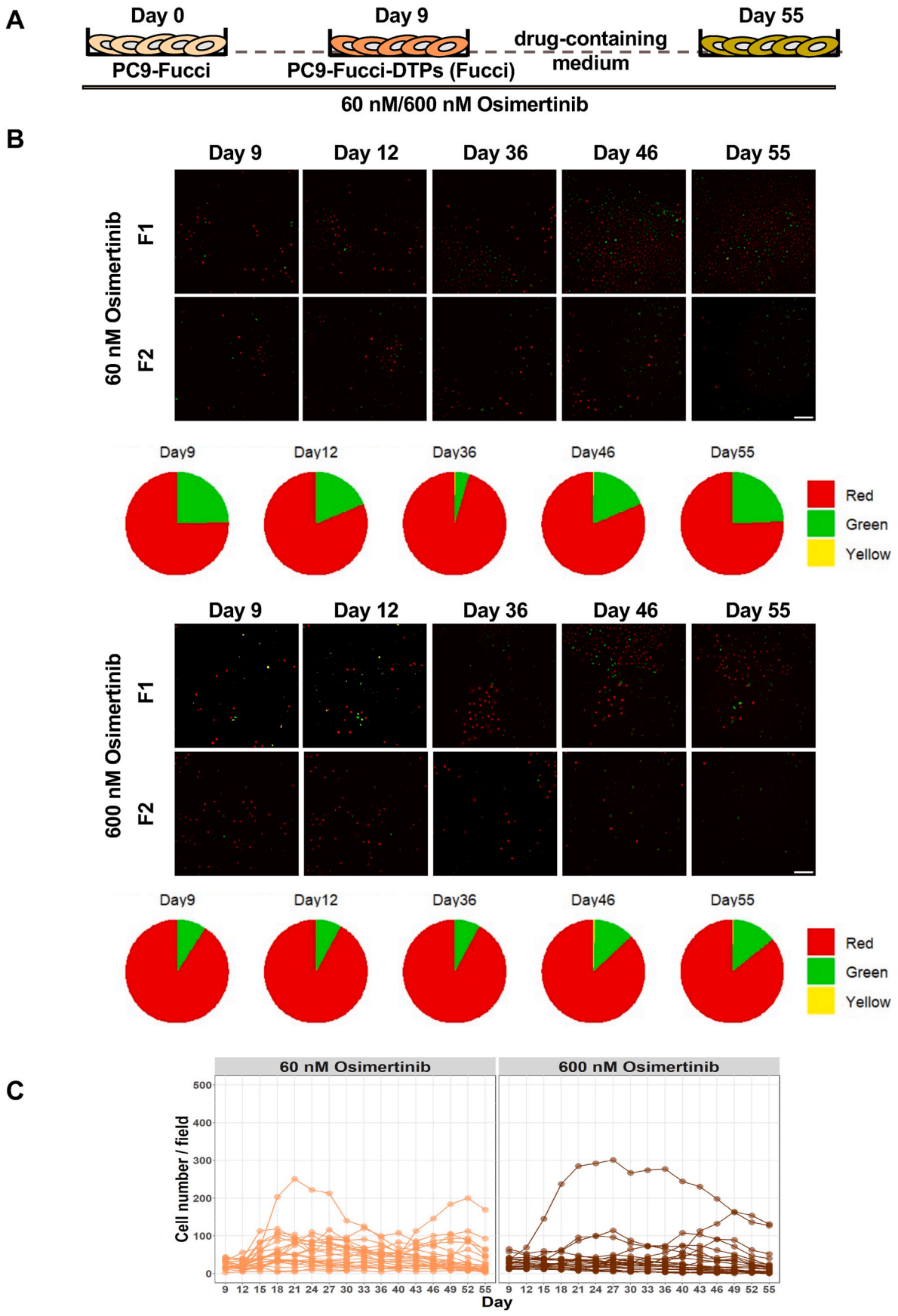

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Fig. 4. Variation in cell number in drug continue state post DTPs formation.
(A) The process of drug treatment after DTP formation. After the formation of DTPs, cells were cultured continuously in a medium containing 60 or 600 nM of osimertinib. Drug-containing medium was changed every three days. (B) Representative images of cells under continued exposure to 60 or 600 nM of osimertinib in the field of view (F1, F2) after formation of DTPs. Images were acquired with CellVoyager CV8000 High-Content Screening. Scale bars: 200 μm. The percentage of green, red, and yellow cells for each day is shown below each image. Image analysis was performed using ImageJ and CellProfiler. (C) Line graph shows the change in cell number under conditions of continuous drug exposure after DTPs formation. Each line represents the number of cells in each field of view for each group, totaling 21 fields of view. The experiment was repeated with similar results. DTPs, drug-tolerant persister cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

Traditionally, DTPs have been regarded as a subpopulation of cells that undergo cell cycle arrest. However, Oren et al. revealed the existence of a subset of proliferatively competent cells among DTPs [25]. To specifically investigate the cell cycle changes in DTPs during and after their formation in drug-containing and drug-free media, the effect of drug treatment on the cell cycle dynamics of DTPs was evaluated. Cell cycle diversity was observed when DTPs were established and proliferation after DTPs formation was observed in drug-free and drug-containing media.

This phenomenon may be attributed to the inherent differences in drug tolerance among DTPs. In this study, DTPs exhibited varying proliferative restoring capabilities; some cells possessed robust restorative power upon drug cessation, whereas others were weaker (Fig. 3). In contrast, under continuous drug treatment, some cells began to proliferate after 36 days of treatment, while others did not (Fig. 4). This diversity highlights the heterogeneity of DTPs and may stem from preexisting differences among cells. Based on previous studies, these differences may have occurred before the formation of DTPs and were further amplified by the drug effects. Seth et al. found that the maintenance of pancreatic tumors relied on a functional hierarchical structure in which different tumorigenic cells exhibited varying proliferative capacities [30]. This phenomenon suggests that even before treatment, there are preexisting differences in the proliferative potential of tumor cells. Such intrinsic cellular heterogeneity may contribute to diverse response patterns or development of resistance following treatment. Goyal et al. conducted drug treatment and analysis of barcoded cells, and found that the resistance potential of clones was determined by intrinsic rather than predetermined by external factors [31]. These studies are consistent with the results of this study. To investigate the reasons for the diversity of DTPs, details of cell cycle dynamics and diversity of single cell-derived clone are required. In the future, we will focus on establishing single-cell-derived clones and evaluating the differences between clones before drug exposure, as well as their impact on the formation of DTPs and their subsequent effects. Meanwhile, we will focus on the cell cycle dynamics during and after DTPs formation in different cell lines to further evaluate the impact of interclonal heterogeneity on DTPs formation.

To better understand the nature of DTPs and cell cycle dynamics in the post-DTPs state, cell sorting based on fluorescence intensity followed by culture would be a valuable approach. However, sorting DTPs is technically challenging because they are fragile and only a small number of cells are generated in each experiment. As a result, these cells may not withstand the physical stress of sorting, and their reattachment after sorting can be inconsistent. Additionally, there is a risk of introducing bias during the reattachment phase. Despite these challenges, sorting cells at the DTPs stage or during the recovery phase after drug removal could help uncover important characteristics of these populations. In particular, comparing the behavior of green and red cells after sorting may provide insights into their proliferative capacity and fate.

Previous studies have reported that DTPs share several phenotypic and functional characteristics with cancer stem cells (CSCs), as described in the literature [24,32,33]. In particular, CSCs are known to retain strong tumor-initiating capacity even when arrested in the cell cycle [34,35]. While there is a view that CSCs are not necessarily essential for tumor origin considering the plasticity observed in cancer [35], it is still important to explore whether DTPs with green or red fluorescence, which reflect different cell cycle states, differ in their tumor-initiating ability. Although our findings showed that cells began proliferating after drug withdrawal, particularly when transitioning into the green phase, it remains unclear whether this proliferative shift is directly linked to tumor initiation. To better understand this relationship, additional studies would be necessary. For instance, assessing the expression of CSC markers in green and red cell populations, as well as evaluating tumor-initiating potential using *in vivo* models, could help determine whether the *in vitro* observations presented here are applicable under physiological conditions. These investigations may further clarify the link between cell cycle status and tumorigenic capacity and address a limitation of the current study.

As the phenomenon of continued culture in a drug-containing medium leads to the repopulation of some cells (Fig. 4), the presence of osimertinib-resistant cells in DTPs should also be further assessed. To confirm the existence of osimertinib-resistant cells, it will be essential in the future to investigate the presence of resistant genetic mutations, such as EGFR-T790 M and EGFR-C797S. To explore the precise mechanism of DTPs in clinical settings according to the osimertinib blood concentration in patients [36], *in vivo* experiments with relatively high concentrations of osimertinib exposure will be conducted. As a limitation of this study, further validation is required across different drug types and cancer models. In this study, we evaluated the effects of osimertinib on PC9 cells. To enhance the generalizability of our findings, additional investigations should be conducted using melanoma models with BRAF inhibitors and breast cancer models with anti-HER2 therapies.

This study provides a foundation to explore the crucial conditions for the formation of DTPs, provide new mechanistic insights into DTPs, and guide the rational use of drugs in clinical settings in the future.

CRediT authorship contribution statement

Mo Zhou: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Yuya Haga:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. **Akihide Nishimura:** Validation, Writing – review & editing. **Suzuno Tanahashi:** Validation, Writing – review & editing. **Kazuma Higashisaka:** Supervision, Writing – review & editing. **Yasuo Tsutsumi:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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