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Potential use of human pluripotency-related gene expression reporter cell line for screening small molecules to enhance induction of pluripotency

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The reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) is a crucial development in regenerative medicine, providing patient-specific cells for therapeutic uses. Traditional methods often utilize viral vectors and transcription factors that pose tumorigenic risks, rendering them unsuitable for clinical applications. This study explored the use of chemicals as a non-tumorigenic alternative for cell reprogramming. Utilizing CRISPR/Cas9 technology, we previously created iPSCs expressing *OCT4*-EGFP and *NANOG*-tdTomato, and derived *OCT4*-EGFP and *NANOG*-tdTomato fibroblastic cells (ON-FCs). These cells were reprogrammed using episomal vectors, and their pluripotency was validated by fluorescence and FACS analyses. High-content screening was employed to assess small molecules that improve reprogramming efficiency, confirming the usefulness of ON-FCs as a dual reporter cell line for identifying small molecules effective in generating human iPSCs. This study underscores the utility of a dual reporter system and high-content screening in identifying effective reprogramming chemicals, establishing a scalable platform for high-throughput screening. Discovering new chemicals that can reprogram iPSCs would provide a non-tumorigenic method to advance the field of regenerative medicine. [BMB Reports 2025; 58(4): 183-189]

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INTRODUCTION

The reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) has revolutionized regenerative medicine by providing a source of patient-specific cells for therapeutic applications (1). However, traditional methods for generating iPSCs often employ viral vectors and transcription factors, which carry tumorigenic risks and are inappropriate for clinical use (2). Thus, there is an urgent necessity to develop safer and more effective reprogramming techniques.

Chemical reprogramming offers a promising alternative, as it can potentially eliminate the risks associated with viral vectors and provide a non-tumorigenic approach suitable for clinical research (3, 4). Numerous studies have utilized chemicals to successfully generate functional cells from various lineages, including cardiomyocytes (5), neural cells (6-9), and hepatocytes (10). Traditional methods for assessing the effects of these chemicals, such as alkaline phosphatase (AP) staining and the use of pluripotency-specific antibodies, are time-consuming and poorly suited for large-scale screening.

To address these limitations, innovative tools are essential for enabling large-scale screening and early detection of chemical effects during reprogramming. In our previous study, we employed the CRISPR/Cas9 technique to generate iPSCs expressing *OCT4*-EGFP and *NANOG*-tdTomato (11). This dual reporter system facilitates real-time visualization of pluripotency markers, significantly simplifying the analysis. Subsequently, we differentiated the reporter iPSCs to produce *OCT4*-EGFP and *NANOG*-tdTomato fibroblastic cells (ON-FCs) (11). These ON-FCs enable observation of *OCT4* and *NANOG* expression through EGFP and tdTomato, respectively, under a fluorescence microscope, providing effective tools for monitoring progression in reprogramming.

In this study, we investigated the potential use of ON-FCs as a dual reporter cell line for reprogramming and high-content

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screening (HCS) to identify chemicals that enhance reprogramming efficiency (Fig. 1) (12). Specifically, we aimed to evaluate the effectiveness of ON-FCs as a tool for real-time monitoring of reprogramming processes. Additionally, we explored the scalability of using ON-FCs in HCS platforms, which are essential for the rapid large-scale screening required to discover novel reprogramming compounds.

RESULTS

Characterization of ON-FCs derived from *OCT4*-EGFP and *NANOG*-tdTomato dual-reporter hiPSCs

Teratoma-derived single cells from *OCT4*-EGFP/*NANOG*-tdTomato dual-reporter hiPSCs were cultured in fibroblast cell culture medium. After 8 passages, the fibroblast cells, ON-FCs, dominated the culture dish. These ON-FCs were morphologically similar to BJ cells but distinct from H9 cells (Fig. 2A). The expression of fibroblast-specific genes (*COL3A1*, *DCN*) and pluripotency genes (*OCT4*, *SOX2*, *NANOG*), analyzed using RT-PCR (Fig. 2B) and qPCR (Fig. 2C, D), confirmed that ON-FCs and BJ cells displayed similar expression patterns. ON-FCs did not exhibit *OCT4*-EGFP/*NANOG*-tdTomato fluorescence (Supplementary Fig. 1).

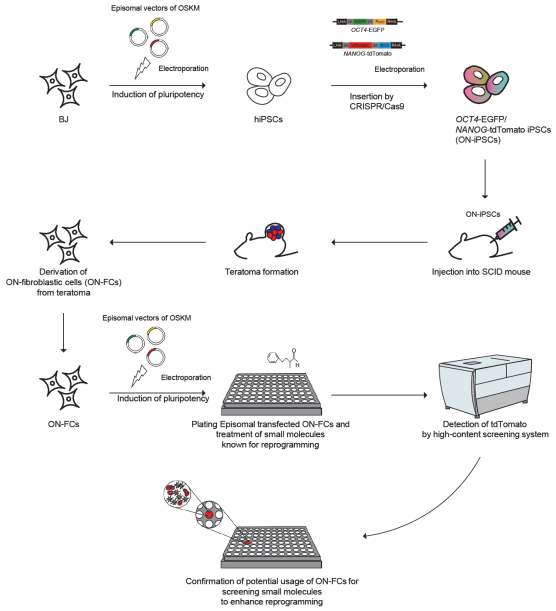

Fig. 1. Generation and application of *OCT4*-EGFP/*NANOG*-tdTomato fibroblastic cells (ON-FCs) to confirm their potential for identifying small molecules that enhance reprogramming efficiency through high-content screening (HCS). This schematic illustrates the derivation and application of *OCT4*-EGFP/*NANOG*-tdTomato-expressing fibroblastic cells (ON-FCs) from teratoma-derived ON-iPSCs following CRISPR/Cas9 insertion. Teratoma formation is induced by injecting ON-iPSCs into SCID mice, after which ON-FCs are derived from the teratoma tissues. These ON-FCs undergo episomal vector transfection with OSKM factors to induce pluripotency, and reprogramming efficiency is assessed through tdTomato signal detection in a HCS platform, confirming their potential to identify small molecules that enhance reprogramming efficiency.

Reprogramming of ON-FCs and expression of *OCT4*-EGFP and *NANOG*-tdTomato

To verify the expression of *OCT4*-EGFP and *NANOG*-tdTomato during the reprogramming of ON-FCs, we introduced episomal vectors via electroporation and monitored the expression of the fluorescent proteins. We observed EGFP and tdTomato fluorescence indicating *OCT4* and *NANOG* expression on reprogramming days 4, 8, and 12 (Fig. 3A). FACS analysis revealed an increase in tdTomato-positive and EGFP-positive subsets on the same days (Fig. 3B). These findings confirmed that the expression of *OCT4* and *NANOG* increased during reprogramming, causing a rise in fluorescence in ON-FC lines.

Reprogramming of ON-FCs into ON-iPSCs

By reprogramming ON-FCs, we derived iPSCs expressing *OCT4*-EGFP and *NANOG*-tdTomato fluorescence, hereafter referred to as ON-iPSCs. Morphologically, ON-iPSCs were indistinguishable from H9 cells. Using RT-PCR and qPCR, we analyzed the expression of fibroblast-specific and pluripotency genes. The gene expression profile of ON-iPSCs was significantly different from that of BJ cells and similar to that of H9 cells (Supplementary Fig. 2). Live cell gating was performed based on the FSC-SSC subsets, as illustrated in Supplementary Fig 3.

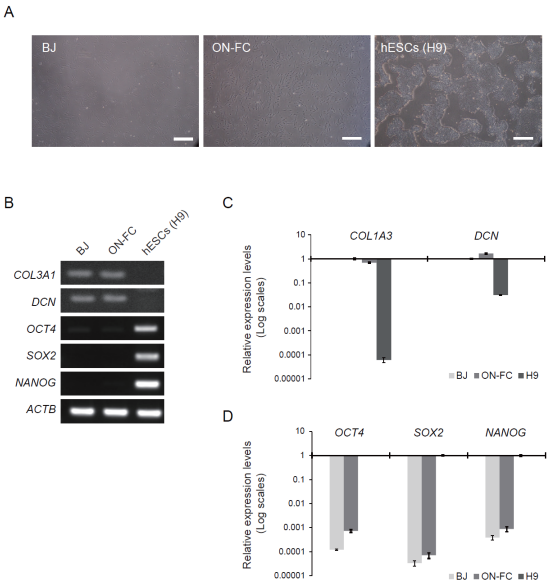

Fig. 2. ON-FC characterization. (A) Morphologies of BJ, ON-FC, and H9 cells. Scale bars = 100 μm. (B) RT-PCR analysis of mRNA expression of fibroblast-specific marker genes (*COL3A1, DCN*) and pluripotency marker genes (*OCT4, SOX2, NANOG*). *ACTB* mRNA was used as a loading control. (C, D) qPCR analysis of mRNA levels of (C) fibroblast-specific marker genes and (D) pluripotency marker genes.

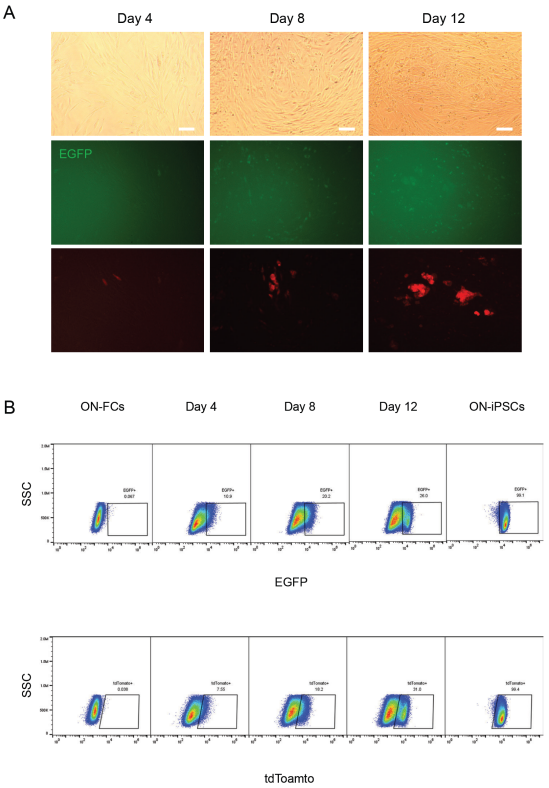

Fig. 3. Verification of ON-FC reprogramming efficiency. (A) Bright field (BF) and expression of EGFP and tdTomato during ON-FC reprogramming on days 4-12. Scale bars = 100 μm. (B) The percentages of EGFP- and tdTomato-positive cells during reprogramming days 4-12, as well as in ON-FCs and ON-iPSCs, were analyzed using FACS.

This unique expression profile, coupled with morphological similarities to H9 cells, confirmed the ability of ON-FCs to be reprogrammed and acquire pluripotency.

Reprogramming ON-FCs with the HCS system

We evaluated the performance of the ON-FC reporter system during reprogramming by introducing small molecules known to enhance the efficiency of reprogramming and employing HCS. Reprogramming was conducted in 96- and 384-well plates, with small molecules introduced 2 days after seeding the cells. To assess the impact of small molecules on enhancing reprogramming efficiency, we quantified the number of cells expressing *NANOG*-tdTomato on reprogramming day 9 (Fig. 4A). The ratio of tdTomato-positive cells to live cells was determined using Hoechst staining, which labeled the live cells (Fig. 4B, C). HCS was employed to quantify the proportion of tdTomato-positive cells relative to live cells in both 96- and 384-well plates (Fig. 4D, E). Our AP staining produced comparable results (Supplementary Fig. 4), confirming that HCS can effectively replace AP staining.

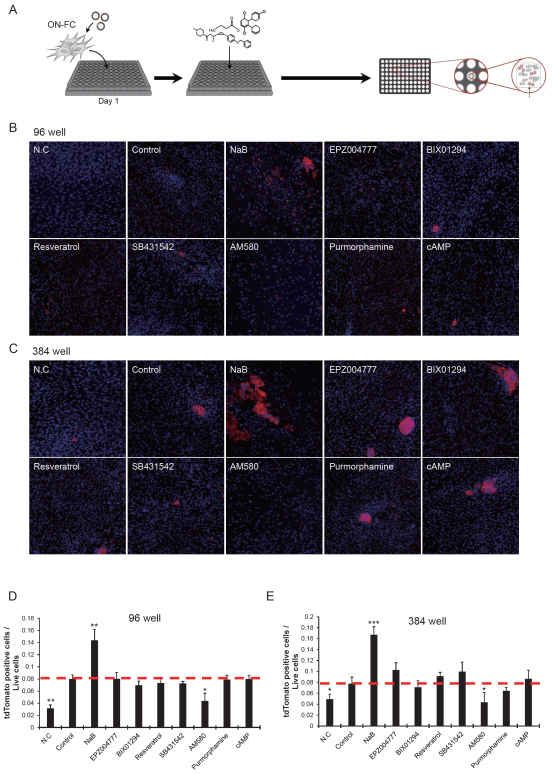

Fig. 4. Re-identification of chemical effects using a high-content screening system. (A) A diagram of the high-content screening system and the method for counting *NANOG*-tdTomato positive cells. (B, C) Fluorescence images of *NANOG*-tdTomato and Hoechst in 96- and 384-well plates on day 9 post-chemical treatment. (D) Ratio of tdTomato-positive cells to live cells on day 9 post-chemical treatment in 96-well plates. Data represent mean ± SEM (n = 6). *P < 0.05, **P < 0.01. (E) Ratio of *NANOG*-tdTomato positive cells to live cells on day 9 post-chemical treatment in 384-well plates. Data represent mean ± SEM (n = 9). *P < 0.05, ***P < 0.001. N.C: negative control (non-reprogrammed cells); Control: reprogrammed cells (not treated with chemicals).

DISCUSSION

The use of small molecules in reprogramming offers significant advantages, particularly in reducing the risk of tumorigenicity and in its applicability to clinical research involving human iPSCs (3, 4). Additionally, screening these chemicals enhances our understanding of the mechanisms involved in cellular reprogramming, providing crucial insights for advancing regenerative medicine (13, 14).

Traditional methods for evaluating the effects of small molecules on reprogramming, such as counting iPSC colonies through AP staining or using pluripotency-specific surface markers, are well-established and straightforward for assessing the induction of pluripotency (15, 16). However, these methods are inherently time-consuming and labor-intensive, rendering them impractical for large-scale screening efforts. There is an urgent

need for more efficient and scalable approaches to advance our capability to rapidly identify and characterize novel reprogramming factors. Thus, it is critical to develop high-throughput screening techniques that can swiftly and effectively evaluate the reprogramming potential of a vast array of chemical compounds, crucial for the progress of stem cell research and its clinical translation (17).

To overcome these limitations, it is essential to develop tools that facilitate large-scale screening and early detection of the effects of chemicals on reprogramming. In this study, we introduce a novel approach that combines a pluripotent reporter cell line with HCS to create a scalable platform for identifying efficient reprogramming small molecules. This method is adaptable to both 96- and 384-well plates, enhancing its suitability for high-throughput screening and early detection of chemical effects during reprogramming. By coupling HCS with a pluripotent reporter system, our approach enables the rapid and efficient assessment of numerous chemical compounds, significantly advancing beyond traditional methods. This scalable platform not only optimizes the identification of effective reprogramming chemicals but also improves our capacity to explore the dynamics of reprogramming on a larger scale, ultimately accelerating the advancement of regenerative medicine research and its clinical applications.

NANOG is expressed during the early stages of reprogramming, whereas *OCT4* expression becomes more prominent in fully reprogrammed iPSCs (18, 19). Consequently, in our study, we focused on *NANOG* expression to screen for chemicals that enhance reprogramming efficiency, finding this method more effective than depending on *OCT4* expression. By concentrating on *NANOG*, our goal was to identify compounds that boost the early stages of reprogramming. This strategic focus allows for the early detection of reprogramming activity, potentially leading to the discovery of chemicals that not only promote, but also accelerate, the reprogramming process. Thus, our approach utilizes the temporal expression patterns of a crucial pluripotency marker to refine screening, enhancing the probability of identifying potent agents that improve reprogramming efficiency.

To examine the effects of chemicals on living cells, we employed HCS to detect tdTomato fluorescence. The HCS system, an automated fluorescence imaging platform, facilitates large-scale fluorescence detection in living cells, enabling high-throughput analysis. In our study, we treated a reporter cell line undergoing reprogramming using eight chemicals known as signaling pathway regulators and epigenetic modulators. Reprogramming within both 96- and 384-well plates was achieved, enabling efficient screening of these chemicals. These findings align with previous studies (20-28) that have demonstrated the positive effects of these compounds on reprogramming. Through HCS, we were able to swiftly and precisely evaluate the impact of these chemicals, delivering strong evidence to support the effectiveness of our screening approach. This method not only verifies the utility of the selected chemicals but also highlights the potential of HCS as an influential tool in advancing reprogramming research.

Our chemical screening approach not only validates the utility of established reprogramming chemicals but also demonstrates that our cell line and platform can be effectively utilized to identify new chemicals with reprogramming capabilities. This method offers a promising avenue for discovering novel candidates for the chemical reprogramming of iPSCs, providing a non-tumorigenic and clinically relevant approach. Utilizing the capabilities of our pluripotent reporter cell line, future research can expand the screening to include a wider range of chemicals, potentially revealing new pathways and mechanisms that enhance reprogramming efficiency.

The approach presented here underscores the versatility and robustness of our screening platform, adaptable for uncovering previously uncharacterized compounds with significant reprogramming potential. Efficiently screening large libraries of chemicals will facilitate the discovery of novel reprogramming agents that may exceed current standards in efficacy and safety. Further, understanding how these new chemicals influence reprogramming offers deeper insights into the fundamental biology of cell fate conversion, thereby paving the way for innovative therapeutic strategies in regenerative medicine (29).

While our study underscores the effectiveness of HCS as a high-throughput screening platform for identifying reprogramming-enhancing small molecules, several potential challenges must be considered for its broader acceptance and clinical translation. A primary concern in any high-throughput screening system is its reproducibility across various experimental conditions and laboratories. Although we observed consistent trends using both 96- and 384-well plate formats, variability in fluorescence intensity and signal quantification may arise due to differences in imaging conditions, cell density, and reagent batch effects. The standardization of imaging parameters and automation of data analysis pipelines could enhance the reliability of our system. Moreover, while our HCS platform significantly reduces the time and labor required for traditional reprogramming assessments, the cost associated with high-content imaging systems, fluorescence-compatible plates, and image processing software may pose barriers to widespread adoption, especially in resource-limited research environments. Addressing these limitations is essential for enhancing the accessibility and utility of this approach.

Identifying novel small molecules that enhance reprogramming efficiency holds significant clinical implications. Chemical reprogramming presents a promising alternative to transgene-based methods, reducing the risks associated with genomic integration and tumorigenicity, and thus improving the safety profile of iPSC-based therapies.

In conclusion, our study successfully generated ON-FCs and established a robust HCS platform for screening reprogramming chemicals. This method's potential to identify candidate chemicals for reprogramming iPSCs further highlights its utility and impact. By providing an efficient and scalable tool for

discovering new reprogramming small molecules, our platform can significantly contribute to developing safer, more effective reprogramming protocols, ultimately enhancing iPSCs' clinical applicability in future cell therapies.

MATERIALS AND METHODS

Derivation of ON-FCs from *OCT4*-EGFP and *NANOG*-tdTomato dual-reporter hiPSCs

ON-FCs were generated using methods established in a previous study (11). The overall experiment was performed based on the schematic diagram shown in Fig. 1. This research was approved by the Konkuk University Institutional Animal Care and Use Committee (approval no. KU24171). Approximately 4.0×10^6 *OCT4*-EGFP and *NANOG*-tdTomato dual-reporter hiPSCs suspended in mTeSR medium (Stemcell Technology) containing Matrigel were injected subcutaneously into the dorsolateral flank of an 8-week-old male NCr nude mouse (DBL). The sex of the mouse does not influence on the formation of the teratoma. The teratoma was harvested 6-8 weeks post-injection, finely chopped, and digested with collagenase and hyaluronidase. This digestion allowed fibroblast cells to spread and proliferate in human fibroblast cell culture medium (DMEM with 10% fetal bovine serum (FBS), 1× non-essential amino acid (NEAA), 1× penicillin/streptomycin; all from Welgene or Corning) at 37°C and 5% CO_2. The cells were passaged using 0.05% trypsin until a homogenous fibroblast cell population was obtained.

Cell cultures

BJ fibroblast cells and ON-FCs were cultured in DMEM supplemented with 10% heat-inactivated FBS, 0.1 mM NEAA, and 1% penicillin/streptomycin. H9 human embryonic stem cells and iPSCs were cultured on Matrigel-coated dishes containing mTeSR medium.

Induction of pluripotency in human fibroblasts

Human fibroblasts were reprogrammed into iPSCs by methods established in a previous study (30). Human iPSCs were generated by electroporation of episomal vectors encoding reprogramming factors (pCLXE-h*OCT4/shp53*, pCLXE-h*SOX2/KLF4*, and pCLXE-h*L-MYC/LIN28A*; Addgene plasmids #27077, #27078, #27080, respectively). Fibroblasts (1.5×10^6) were isolated from the culture dish by treatment with 0.25% trypsin and were electroporated with the three episomal vectors using an Amaxa 4D-Nucleofector kit for P2 primary cell solution (Lonza). Transfected fibroblasts were plated into Matrigel-coated wells in reprogramming medium (E7 medium, Stemcell Technology).

RT-PCR and qPCR

Total RNA was isolated using the RNeasy Kit (Qiagen), following the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RT-PCR was conducted using Ex Taq Polymerase (TaKaRa). qPCR was carried out using SYBR Green (Enzynomics), in a LightCycler 1536 Real-Time PCR System (Roche). Expression levels were normalized to those of the housekeeping gene, *ACTB*.

Fluorescence-activated cell sorting

To select the time point when pluripotent reporter somatic cells become reprogramming positive, on reprogramming days 4, 8, and 12 the cells were dissociated. The pelleted cells were resuspended in 3 ml of cold PBS containing 2% FBS and filtered through a 40 μm cell strainer (BD Falcon) to remove clumps and ensure single-cell suspension. The cells were kept on ice until sorting. *OCT4*-EGFP and *NANOG*-tdTomato were used as the marker for pluripotent cells. No additional antibodies or stains were used in this analysis. FACS was performed using a FACS Aria III system (BD Biosciences). Forward scatter (FSC) and side scatter (SSC) were used to gate single, live cells, excluding debris and doublets. The gating strategy is detailed in Supplementary Fig. 3.

Small molecule screening using HCS system

To determine whether ON-FCs could be used for HCS to identify small molecules that enhance the efficiency of reprogramming, we used small molecules known to be able to reprogram somatic cells into iPSCs (Table 1). Electroporated ON-FCs were seeded onto Matrigel-precoated 96- or 384-well Fluotrac plates (Greiner Bio One) at densities of 4.0×10^3 cells/well in 200 μl E7 medium or 2.0×10^3 cells/well in 100

Table 1. Small molecules used for high-content screening system

Small molecule	Function	Concentration	Reference
Sodium butyrate	Histone deacetylase (HDAC) inhibitor	500 μM	(20)
EPZ004777	H3K79me2 decrease, H3K79 methyltransferase (DOT1L) inhibitor	300 nM	(21)
BIX01294	Histone methyltransferase inhibitor	100 nM	(22)
Resveratrol	SIRT1 activator	10 nM	(23)
SB431542	ALK 4, 5, 7 inhibitor	2 μM	(24, 25)
AM580	Retinoic acid receptor (RARα) agonist	10 nM	(26)
Purmorphamine	Sonic hedgehog (Shh) agonist	100 nM	(27)
cAMP	Protein kinase A (PKA) activator	1 μg/ml	(28)

μl E7 medium, respectively. E7 medium (negative control) and E7 medium with small molecules were refreshed every 2 days. On reprogramming day 9, untreated and treated cells were stained with Hoechst dye in PBS for 20 min at room temperature. The fluorescence signal of tdTomato in live cells was imaged using an Operetta CLS High-Content Analysis System (PerkinElmer). Cells with the intensity of tdTomato fluorescence exceeding 500 AU were detected using Harmony software (PerkinElmer). Statistical analysis was performed by comparing each sample to the control group using Student's t-test.

Software analysis algorithm

Cells were segmented on the basis of a common threshold of Hoechst at 0.10, splitting coefficient 7.0, an individual threshold 0.40, and an area over 30 μm^2. Cytoplasms of the cells were calculated using the "Find cytoplasm" function based on tdTomato fluorescence with an individual threshold of 0.05. Each fluorescence intensity was measured using the "Calculate intensity properties" function, and cells with >500 gray values were selected as the target population. Morphological properties of the cells were elicited with the information on cell area, roundness, width, length and width/length ratio.

Equipment setting for high-content analysis system

A 384-well plate was scanned using a 10× objective lens (Air NA 0.3, Zeiss) or 20× lens (Air NA 0.8, Zeiss) with two-peak autofocus and binning 2 in spinning-disk confocal mode. Excitation bandwidth was 530-560 nm and emission was 570-650 nm for tdTomato fluorescence.

Alkaline phosphatase staining

Cells were fixed in 4% paraformaldehyde for 15 min at room temperature and stained for AP with the Leukocyte Alkaline Phosphatase kit (Stemgent) following the manufacturer's instructions.

Statistical analysis

The values are represented as mean ± SEM. All statistical analyses were carried out using GraphPad Prism 5.0 (GraphPad Software). A *t*-test was used to assess statistical significance. A P-value below 0.05 was deemed statistically significant.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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