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LETTER TO THE EDITOR

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Bromodeoxyuridine promotes full-chemical induction of mouse pluripotent stem cells

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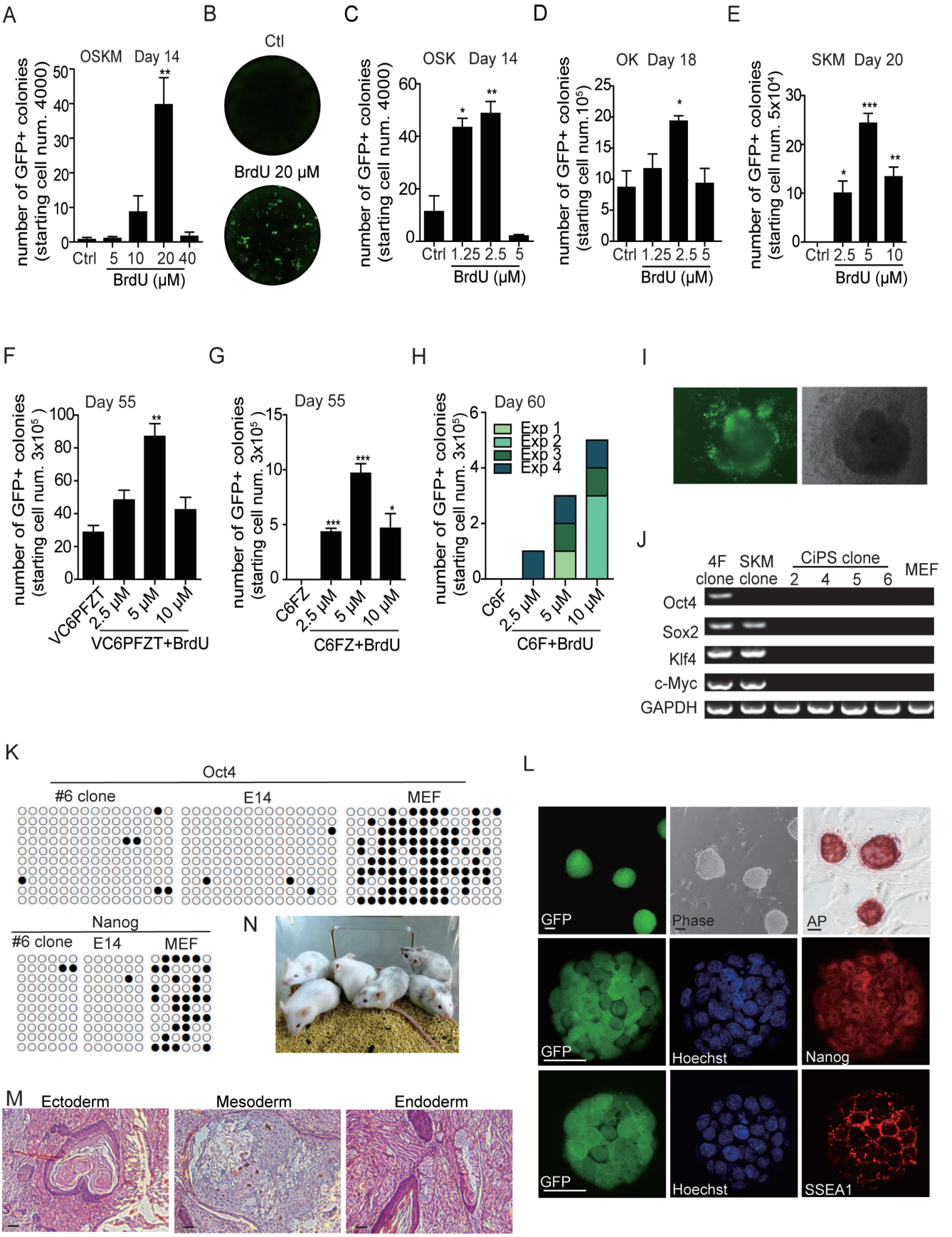
Dear Editor,

Direct reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) with transcription factors (e.g., Oct4 (O), Sox2 (S), Klf4 (K), and c-Myc (M)) greatly expands our understanding of cell fate control. iPSCs resemble embryonic stem cells (ESCs) but without immune rejection and ethic issues, and are therefore considered as a promising source for cell replacement therapy. Patient-derived iPSCs can also be differentiated into disease-associated cell types and be used for drug screening [1]. However, iPSC applications are hindered by safety concerns about the possible genetic alterations caused by the use of exogenous pluripotency-associated factors. Many efforts have been taken to make iPSCs more amendable in clinical applications by using non-integrating gene delivery approaches [2], or cell membrane-permeable proteins [3, 4] to induce the reprogramming.

Small-molecule compounds have also been found to be extremely useful in facilitating iPSC generation and can replace several reprogramming factors [5]. Several combinations of small-molecule compounds have been reported to allow iPSC generation with only Oct4 [6, 7]. However, complete chemical-mediated reprogramming of somatic cells into the pluripotent state has been proved to be extremely difficult. To date, only one study reported a seven- or four-chemical cocktail that can induce reprogramming [8]. Here we report that the commonly used biological reagent, bromodeoxyuridine (BrdU), is able to enhance Yamanaka factor-mediated reprogramming. More interestingly, BrdU can replace Oct4, the most critical factor in iPSC generation. Further studies demonstrate that BrdU promotes full-chemical induction of mouse iPSCs using several chemical cocktails, with the minimal combination being BrdU, CHIR99021, Repsox, and Forskolin. These iPSCs resemble ESCs in terms of their gene expression, epigenetic status, *in vivo* differentiation potentials and the ability to generate chimera.

We have previously established a 96-well plate-based screening system (Supplementary information, Figure S1A) for chemicals that could affect OSKM-induced reprogramming of OG2 mouse embryonic fibroblasts (MEFs) stably carrying an *Oct4* promoter-driven GFP reporter. Using this system we identified both LiCl and high osmotic pressure could enhance OSKM-mediated reprogramming of MEFs [9, 10]. Unexpectedly, we also discovered that BrdU, a synthetic nucleoside that is an analog of thymidine and is commonly used in tracing DNA replication, was able to facilitate OSKM-induced reprogramming. About 40 GFP^+ colonies could be observed in BrdU-treated wells (starting from 4 000 MEFs/well), while only 1-2 colonies could be observed in the control well (Figure 1A and 1B). We treated the OSKM-infected MEFs with BrdU for various durations starting from day 3, 6 or 9 post infection (Supplementary information, Figure S1B and S1C). BrdU displayed remarkable effect at the early stage of reprogramming (Supplementary information, Figure S1B) and showed maximum effect if its treatment was maintained from day 3 to day 7 (Supplementary information, Figure S1C). BrdU could not maintain self-renewal of mESCs in LIF-free condition, and in the presence of LIF, BrdU even induced differentiation of mESCs (Supplementary information, Figure S1D). This explained why prolonged treatment of BrdU actually reduced the number of iPSC clones (Supplementary information, Figure S1C). Interestingly, the most effective concentration of BrdU in promoting reprogramming seems to vary according to the starting density of MEFs. The lower the starting MEF density, the lower the most effective concentration of BrdU (Supplementary information, Figure S1E). BrdU not only increased the number of GFP^+ cells, but also speeded up the reprogramming process. GFP^+ cells could be observed in BrdU-treated group as early as day 7 by FACS analysis (Supplementary information, Figure S1F), and more than 30% cells were GFP^+ on day 14 in BrdU group.

To confirm the pluripotency of iPSCs generated with the OSKM and BrdU method, a series of iPSC lines were established. Real-time PCR analysis confirmed the reactivation of the endogenous *Oct4*, *Sox2*, *Nanog* and *Rex1* and the silencing of viral genes in these iPSC lines (Supplementary information, Figure S2A and S2B). These


A
OSKM Day 14
number of GFP+ colonies (starting cell num. 4000)
Ctrl 5 10 20 40
BrdU (μM)
B
Ctl
BrdU 20 μM
C
OSK Day 14
number of GFP+ colonies (starting cell num. 4000)
Ctrl 1.25 2.5 5
BrdU (μM)
D
OK Day 18
number of GFP+ colonies (starting cell num.10^5)
Ctrl 1.25 2.5 5
BrdU (μM)
E
SKM Day 20
number of GFP+ colonies (starting cell num. 5x10^4)
Ctrl 2.5 5 10
BrdU (μM)
F
Day 55
number of GFP+ colonies (starting cell num. 3x10^5)
VC6PFZT 2.5 μM 5 μM 10 μM
VC6PFZT+BrdU
G
Day 55
number of GFP+ colonies (starting cell num. 3x10^5)
C6FZ 2.5 μM 5 μM 10 μM
C6FZ+BrdU
H
Day 60
number of GFP+ colonies (starting cell num. 3x10^5)
Exp 1
Exp 2
Exp 3
Exp 4
C6F 2.5 μM 5 μM 10 μM
C6F+BrdU
I
J
4F clone
SKM clone
CiPS clone 2 4 5 6
MEF
Oct4
Sox2
Klf4
c-Myc
GAPDH
K
Oct4
#6 clone
E14
MEF
Nanog
#6 clone
E14
MEF
N
L
GFP
Phase
AP
GFP
Hoechst
Nanog
GFP
Hoechst
SSEA1
M
Ectoderm
Mesoderm
Endoderm

iPSCs maintained GFP expression and ESC-like morphology, and expressed pluripotency markers, including alkaline phosphatase, Nanog, and SSEA1 (Supplementary information, Figure S2C). Subcutaneous injection of the iPSCs into NOD-SCID mice led to teratoma formation in 4 weeks, with tissues derived from three germ layers (Supplementary information, Figure S2D). We also injected the iPSCs into the ICR blastocysts and chimeric mice were successfully obtained (Supplementary information, Figure S2E).

Next we tested whether BrdU could enhance reprogramming with reduced numbers of transcription factors. BrdU was found to enhance reprogramming of MEFs induced by OSK (Figure 1C) or OK plus a chemical cocktail (3 μM CHIR99021 and 1 μM RepSox, Figure 1D). Most surprisingly, BrdU was found to be able to replace Oct4, and induce iPSC generation with SKM. All three concentrations of BrdU were found to be effective (Figure 1E). More than 20 GFP^+ colonies could be observed in the 5 μM BrdU-treated wells (starting from 50 000 MEFs/well in a 6-well plate), while none was found in the control wells (Figure 1E). Genomic PCR analysis confirmed that the SKM-iPSCs were free of *Oct4* transgene contamination (Figure 1J).

Previous studies have suggested that Oct4 is the most essential of the four Yamanaka factors. Several chemical cocktails were found to be effective in inducing reprogramming only in the presence of Oct4 [6, 7]. To date, only one study demonstrated that a 7-chemical cocktail (VPA (V), CHIR99021 (C), E-616452 (6, Repsox), Parnate (Tranylcypromine, P), Forskolin (F), DZNep (Z) and TTNPB (T)), with C6FZ being the core components, was able to fully replace Yamanaka factors and induce reprogramming of mouse fibroblasts [8]. As BrdU was able to replace Oct4, we asked whether it could be useful in chemical-induced reprogramming. In the chemical-induced reprogramming system, VC6PFT were added from day 0 to day 32, and Z was added from day 16 to day 32. The chemical cocktail was supplemented with various concentrations of BrdU from day 0 to day 32. After day 32, medium containing chemicals was replaced with the 2i-medium. GFP^+ colonies appeared at around day 45. BrdU (5 μM) enhanced 7 chemical-induced reprogramming by approximately threefold (Figure 1F). We failed to observe GFP^+ iPSCs with only 4 chemicals (C6FZ) after a 2-month induction, but in BrdU-treated groups, ~10 GFP^+ colonies could be observed (Figure 1G). Further experiments indicated that Z could also be removed from the cocktail, a C6F-BrdU combination could successfully induce the generation of GFP^+ iPSCs, although the efficiency was extremely low (Figure 1H).

Typical chemically generated iPSC (CiPSC) colonies (Figure 1I) were selected for further analyses. CiPSCs induced by different chemical cocktails were referred to as 7B (VC6PFZT plus BrdU)-, 4B (C6FZ plus BrdU)- or 3B (C6F plus BrdU)-CiPSCs. Genomic PCR analysis confirmed that the 4B-CiPSC clones were free of transgene contamination (Figure 1J). Real-time PCR analysis revealed the reactivation of the endogenous *Oct4*, *Sox2*, *Nanog* and *Rex1* in the CiPSCs (Supplementary information, Figure S3A and S3B). Bisulfite genomic sequencing analyses showed that the *Oct4* and *Nanog* promoters were demethylated in the 4B-CiPSCs similar to ESCs and different from MEFs (Figure 1K). Both the 4B- and 3B-CiPSC clones maintained GFP expression and ESC-like morphology, and expressed pluripotency markers such as alkaline phosphatase, Nanog, and SSEA1 (Figure 1L and Supplementary information, Figure S3C). Karyotyping

Figure 1 BrdU promotes full-chemical induced reprogramming of MEFs. **(A)** Dose-response effect of BrdU on OKSM-induced reprogramming of MEFs. Starting cell density was 4 000 MEFs/well (96-well plate). GFP^+ colonies were counted on day 14. **(B)** Representative images of GFP^+ colonies in a well of a 96-well plate on day 14 after induction. **(C)** iPSC generation with OSK and various concentrations of BrdU. GFP^+ colonies were counted on day 14. **(D)** iPSC generation with OK plus 3 μM CHIR99021, 1 μM RepSox and various concentrations of BrdU. GFP^+ colonies were counted on day 18. **(E)** iPSC generation with SKM and various concentrations of BrdU. GFP^+ colonies were counted on day 20. Data in **A**-**E** are presented as mean ± SEM of a representative experiment (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 (versus control). **(F)** MEFs were treated with a combination of seven small molecules including 0.5 mM VPA (V), 10 μM CHIR99021 (C), 10 μM RepSox (6), 5 μM parnate (P), 50 μM Forskolin **(F)**, 50 nM DZNep (Z) and 1 μM TTNPB (T) supplemented with BrdU or not. GFP^+ colonies were counted on day 55. **(G)** MEFs were treated with a combination of 4 small molecules including 10 μM CHIR99021 (C), 10 μM RepSox (6), 50 μM Forskolin (F) and 50 nM DZNep (Z) supplemented with BrdU or not. GFP^+ colonies were counted on day 55. Data in **F** and **G** are presented as mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 (versus control). **(H)** MEFs were treated with a combination of three small molecules including 10 μM CHIR99021 (C), 10 μM RepSox (6) and 50 μM Forskolin (F) supplemented with BrdU or not. GFP^+ colonies were counted on day 60. Four independent experiments results were shown. **(I)** Morphology of a typical GFP^+ colony induced by C6FZ and BrdU (5 μM) on day 55. **(J)** PCR analysis to confirm the absence of OSKM integration in CiPSC clones generated with C6FZ and 5 μM BrdU (4B-CiPSC, #2, #4, #5 and #6). A clone generated with SMK and BrdU was also tested. MEFs and an OSMK-clone were used as controls. **(K)** DNA methylation profile of the *Oct4* and *Nanog* promoters in 4B-CiPSC clone #6 (4B-CiPSC-6). E14 mESCs and MEFs were used as controls. **(L)** GFP expression, colony morphology, AP staining and immunofluorescence staining of pluripotency markers Nanog and SSEA-1 in 4B-CiPSC-6. Scale bar, 50 μm. **(M)** H&E stained sections of teratoma formed by 4B-CiPSC-6. Scale bar, 50 μm. **(N)** Chimeric mice generated with 4B-CiPSC-6.

analysis of 4B-CiPSC clone 6 (4B-CiPSC-6) revealed a normal mouse karyotype (40, XY) (Supplementary information, Figure S3D). The 4B-CiPSC-6 clone was then induced to form embryoid bodies (EBs) *in vitro*. qRT-PCR analysis revealed the upregulation of lineage-specific genes in CiPSC-derived EBs similar to E14-derived EBs (Supplementary information, Figure S3E), and immunofluorescence staining identified cells in all three germ layers (Supplementary information, Figure S3F). Both the 4B- and 3B-CiPSC clones were able to form teratomas in NOD-SCID mice and differentiate into three germ layers *in vivo* (Figure 1M and Supplementary information, Figure S3G). Furthermore, the 4B-CiPSC-6 clone was able to produce chimeric mice when injected into ICR blastocysts (Figure 1N).

BrdU is a thymidine analog that is incorporated into DNA of dividing cells, and is thus used for birth dating and monitoring cell proliferation. Currently BrdU labeling is the most used technique for studying adult neurogenesis in mammals, including human [11]. It has also been used in cancer patients for diagnostic purposes [12]. The exact mechanisms by which BrdU promotes transcription factor- and chemical-induced reprogramming remain unclear. A previous report indicated that BrdU could increase the multipotency of human mesenchymal stem cells [13]. However, our data demonstrate that BrdU is detrimental for ESC self-renewal. Previous studies have demonstrated that BrdU incorporation could affect DNA structure [11], and may thus induce epigenetic changes necessary for the reprogramming. However, such DNA modification may also lead to DNA instability, increasing the risk of sister chromatid exchanges, mutations and double-strand breaks [11] and cause toxicity to the cells. We therefore tested BrdU with the *thymidine kinase* (*TK*) gene mutation assay, a classical assay used to evaluate cytotoxicity and genotoxicity of developing drugs [14, 15]. In this assay, we used the human lymphoblastoid cell line TK6, which is heterozygous at the *TK* locus ($TK^{+/-}$) and thus the wild-type allele serves as a target for mutation. As demonstrated in Supplementary information, Table S1, BrdU at 25 μM, a concentration higher than that was used for reprogramming, did not affect the mutation frequency, although slightly reduced the relative suspension growth. At higher concentrations (50-200 μM), BrdU indeed significantly increased the mutation frequency (Supplementary information, Table S1). These results indicate that BrdU at reprogramming-effective concentrations will not cause genotoxicity to cells, further confirming the karyotyping result (Supplementary information, Figure S3D).

In summary, we demonstrate that BrdU can replace Oct4, the most critical factor in iPSC generation, and promotes full-chemical induction of mouse iPSCs with the minimal combination being BrdU, CHIR99021, Repsox and Forskolin. Since BrdU has already been used in patients [12], this combination may lay a foundation for full-chemical induction of human iPSCs and may eventually provide a safer strategy to generate clinically applicable iPSCs.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)