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Research Articles

A Highly Optimized Protocol for Reprogramming Cancer Cells to Pluripotency Using Nonviral Plasmid Vectors

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Abstract

In spite of considerable interest in the field, reprogramming induced pluripotent stem cells (iPSCs) directly from cancer cells has encountered considerable challenges, including the extremely low reprogramming efficiency and nstability of cancer-derived iPSCs (C-iPSCs). In this study, we aimed to identify the main obstacles that limit cancer cell reprogramming. Through a detailed multidimensional kinetic optimization, a highly optimized protocol is established for reprogramming C-iPSCs using nonviral plasmid vectors. We demonstrated how the initial cancer cell density seeded could be the most critical factor ultimately affecting C-iPSCs reprogramming. We have consistently achieved an unprecedented high C-iPSC reprogramming efficiency, establishing stable colonies with typical iPSC morphology, up to 50% of which express the iPSC phenotypic (Oct3/4, Sox2, Nanog) and enzymatic (alkaline phosphatase) markers. Furthermore, established C-iPSC lines were shown to be capable of forming teratomas *in vivo*, containing cell types and tissues from each of the embryonic germ layers, fully consistent with their acquisition of pluripotency. This protocol was tested and confirmed in two completely unrelated human lung adenocarcinoma (A549) and mouse melanoma (B16f10) cancer cell lines and thus offers a potentially valuable method for generating effectively virus-free C-iPSCs for future applications.

CLASSIFICATION OF STEM CELL BASED ON POTENCY

CELL TYPE	DESCRIPTION	EXAMPLES
Totipotent	Each cell can develop in to new individual	Cells from early (1-3) days embryo
Pluripotent	Cells can form (over) cell types	Some cells of blastocyst (5 -14 days)
Multipotent	Cells differentiated but can form a number of other types	Fetal tissue cord blood and adult stem cells
Oligo potent	Ability to differentiate in to few cells	Adult lymphoid or myeloid cell
Unipotent	Ability to produce cells there own type , self renewal	Adult muscle stem cells

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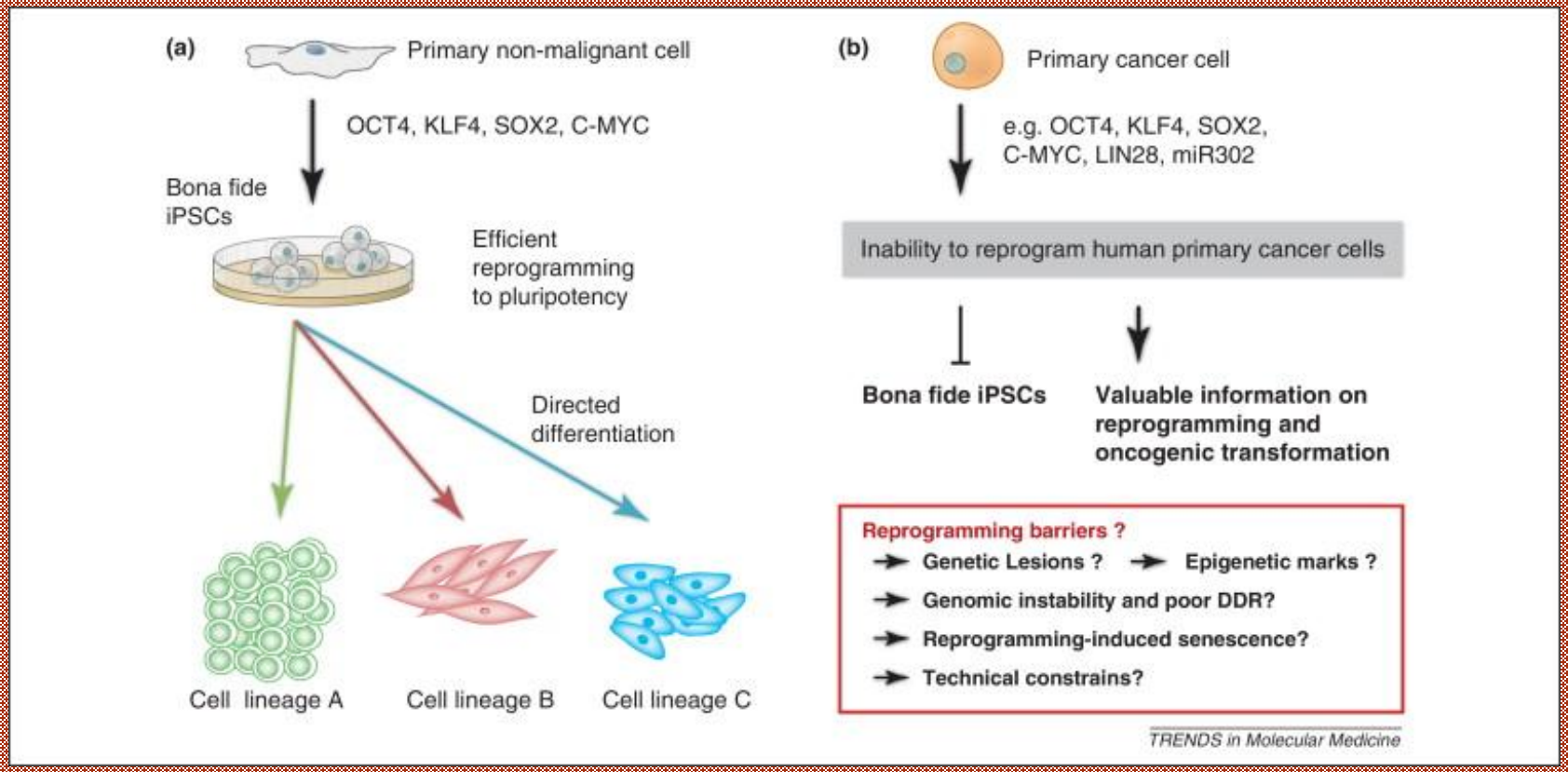
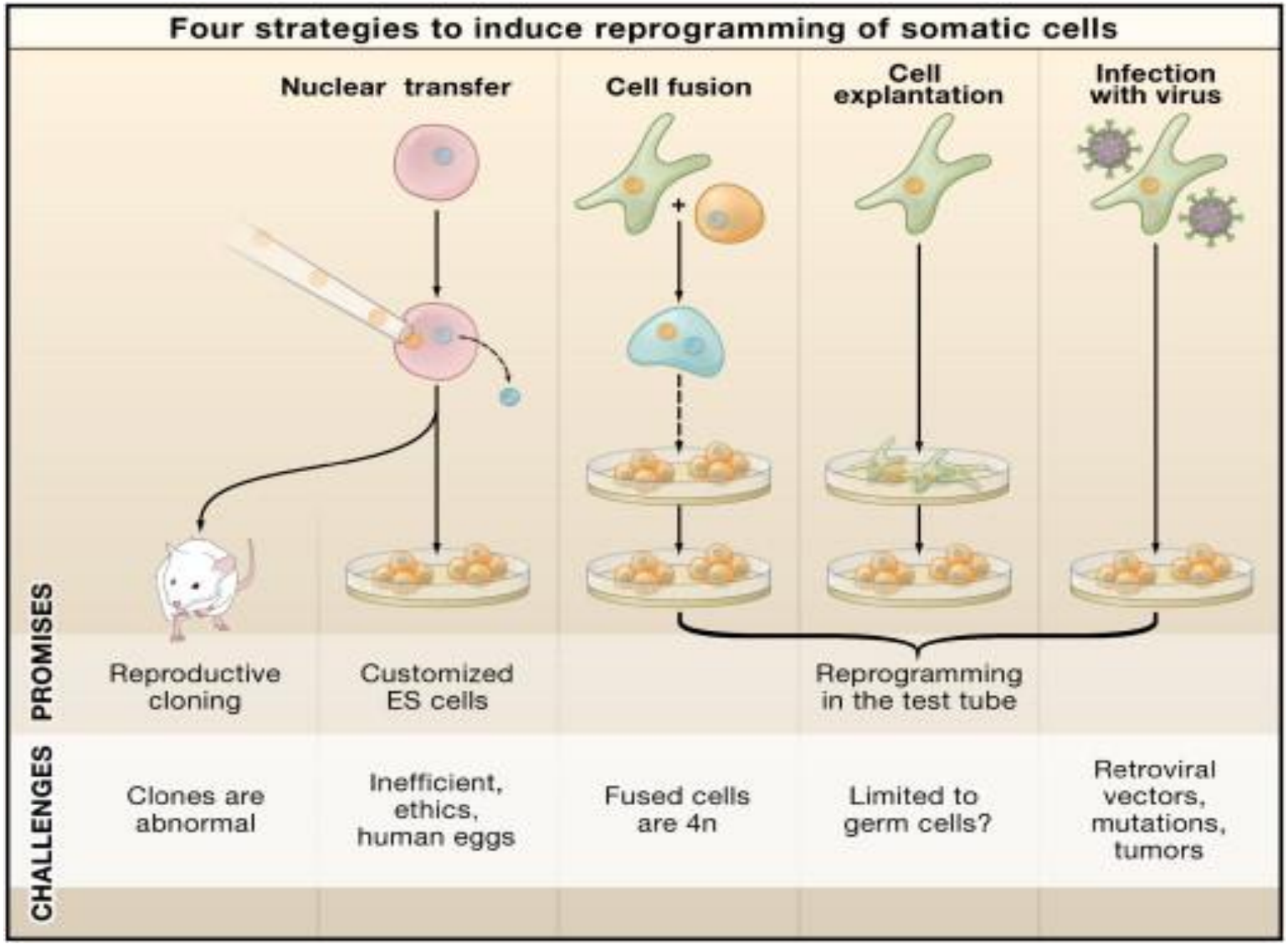
REPROGRAMING?





(1) Nuclear transfer(NT) injection of a somatic nucleus into an enucleated oocyte, can give rise to genetically matched embryonic stem (ES) cells ("somatic cell nuclear transfer," SCNT).

(2) a somatic cell is fused with an embryonic stem cell (ESC).

(3) Explantation of somatic cells in culture selects for immortal cell lines that may be pluripotent or multipotent.

(4) Transduction of somatic cells with defined factors can initiate reprogramming to a pluripotent state.

Materials and Methods

Cell lines and maintenance:

The A549 human alveolar adenocarcinoma cell line: RPMI-1640 containing 10% FBS
The B16f10 cell line : DMEM containing 10 % FBS.

The reprogrammed A549-iPSCs and B16f10-iPSCs both of which contained 15% KnockOut Serum Replacement .

Plasmid vectors and transfection reagents

three plasmid vectors:

These included pCXOKS-2A (encoding Oct-3/4, Sox2, and Klf4) and pCXcMyc (encoding c-Myc) from Addgene for iPSC induction

pIRES2-EGFP from Clontech for the assessment of cell transfection efficiency.

tion reagent (P:TR) ratio], and detailed time kinetics.

We tested and compared these parameters by reprogramming two very different cancer cell types, the human lung adenocarcinoma (A549) and mouse melanoma (B16f10) cell lines, respectively. We show here that although correct timing and an optimized transfection reagent concentration/ratio are very important kinetic factors to be considered, the initial cancer cell density is the most critical determining factor, which is also very much cancer cell type–dependent.

Initial assessment for cancer cell transfection efficiency using the plasmid vectors

A protocol for cell transfection using the virus-free plasmid vectors previously described by Okita et al. (2010) was adopted, but modified for cancer cell transfection in the present study. To optimize for transfection efficiency, an initial assessment was carried out using the pIRES2-EGFP plasmid encoding a fluorescent protein for tracking. Briefly,

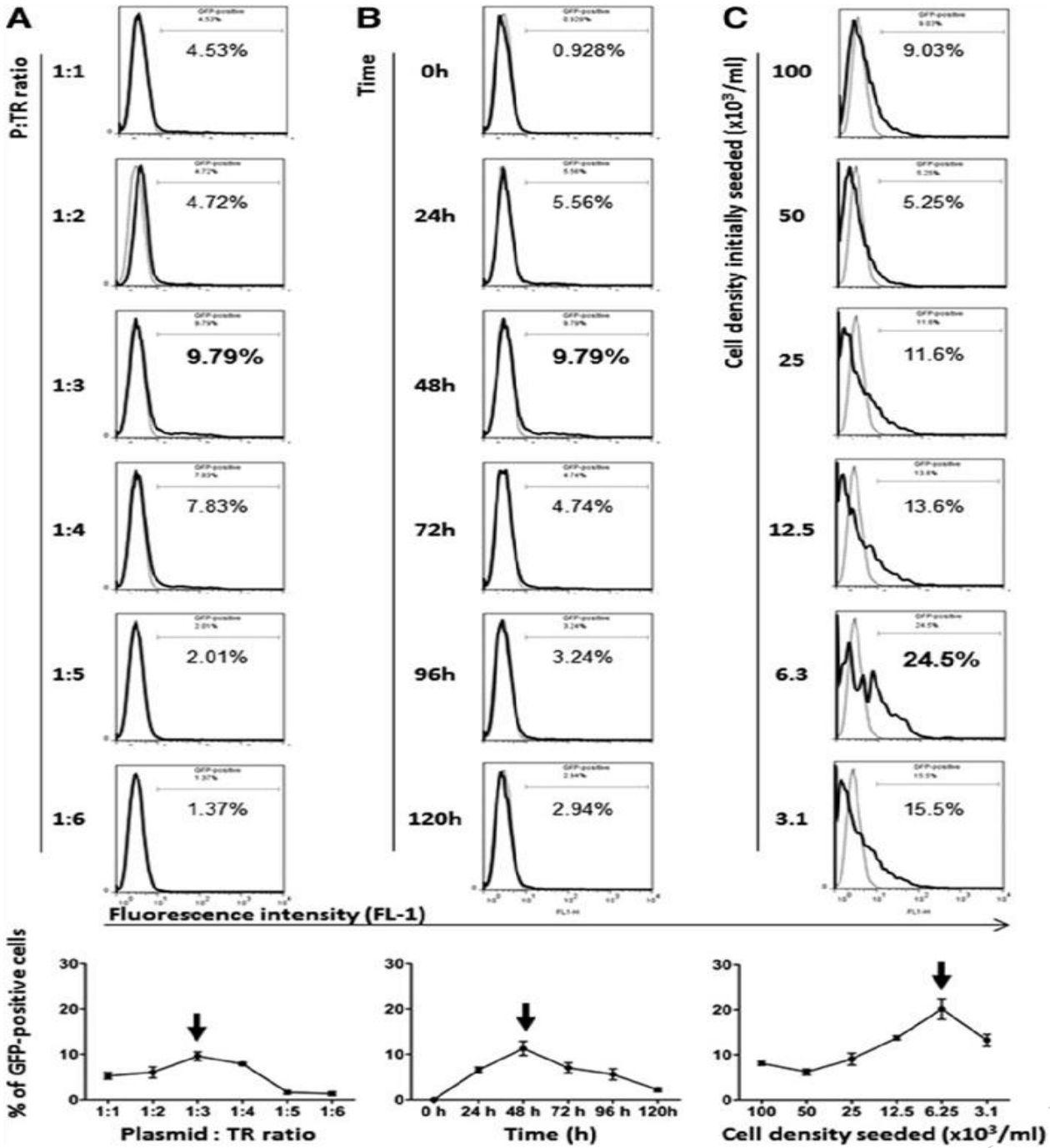
cancer cells were prepared in six-well plates containing 2 mL per well of fresh medium A549 or B16f10 (RPMI-1640 or DMEM containing 10% FCS) respectively. To prepare for the DNA/X-tremeGENE complexes, for each well, 50 μL of Opti-MEM were transferred into a 1.5-mL test tube. The pIRES2-EGFP plasmid was then added (0.5 μg/0.5 μL), together with the X-tremeGENE Transfection Reagent (TR), at different P:TR ratios (1:1 to 1:6 in volume). After gentle mixing and incubation for 20 min at room temperature, the DNA/X-tremeGENE complex preparation was added to the cancer cell cultures at a 1:1 ratio and incubated overnight at 37°C, 5% CO_2.

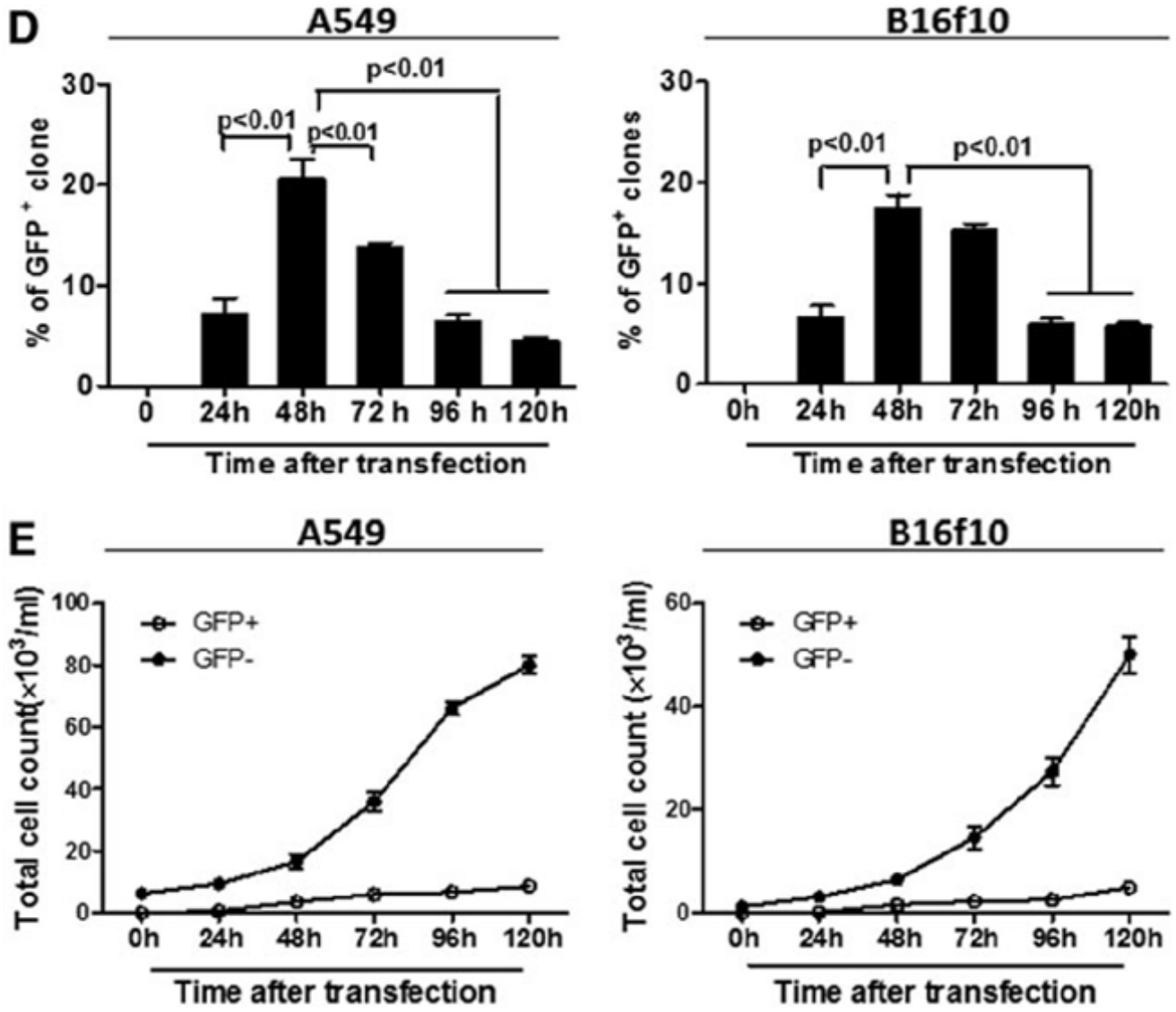
Following the transfection procedures, at different time points, samples of the transfected cells were collected, washed with fluorescence-activated cell sorting (FACS) buffer and analyzed by flow cytometry to detect and to quantify for the frequency of green fluorescent protein–positive (GFP^+)

with more defined morphology could be observed in the cultures. The cells were subsequently analyzed and their expression of iPSC enzymatic and phenotypic markers confirmed, as described below.

AP staining for initial iPSCs identification

The induction of iPSC-like cells was initially identified by their expression of AP using the StemTAG™ Alkaline Phosphatase Staining Kit (Cell Biolabs, Inc). According to the protocol, the cells were washed with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), and fixed at room temperature for 2 min in the fixative solution. After two washes in PBS-T, freshly prepared substrate StemTAG™ Alkaline Phosphate Staining Solution was added and incubated at room temperature for 30 min in the dark. The stained cells were then washed twice with PBS and analyzed by light microscopy. The numbers of AP^+ and AP^- colo-


A
P:TR ratio
1:1
4.53%
1:2
4.72%
1:3
9.79%
1:4
7.83%
1:5
2.01%
1:6
1.37%
B
Time
0h
0.928%
24h
5.56%
48h
9.79%
72h
4.74%
96h
3.24%
120h
2.94%
C
Cell density initially seeded (x10³/ml)
100
9.03%
50
5.25%
25
11.6%
12.5
13.6%
6.3
24.5%
3.1
15.5%
Fluorescence intensity (FL-1)
% of GFP-positive cells
30
20
10
0
1:1 1:2 1:3 1:4 1:5 1:6
Plasmid : TR ratio
0 h 24 h 48 h 72 h 96 h 120h
Time (h)
100 50 25 12.5 6.25 3.1
Cell density seeded (x10³/ml)


D
A549
B16f10
% of GFP+ clone
% of GFP+ clones
p<0.01
p<0.01
p<0.01
p<0.01
p<0.01
0
10
20
30
0
24h
48h
72 h
96 h
120h
0h
72h
Time after transfection
E
A549
B16f10
Total cell count(×10³/ml)
Total cell count (×10³/ml)
GFP+
GFP-
0
20
40
60
80
100
0h
24h
48h
72h
96h
120h
Time after transfection

AP staining for initial iPSCs identification

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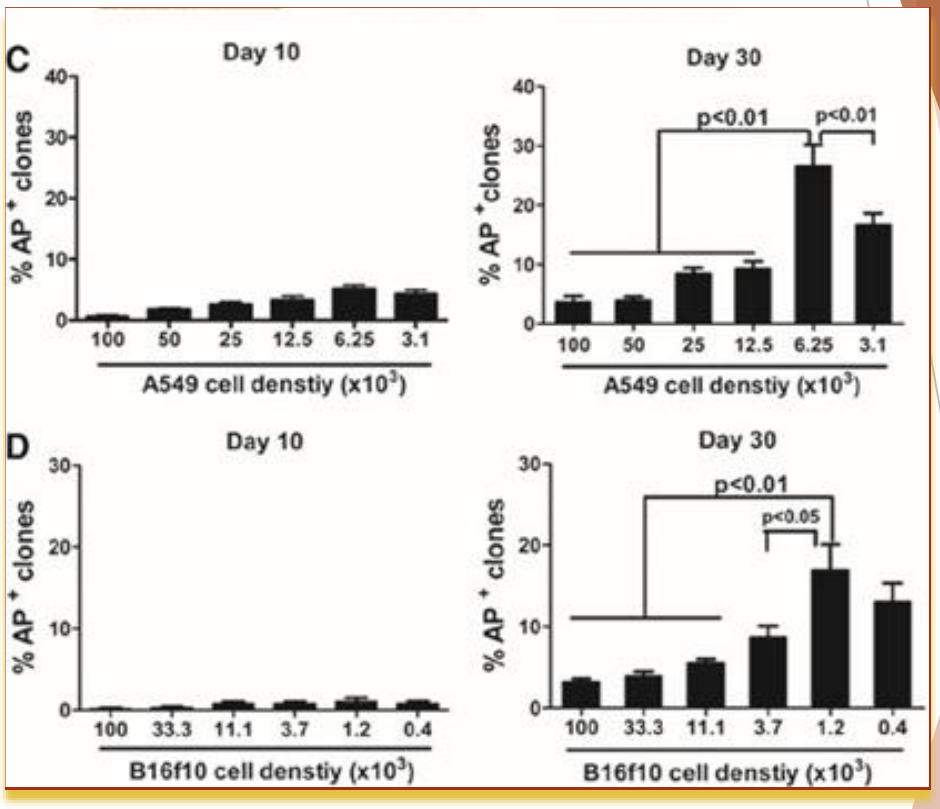
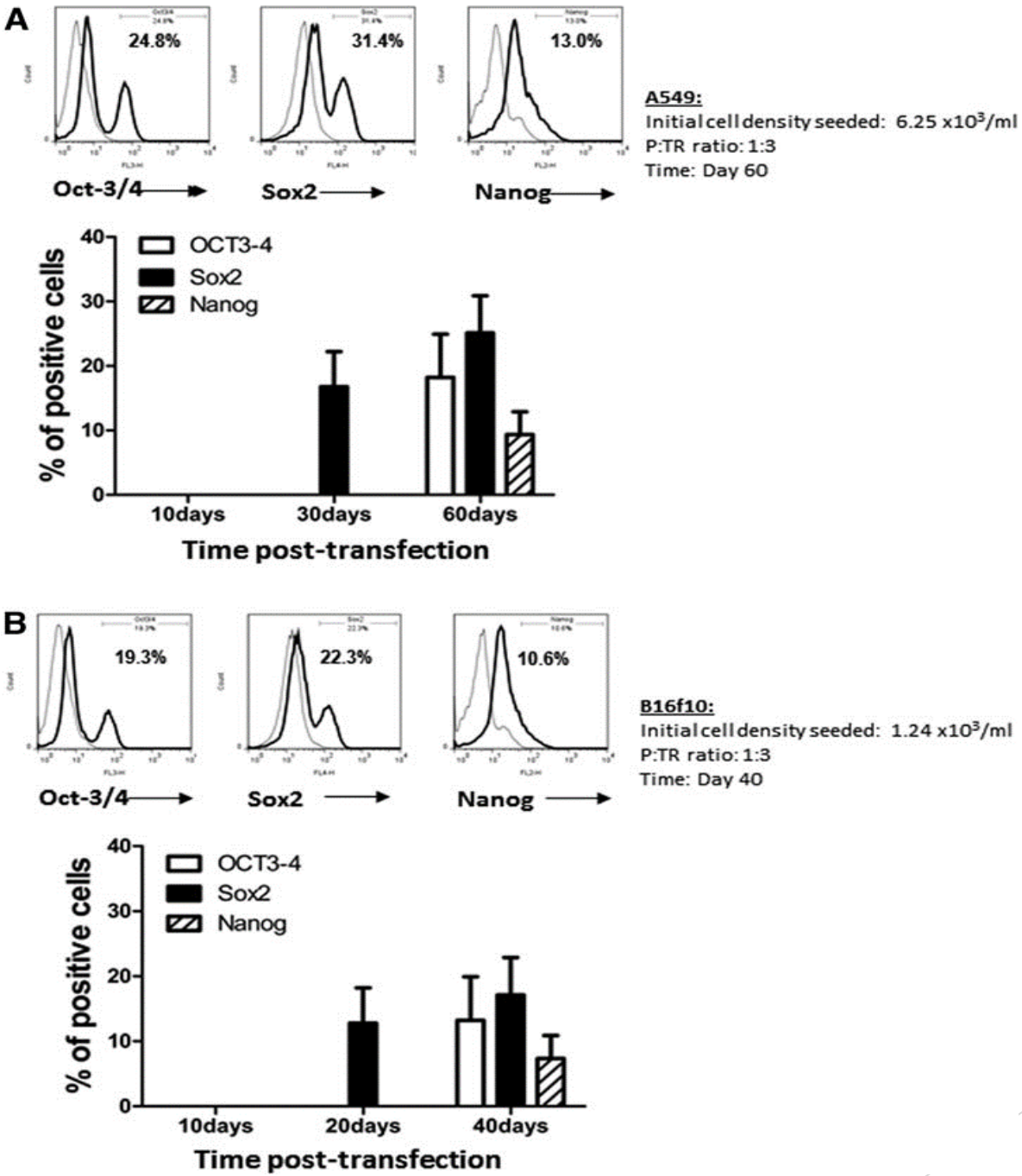


nies in 10 randomly selected views of each well were counted twice under an inverted microscope (X-400; Olympus, Japan) and shown as mean values and percentages of the AP^+ colonies over the total numbers of colonies.

Flow cytometry analysis

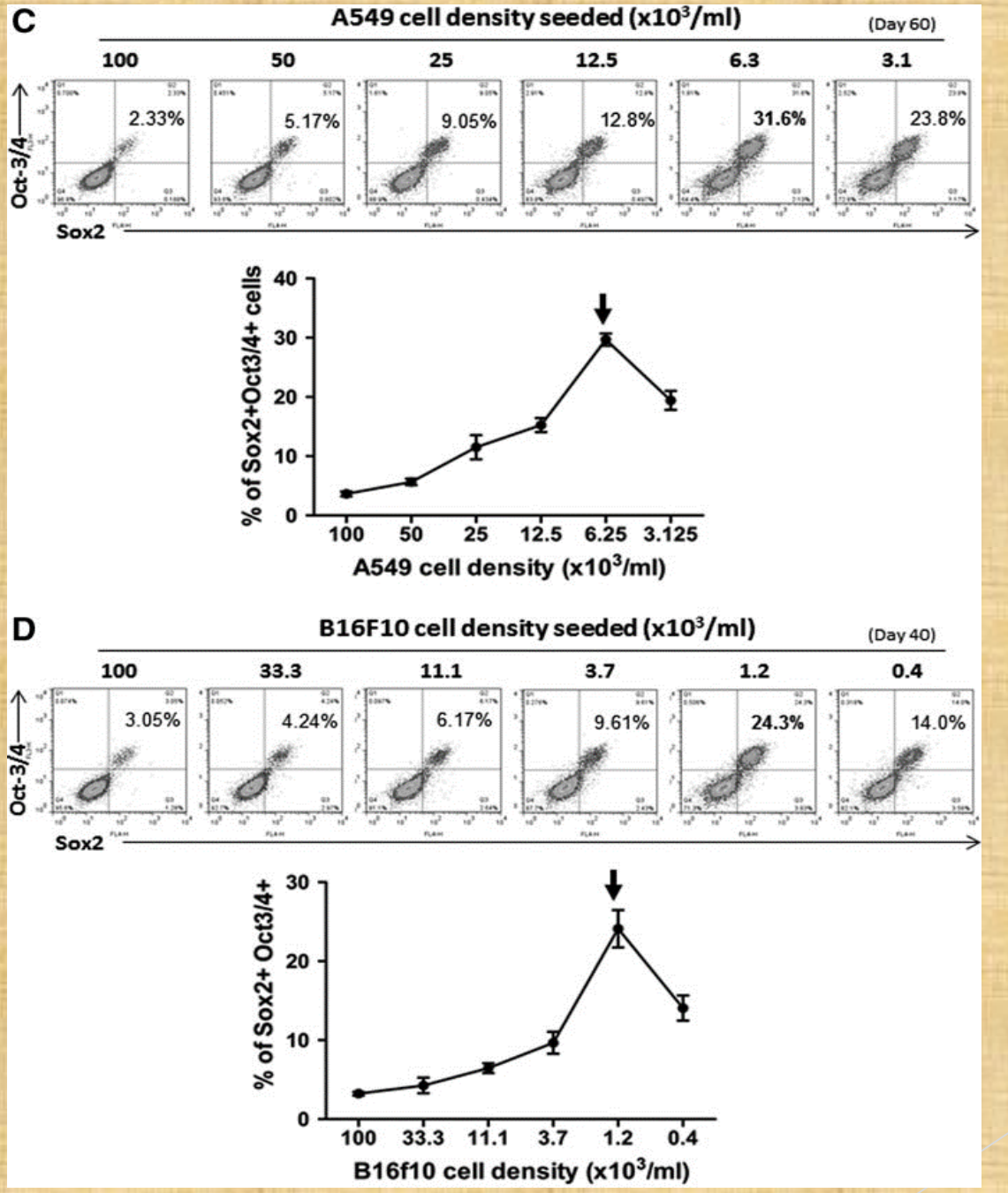
Flow cytometry analysis was carried out throughout for quantification and phenotypic analysis of the GFP-transfected cells and the induced C-iPSCs. For phenotypic analysis, the cells were stained with the BD Stemnow™ human and mouse Pluripotent Stem Cell Transcription Factor Analysis Kits according to manufacturer's recommendations (BD Biosciences, cat. no. 560589, 560585). Briefly, single-cell suspensions were prepared and fixed with the BD Cytofix Fixation Buffer [4% paraformaldehyde (PFA)] and incu-

To confirm these results further phenotypically, the iPSC-like cells were harvested and stained for their expression of iPSC markers. Flow cytometry analysis revealed that these cancer-derived iPSC-like cells expressed several typical pluripotency markers, including Sox2, Oct-3/4, and Nanog (Fig. 4). The expression of these transcription factors, in terms of frequency and level, also increased in a time-dependent manner. Sox2, being an early marker of pluripotency, was clearly detectable at days 20–30, followed by the appearance of Oct-3/4 and Nanog at a relatively later stage (Fig. 4A, B). By day 60 and day 40 for the A549 and B16f10 lines, respectively, many of these cells, as high as 31.6% and 24.3% from those cultures with highly optimized reprogramming conditions (Fig. 4C, D, arrows), were found to be



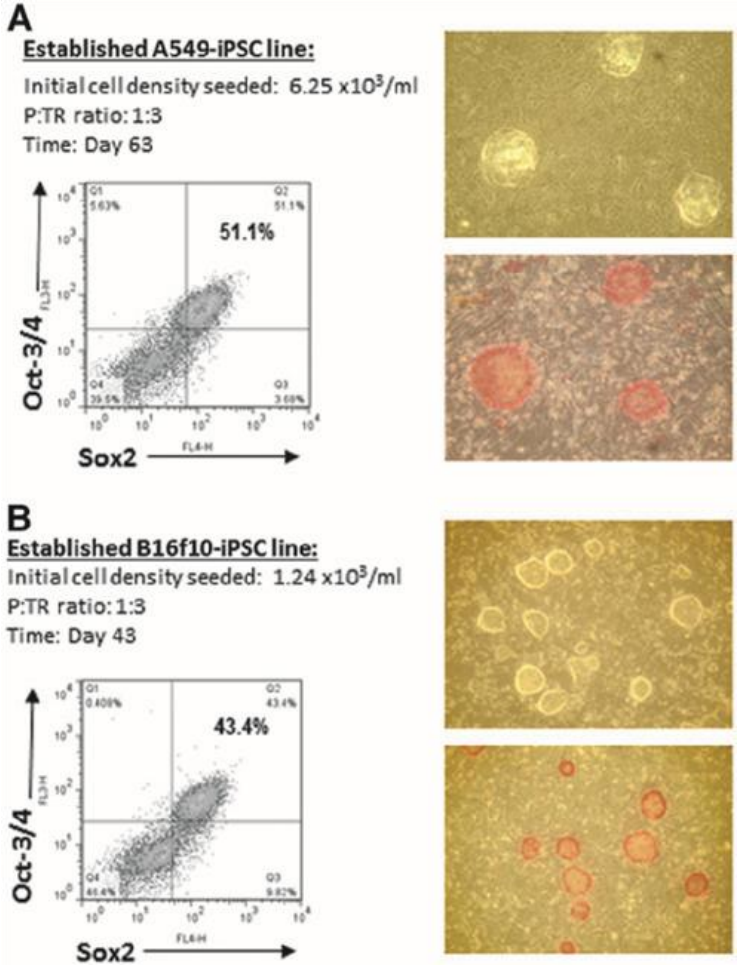
A549:
Initial cell density seeded: 6.25 x10^3/ml
P:TR ratio: 1:3
Time: Day 60

B16f10:
Initial cell density seeded: 1.24 x10^3/ml
P:TR ratio: 1:3
Time: Day 40


C
A549 cell density seeded (x10³/ml)
(Day 60)
100
50
25
12.5
6.3
3.1
2.33%
5.17%
9.05%
12.8%
31.6%
23.8%
Oct-3/4
Sox2
% of Sox2+Oct3/4+ cells
40
30
20
10
0
100
50
25
12.5
6.25
3.125
A549 cell density (x10³/ml)
D
B16F10 cell density seeded (x10³/ml)
(Day 40)
100
33.3
11.1
3.7
1.2
0.4
3.05%
4.24%
6.17%
9.61%
24.3%
14.0%
Oct-3/4
Sox2
% of Sox2+ Oct3/4+
30
20
10
0
100
33.3
11.1
3.7
1.2
0.4
B16f10 cell density (x10³/ml)

The present study aimed to develop a highly optimized method for reprogramming iPSCs directly from cancer cells using the nonviral plasmid vector approach. To maximize reprogramming efficiency, the underlying basis of the variable susceptibility of different cancer cells to reprogramming needs to be better understood. Due to the transformed nature of cancer cells, conferring on them rapid and un- cancer cells seeded at high density can quickly become overconfluent. The growth of those newly induced iPSC colonies at their initial low frequency may be hindered by the majority of those nontransformed , particularly after the prolonged culture time required for iPSC reprogramming. On the other hand, if cells are seeded at too low a density, they may senesce, being less amenable to reprogramming. Therefore, it is important to understand the problems involved to remove the barriers preventing efficient C-iPSC induction.

Using a protocol previously developed by Kaji et al. (2009) for iPSCs induction from normal somatic adult cells using the nonviral approach, we aimed to achieve its optimization for efficient reprogramming of C-iPSCs in the present study. A detailed kinetic analysis was carried out, taking into account a combination of parameters considered to be most critical for C-iPSCs induction. The efficiency for cancer cell transfection and iPSC induction was found to be critically dependent on the initial cell density seeded, ratio of plasmid DNA (Oct-3/4, Sox2, Klf4, and c-Myc), as well as transfection reagent concentrations [plasmid to transfection reagent (P:TR) ratio], and detailed time kinetics.



DISCUSSION:

the initial cell density seeded in culture is one of the most critical parameters to consider to achieve a high reprogramming efficacy for C-iPSCs induction and establishment

as high as 50% of the cells or more with typical iPSCs morphology and phenotypic markers could be consistently achieved.