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EXPRESSION OF SSEA-1 IN DIFFERENT CLONES OF REPROGRAMMED MURINE EMBRYONIC FIBROBLASTS

Murine embryonic fibroblasts were reprogrammed with Sleeping Beauty transposon system towards proliferating cells that formed embryonic stem cell-like colonies on feeder-coated plates. The emerged colonies were picked-up and expanded. Passaged further clones demonstrated typical for embryonic stem cell morphology. Fluorescent-labeled antibody staining for one of the most reliable extracellular pluripotency markers – stage-specific embryonic antigen 1 (SSEA-1) was performed. The percentage of SSEA-1 positive cells in obtained clones was estimated via flow cytometry. The SSEA-1 expression level varied among the clones but appeared to be comparable to it's rate in established murine embryonic stem cell and induced pluripotent stem cell lines.

Keywords: murine embryonic fibroblasts, reprogramming, pluripotency, stage-specific antigen-1.

Introduction

The era of induced pluripotency started in 2006 with pioneer work of Jamanaka *et al*. First iPS colonies in murine and human systems were obtained due to the stable integration of only 4 pluripotency genes – Oct4, Sox2, Klf4 and c-Myc (OSKM) into the genome of somatic cells. Obtained colonies were called induced pluripotent stem cells (iPSCs), as they performed the characteristics of pluripotent embryonic stem (ES) cells – expression of pluripotency markers on various levels, *in vivo* and *in vitro* differentiational potential *etc*. [1]. This finding gave rise to the development of various reprogramming strategies to improve the process of reprogramming in terms of efficiency and quality. Various constructs exploited as gene-delivery systems to perform stable integration – lentiviruses [2] or transient expression of pluripotency markers – adenoviruses [3] or episomes [4] *etc*.

To make the efficiency of reprogramming higher (especially for "hard to transfect cells"), transposon-based methods were developed and tuned to be promising. Among the emerged ones, most famous are Piggy-Bac transposon- and Sleeping-beauty transposon-based gene delivery systems [5]. Transposon-based system usually consists with two plasmids. The first one is transposon itself. It harbors gene of interest (in our case the reprogramming cassette with OSKM genes) flanked by recognition sites for transposase. The second plasmid encodes transposase that catalyses the insertion of the gene of interest into the genome. First transposon-based successful reprogramming of somatic cells towards induced pluripotent cells was performed on PiggyBac system by Woltjen et al in 2009 [6].

Sleeping beauty (SB) transposones have several advantages as a gene-delivery system, among which are: activity of transposase, cargo size, no tendency for the transposition into coding sequences *etc* [7]. Moreover, the construct of SB transposon system harbors the means for further removal of the integrated cassette to generate integration-free iPS cell lines. Hence, this system was used to reprogram murine embryonic fibroblasts towards induced pluripotent stem cells.

There is a conventional panel of tests to check the newly reprogrammed clones for pluripotency. Among them are morphology, the expression of pluripotency markers on mRNA and protein level (both intra- and extracellular), methylation of promoters, *in vivo* and *in vitro* differentiational potential *etc*. One of the most reliable pluripotency markers is stage specific antigen antigen 1 (SSEA-1), expressed at morula stage, teratoma cells, undifferentiated murine ES cells and cease to express after induction of differentiation [8]. Due to its extracellular expression, the percentage of the positive for SSEA-1 cells can be estimated. Therefore, generated from reprogrammed murine embryonic fibroblasts clones were checked for SSEA-1 expression to choose the most promising ones for further characterization.

Materials and methods Cell culture

Murine embryonic fibroblasts (MEFs) were cultured in DMEM Glutamax high glucose, supplemented with 10 % fetal bovine serum (FBS), 1x non-essential amino-acids (NEAA), 50mM β-mercaptoethanol and 2 % penicillin/streptomycin. During reprogramming, the medium was changed for ES medium (DMEM Glutamax high glucose,

15 % FBS, 1x NEAA, 50mM β-mercaptoethanol, Leukemia inhibitory Factor (LIF), 1 U/μl) supplemented with ascorbic and valproic acid to accelerate the reprogramming process. Cells were kept at absolute humidity in incubator with 5 % of CO_2 in the atmosphere. Passaging was performed with Trypsin-EDTA.

Reprogramming of murine embryonic fibroblasts

Plasmids of Sleeping Beauty transposon-besed gene-delivery system were kindly provided by Zs. Izsvak and Z. Ivics, Max Max-Delbrück-Centrum für Molekulare Medizin (MDC), Berlin. Those plasmids were 1) transposase encoding pCMV(CAT) T7-SB100x, 2) reprogramming cassette harboring pT2/RMCE-OSKM-puDTk(PB ITR deleted) or 3) pT2/RMCE-OSKML-puDTk(PB ITR deleted) and 4) pT2venus – venus fluorescent protein flanked by sites to transposase. Freshly generated MEFs (passage 1) from 13,5 day embryos of c57Bl/6J mice were transfected with the pare of plasmids – transposase and reprogramming cassette or pT2venus as control. The transfection was performed on Neon Invitrogen transfection system following the Invitrogen protocol for MEF transfection. On Day 2 transfected MEFs were plated on feeder cells (inactivated murine embryonic fibroblasts) and the medium was changed for murine ES medium. The cells were observed daily for the emergence of colony-like structures under the benchtop stereomicroscope (Leica Stereomicroscope).

Pick-up of the colonies

Emerged on the 8 day after transfection colonies were cultured to grow till the condition to be ready for pick-up. In 3 more days 3D round colonies were picked-up using stereomicroscope (Leica Stereomicroscope) and passaged into 24-well feeder-coated plates for further expansion.

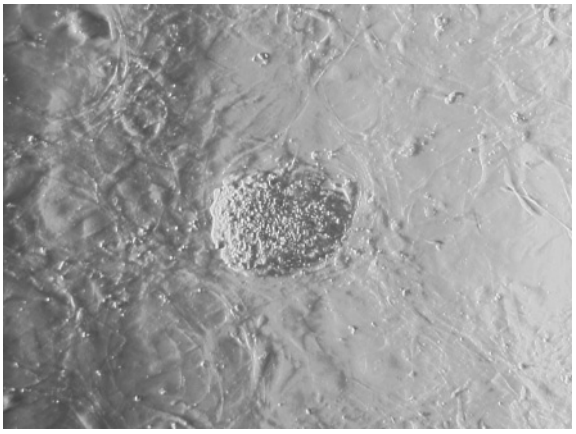
SSEA-1 staining

Antibody staining was performed on trypsinised cells of the clones with SSEA-1 antibody for specific binding and mouse IgM non-conjugated as isotype control (Santa Cruz Biotechnology) and labeled with FITC- or Alexa-fluor-555-conjugated anti-IgM (Santa Cruz Biotechnology). The percentage of SSEA-1 – positive cells was measured via Flow Cytometry on fluorescence intensity FACS SCAN (BD Biosciences), and the data analysis was carried out with CellQuest software (BD).

Results

The first signs of reprogramming – colony like structures, potential iPS colonies – started occurring on the Day 8 after transfection. No colonies appeared in MEFs, tranfected with transposase and venus-coding plasmid. Emerged colony-like structured are shown on Figure 1 (*1*). The colonies were allowed to grow for 3–4 days more to reach the appropriate for picking-up size like on Figure 1 (*2*) and then picked-up and passaged each one separately.

1



2

Fig. 1. Colony-like structures on feeder-coated plates during the reprogramming of murine embryonic fibroblasts, x5: *1* – on day 8, x2,5 and *2* – day 11

Twenty tree clones that demonstrated nice ES-like morphology and best proliferation rate (numbered clone 1–23) were regularly passaged up to passage 10 for complete reprogramming and expanded for pluripotency marker analysis. Alkaline-phosphatase staining was performed. All the clones were positive. The intensity of staining was comparable to established ES and iPS cell lines (data not shown).

On the passage 10 reprogrammed murine embryonic fibroblasts performed nice ES-like morphology like on Figure 2 (*1–4*), compared to established murine embryonic stem cell line aPIG44 and induced pluripotent stem cell line AT25 on Figure 2 (*5*) and (*6*).

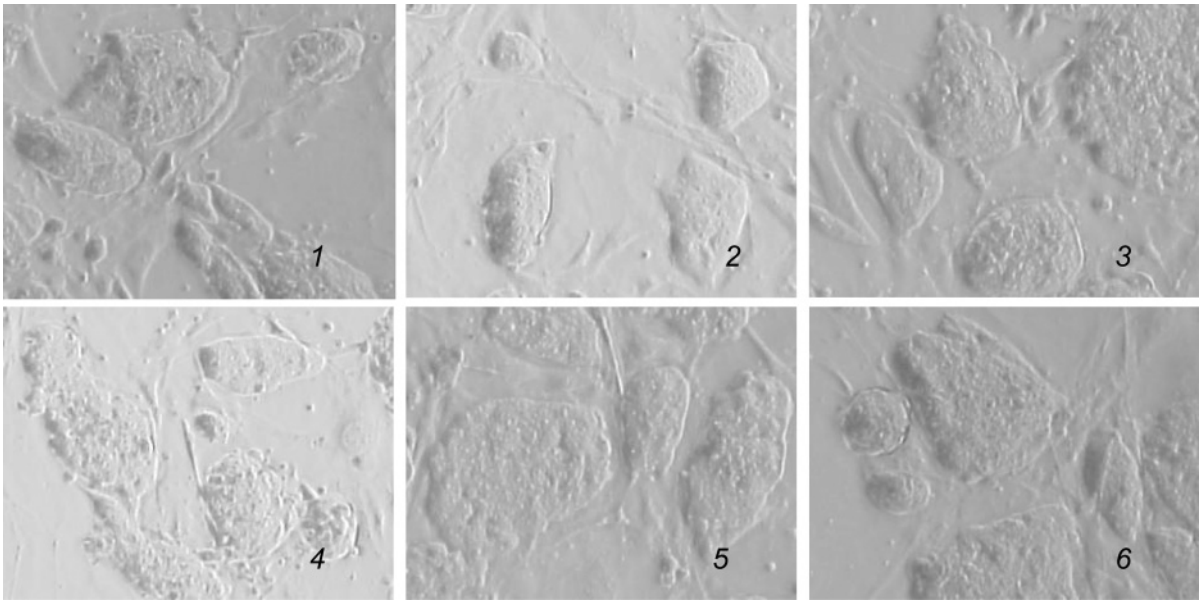


Fig. 2. ES-like morphology of some obtained clones, x10: *1* – clone 6, *2* – clone 7, *3* – clone 9, *4* – clone 22 and established ES and iPS cell lines: *5* – aPIG44 and *6* – AT25

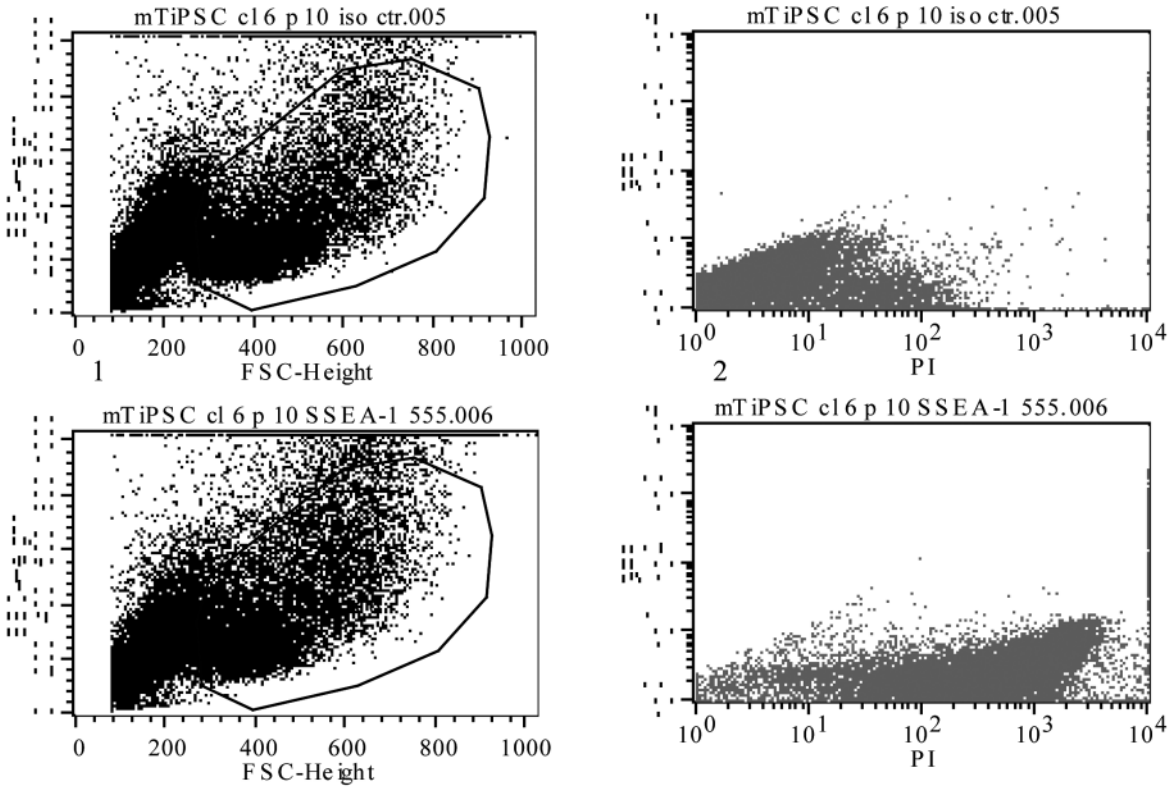
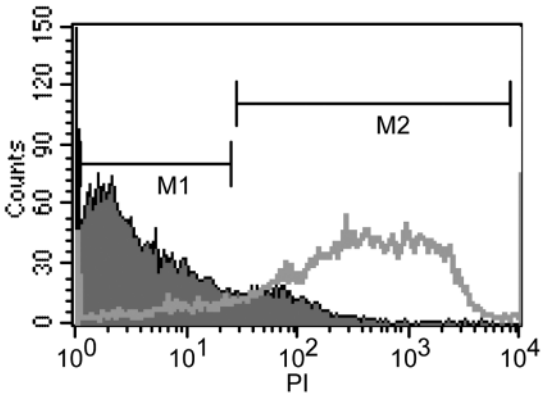


Fig. 3. FACS data for SSEA-1 staining of reprogrammed murine embryonic fibroblasts on passage 10: *1* – gated populations of live cells in isotype control and SSEA-1 stained samples; *2* – detection of the signal from SSEA-1 positive cells in isotype control and SSEA-1 stained sample

After passage 10 various clones of reprogrammed murine fibroblasts were checked for the expression levels of extracellular pluripotency marker SSEA-1. The example of FACS measurements and analysis is shown on the clone 6 SSEA-1 staining data. The population of the live cells was gated appropriately – Fig. 3 (*1*), the same gates were used for all clones and cell lines. Only very few cells look posi-



Histogram Statistics

File: mTiPSC cl 6 p 10 SSEA-1 555.006
Sample ID: mTiPSC cl 6 p 10 SSEA-1 555
Tube: Untitled
Acquisition Date: 25-Feb-70
Gated Events: 15740
X Parameter: PI (Log)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: G1
Total Events: 22099

Marker	Left, Right	Events	% Gated	% Total	Mean	CV	Median	Peak Ch
All	1, 9910	15740	100.00	71.22	813.55	171.55	349.12	9910
M1	1, 25	1533	9.74	6.94	10.68	67.32	9.06	6
M2	29, 8354	13826	87.84	62.56	769.63	120.28	417.92	250

Fig. 4. Example of FACS data analysis of SSEA-1 positive cells

tive due to the unspecific binding of isotype control, a large amount of positive cells was detected in SSEA-1 stained samples Fig. 3 (*2*).

The analysis of the obtained FACS data was performed on CellQuest software (BD). The example is shown on Fig. 4.

SSEA-1 is a surface marker that can be expressed in a very wide range even in established ES/iPS cell lines, rarely can be detected in 100 % of the cells in the cell line, which is confirmed by the results in Table 1. Coming to numbers, SSEA-1 staining analysis showed wide range of expression levels of this marker. Even in the established embryonic stem cell line aPIG44 and induced pluripotant stem cell line AT25 the percentage of SSEA-1 positive cells was 93 % and 91 %, respectively. The expression of SSEA-1 in different clones of reprogrammed murine embryonic fibroblasts ranged from almost 60 % in clone 20 and up to 94 % of SSEA-1 positive cells in the clones 9 and 17. No positive signal of SSEA-1 expression was detected in murine embryonic fibroblasts – the cell line of origin. So, the expression of pluripotency marker SSEA-1 emerged due to the certain intracellular events after the transfection with reprogramming cassette. Based on the FACS data clones 5, 7, 11, 17 and 22 appeared to be more promising in terms of reprogramming towards pluripotency.

Table 1. **Expression of SSEA-1 in the clones of reprogrammed murine embryonic fibroblasts and establishes ES and iPS cell lines**

Cell line / clone	% of SSEA-1 positive cells	Cell line / clone	% of SSEA-1 positive cells
Clone 5	91,36	Clone 13	82,09
Clone 6*	87,84	Clone 17	94,00
Clone 7	71,56	Clone 20*	59,57
Clone 8*	63,41	Clone 22	90,15
Clone 9	94,32	AT25*	91,54
Clone 10*	75,57	aPIG44*	93,07
Clone 11	90,20	MEF	0,66
Clone 12*	66,38		

* labeled with Alexa fluor-555 conjugates secondary antibody

Discussion

Sleeping Beauty transposon system appeared to be helpful tool for murine fibroblast reprogramming. It took about 8 days after transfection for the first colonies to appear. The whole procedure of reprogramming appeared to be quite effective and easy, as no special manipulations or supplements

were applied. Colony like structures that emerged proliferated and demonstrated nice ES-like morphology.

The measurement of the expression of pluripotency marker SSEA-1 is good start point to quantify the pluripotency of freshly obtained clones. SSEA-1 marker was not expressed in the cell line of origin, but was highly expressed (although in various range) freshly generated clones. This can be the evidence of dramatic change in the cell fate, such as reprogramming towards pluripotency.

The obtained clones should further be checked for other crucial pluripotency characteristics, such as the expression of various markers and differentiation potential. Based on the obtained data proliferating clones can be considered potentially reprogrammed towards iPS murine embryonic fibroblasts and several best clones can be chosen for further studies.

Conclusions

Transfection of murine embryonic fibroblasts with Sleeping Beauty transposon system caused reprogramming of somatic cells to proliferating colonies. The obtained clones demonstrated typical for murine embryonic and induced pluripotent stem cells morphology.

SSEA-1 pluripotency marker was detected in reprogrammed cells and was not expressed in the cells of origin – murine embryonic fibroblasts.

The expression of extracellular pluripotency marker SSEA-1 in obtained clones was comparable to its rate in established murine embryonic and induced pluripotent stem cell lines.

According to the results of SSEA-1 staining, clones 5, 9, 11, 17 and are most promising and can be taken for further analysis for pluripotency.

Acknowledgements

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ЕКСПРЕСІЯ SSEA-1 У РІЗНИХ КЛОНАХ РЕПРОГРАМОВАНИХ МИШАЧИХ ФІБРОБЛАСТІВ

Ембріональні фібробласти миші було репрограмовано за допомого системи транспозонів Sleeping Beauty у проліферуючі клітини, що формували на фідерах колонієподібні структури, подібні до колоній ембріональних стовбурових клітин. Отримані окремі колонії відібрано для подальшої експансії. При пасажуванні клони демонстрували типові для ембріональних та індукованих плюрипотентних стовбурових клітин морфологію та проліферацію. Проведено забарвлення клонів за допомогою флуоресценто мічених антитіл для виявлення одного із найбільш надійних маркерів плюрипотентності – стадій-специфічного антигену 1 (SSEA-1). За допомогою проточної цитофлуориметрії було кількісно оцінено рівень експресії цього маркера. Рівень експресії варіював в отриманих клонах, проте не поступався такому ж у вивчених індукованих плюрипотентних та ембріональних стовбурових клітинних лініях.

Ключові слова: ембріональні фібробласти миші, репрограмування, плюрипотентність, стадієспецифічний ембріональний антиген-1.

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