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Induced Pluripotent Stem-Like Cells Derived from Ban, a Vietnamese Native Pig Breed

Nguyen Viet Linh[1,2], Nguyen Thi Nhung[1,2]

[1] Institute of Biotechnology, Vietnam Academy of Science and Technolgy, Hanoi, Vietnam
[2] Graduate University of Science and Technology, Vietnam Academy of Science and Technology, Hanoi, Vietnam
Email: nvlinh@ibt.ac.vn

***Abstract*—** *Induced pluripotent stem cells (iPSc) is a promising technology for applying in bio-medicine and biodiversity conservation. In the present study, we isolate and culture fibroblasts from Ban – a Vietnamese native pig breed and transfer episomal plasmid containing genes Oct3/4, Sox2, Klf4, l-Myc, LIN28 and EBNA1 in order to reprogram cells. We isolated, cultured and cryopreserved successfully 9 primary fibroblast lines from Ban (culture percentage is 90.0%). Plasmids was successfully transferred into Ban fibroblasts with high efficiency. Changes in morphology of fibroblasts into pluripotent stem-like cells showed that they had been reprogrammed under the effect of transferred genes. The pluripotency signal was further proved by in vitro differentiation by formation of embryoid body in all 3 transfected cell lines. The results showed that pluripotent stem-like cells has successfully derived in Ban pigs.*

***Keywords*— Ban pig, fibroblast, gene transfer, iPS-like cells.**

I. INTRODUCTION

Induced pluripotent stem cells is in great concern of scientists for its meanings in research and application. The first success on production of induced pluripotent stem cells was by Takahashi and Yamanaka, 2012 [1], by changes in only 4 genes (Oct3/4, Sox2, Klf4 và c-Myc), fibroblasts (in the highest differentiation level) could be reprogrammed into pluripotent stage, similar to that of embryonic stem cells [1]. Thereafter, researches on iPS cells have been carried out all over the world with plenty of approaches. Nowadays, production of iPS cells is much more effective. Some reported that iPS cells might be achieved by less- or non-genetically modified factors [2].

In pigs, a subject with physiological similarity to human and promising research and application on tissue and organ xenotransplantation, embryonic stem (ES) and iPS cell line establishment is not as stable as that on other species, many unsolved problems such as identification of naïve and prime stages, or expression level of some genes relating to pluripotency such as Oct3/4, Nanog or SSEAs [3].

In the present study, we isolated, cultured and cryopreserved fibroblasts from Ban, a Vietnamese native pig breed, and transfer episomal plasmids with Oct3/4, Sox2, Klf4, l-Myc and EBNA1 into them to derive first induced pluripotent stem-like cells in Ban.

II. MATERIALS AND METHODS

Somatic cell culture

Pieces of ear tissue from Ban pigs were isolated and rinsed carefully by PBS (-) solution before being cut into smaller pieces. Such pieces were then culture in 4 well disks (Nuclon, Denmark) in DMEM (Sigma, USA) supplemented with penicillin/streptomycin and 10% Fetal Bovine Serum (Sigma, USA). After 3 to 10 days, fibroblasts grown from the pieces were passaged and subsequently cryopreserved to be the material for reprograming.

Plasmid transfer into somatic cells

Plasmids pCXLE-hOCT3/4-shp53-F, pCXLE-hSK, pCXLE-hUL and pCXWB-EBNA1 (Addgene) were used to transfer into 3 Ban fibroblast lines by Lonza Nucleofector system. The cells were then re-cultured into new disks containing ES medium with DMEM (Gibco, USA) high glucose, supplemented with antibiotics, 1000 UI/ml LIF (Sigma, USA) and 10uM FGF-2. Monitoring and passaging of cells with changes in morphology were carried out subsequently to maintain the characteristics of cells achieved after plasmid transfer. Another disk of similar cells were transferred by EGFP plasmid to confirm efficiency of plasmid transfer.

Passaging of pluripotent stem-like cells

Passaging of stem-like cells with typical morphology were carried out by mechanical technique of using a pointless needle pulling from a Pasteur pipette to pick up the colony and transferred into centrifuge tube before detachment by gently pipetting. Clusters of several cells were then sub-cultured into new disks pre-coated by gelatin 0.2% in PBS, containing mentioned ES cell medium. Number colonies with typical pluripotent morphology, number of passages of each cell lines were recorded.

Embryoid body formation

To check the pluripotency in vitro, colonies of stem-like cells were detached and transferred into hanging drops of medium similar to ES medium but not supplemented with LIF nor FGF-2. After 3-10 days, embryoid body formation were recorded.

III. RESULTS

Somatic cell culture

Ear samples were collected from a total of 10 Ban pigs. Beside 1 samples contaminated during cell culture, the other 9 samples were successfully used to establish 9 primary fibroblast cell lines, with the quantity of 10-15 million per line, and a total of 104 million (TABLE 1, fig. 1). All was cryopreserved. Some cryovials of such cell lines were thawed to test the viability of cells. All of them results cells with good viability and development competence, able to be used as a material for the subsequent experiment.

Table.1: Collection of fibroblasts at passage 4

Pig number	Number of disks	Quantity of cells (million)
1	10	10
2	12	12
3	10	N/A (contaminated)
4	14	15
5	12	11
6	10	10
7	12	11
8	12	12
9	14	13
10	11	10
Total		104

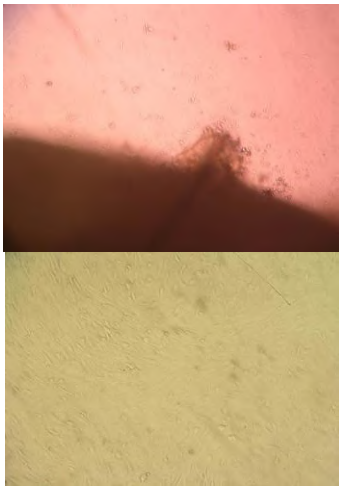

Fig.1: Fibroblasts grown from an ear tissue piece (A) and at passage 2 (B)

Plasmid transfer into somatic cells

Efficiency of plasmid transfer into fibroblasts were confirmed by co-transference of EGFP plasmid. Almost cells were transferred with the plasmid, efficiency reaches to 99.9%. This proves that most fibroblasts were similarly transfected by plasmids containing reprograming factors to be expressed in Ban fibroblasts.

Stem-like cell maintenance efficiency

By mechanical passaging, the efficiency of maintenance in all cell lines based on typical pluripotent morphology was quite high. All three cell lines could keep their morphology until passage 15 (TABLE 2), with the number of colonies with typical morphology (fig. 2) is all higher than 95% in all three cell lines at passages 5, 10 and 15.

Table 2: Stem-like cell maintenance efficiency

Cell line	Percentage of colonies (%) with typical morphology		
	Passage 5	Passage 10	Passage 15
2	96.1 ± 0.4	98.5 ± 0.5[a]	97.4 ± 0.7[a]
4	96.4 ± 0.5	95.2 ± 0.7[b]	95.5 ± 0.5[b]
9	97.3 ± 0.5	97.7 ± 0.2[a]	95.3 ± 0.6[b]

3 replications were carried out, data with different superscripts in each column is significant (P<0.05).

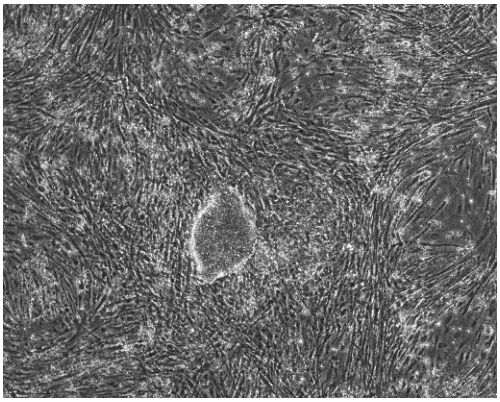

Fig.2: A colony of reprogrammed fibroblasts after 10 days.

Embryoid body formation efficiency

At passage 5, 10, and 15, some colonies of all three cell lines were detached and culture without LIF and FGF-2 to form embryoid body in hanging drops. The results showed that all three cell lines were able to form EB, which is the primary proof of induced pluripotency achieved by transference of episomal plasmids.

IV. DISCUSSION

In pigs, embryonic stem cell lines have been established [4-6]. However, the efficiency was not as high as in other species, few reports showed maintenance of embryonic stem cell lines more than 20 passages [7-12]. Along with ethical issues of using embryonic stem cells in human, iPS cell establishment for medical model and application was preferable. With different reprogramming protocols, many iPS cell lines have been established [13]. However, similarly to ES cell lines, most of the iPS cell lines possesses prime-like properties rather than naïve-like

ones [6, 13-17]. Moreover, there was just one report with germ-line transmission with low efficiency was published [15].

In the present study, we combine a set of episomal plasmids containing effective reprogramming factors (Oct3/4, Sox2, Klf4, l-Myc and EBNA1) and an effective transfer method of using Lonza Nucleofector. The results showed that the transfer efficiency reaches a very high rate. All of the pluripotent stem-like cell lines are in prime type, which is similar to most other researches [15-18]. The reprogrammed cells showed a epithelial-like morphology, forming colonies of cells, however, not LIF dependent. LIF was used to see whether the cells could form any naïve-like colonies, however the results was negative for that.

In vitro differentiation competence is a crucial criterion for confirming pluripotency of cell lines derived from either embryonic or somatic sources. All three cell lines in our study showed this ability to form embryoid bodies in passages 5, 10 and 15 when being cultured without LIF and FGF-2. This could partially prove that the cell lines have been successfully reprogrammed besides changes in morphology. However, further analysis should be carried out to characterize the pluripotency of reprogrammed cell lines from Ban, including in vivo differentiation into teratomas or contribution to germ-line chimera, and trials of further culture of cell lines to passages more than 15.

V. CONCLUSION

Using episomal plasmids, in Ban, a Vietnamese native pig, fibroblasts were successfully reprogrammed into pluripotent stem-like cells with typical morphology which could be maintained to passage 15 and contribute to embryoid body formation which partly proved pluripotency. Further studies should be conducted to confirm the characteristics of produced cells.

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