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Generation of tetraploid complementation mice from embryonic stem cells cultured with chemical defined medium

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Abstract Mouse embryonic stem cells (mESCs) derived from inner cell mass (ICM) of pre-implantation embryos, can maintain undifferentiated state when cultured in N2B27 medium supplemented with GSK3 inhibitor CHIR99021 and MEK inhibitor PD0325901 ("2i") and leukemia inhibitor factor (LIF). Compare to conventional culture medium, all components of this medium are defined. With the N2B27 medium, "2i" and LIF, mESCs can contribute to the germline of the chimeric embryos, however, whether the "all-ES cells" mice can been generated by tetraploid complementation is unclear yet, while the tetraploid complementation serve as a golden standard to assess the pluripotency of ES cells. Here, our study showed that mESCs derived and cultured with the N2B27 complete medium could generate fertile mice by tetraploid complementation. In addition, the survival rate of tetraploid complementation mice produced by inbred mES cell lines is higher than the conventional culture condition, and increased the percentage of Oct4 positive cells contrast to conventional medium either. Therefore, the N2B27 medium supplemented with "2i" and LIF is an alternative choice for the derivation and long-term culture of mouse embryonic stem cells.

Keywords Mouse embryonic stem cells · Pluripotency · Tetraploid complementation · 2i · Chemical defined medium

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1 Introduction

Mouse embryonic stem cells (mESCs) are one type of pluripotent stem cells, which derived from pre-implantation embryos [1], have unlimited self-renewal ability and possess capacity to give rise to all cell types of the mouse. mESCs also could differentiate into many cell types of three germ layers *in vitro*, which have been widely used for the basic research [2]. mESCs are usually maintained on primary embryonic fibroblast feeder cells with culture medium supplemented with leukemia inhibitory factor (LIF) and serum [3] to keep undifferentiated state. mESCs can differentiated when withdrawal of LIF or direct inhibition of STAT3 [4]. Serum is also important to sustain of the self-renewal of mESCs, however, some factors existed in serum also promote differentiation of mESCs, which may make it difficult to illustrate the precise mechanism of self-renewal.

The BMP4 is first identified to support the self-renewal of the mESCs which could replace of the serum, through the downstream Id (inhibitor of DNA binding) genes, or inhibit the phosphorylation of the MAPK [5]. The N2B27 medium facilitate the study of the self-renewal of mESCs, without BMP4 or LIF, the mESCs can be efficiently converted into *Sox1* positive neural precursors [6]. By adding the BMP4 and LIF, or "2i" and LIF can keep the proliferation of mESCs.

In addition, the N2B27 medium supplemented with "2i" can been used to derive the NOD ES cell lines with the germline competency [7]. We also have shown that the N2B27 supplemented with "2i" culture system, the androgenetic embyronic stem (aES) cells could been derived [8]. We also reported that N2B27 supplemented with "2i", knockout serum replacement (KOSR) and vitamin C (Vc) culture system improved the efficiency of rat iPSCs generation from Dark Agouti rat fibroblasts and Sertoli cells [9]. However, it is not clear yet that whether the N2B27 medium with "2i" and LIF can be used to culture the mESCs which can produce "all-ES cells" mice by tetraploid complementation or not.

Here, we used a chemical defined medium (N2B27 complete medium: DMEM/F12 supplemented with N2, B27, "2i" and LIF) to culture mESCs instead of conventional serum and LIF medium. Our results showed that these mESCs could fulfill the most rigorous rule of pluripotency of ESC and give birth to live pups through tetraploid complementation. It is also suggested that the N2B27 complete medium would be suitable for derivation and long-term culture of the mouse embryonic stem cells.

2 Materials and methods

2.1 Animals

B6D2 (C57BL/6×DBA) F1 females (8–10 weeks old) and CD-1 mice were purchased from Beijing Vital River Laboratory Animal Center and housed in a specific pathogen-free environment in the animal facility of the Institute of Zoology. All studies were performed in accordance with the Guidelines of Institute of Zoology, Chinese Academy of Sciences for the Use of Animals in Research.

2.2 Derivation of ES cell lines

Fertilized blastocysts were plated on the 4-well plates pre-coated with feeder cells, proliferating outgrowths were dissociated using manual pipetting or 0.25 % trypsin treatment, and then re-plated on fibroblasts until the stem cells expand enough. Stem cells were cultured as previously described. N2B27 medium supplemented with recombinant LIF and "2i" (3 μmol/L GSK3 inhibitor CHIR99021 and 1 μmol/L MEK inhibitor PD0325901). DMEM/F12 medium supplemented with 15 % KOSR and LIF was used as control medium.

2.3 Immunofluorescence analysis and alkaline phosphatase staining

Cells were fixed with 4 % paraformaldehyde for 30 min and then permeabilized with 0.5 % Triton X-100 for 30 min, followed by blocking with 2 % BSA (Sigma, MO, USA). Cells were incubated with primary antibody overnight at 4 °C and secondary antibody at room temperature for 1 h. The following antibodies were used: anti-SSEA1 (Chemicon, CA, USA), anti-Oct4 (Santa Cruz, CA, USA), and anti-Nanog (Chemicon). Alkaline phosphatase staining was performed with BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime, Jiangsu, China) according to the manufacturer's instructions.

2.4 Fluorescence-activated cell sorting analyses

Briefly, confluent (ESC1, Oct4-EGFP) ES cells were trypsinized to single cells and washed by DMEM (GIBCO, CA, USA) supplemented with 10 % FBS (GIBCO), then filtered with 40 μm cell strainers. GFP negative ESC sample was used as control. Single-cell suspensions were recorded by FACS Vantage-HG. After purification, the GFP positive ES cells were sorted for further culture.

2.5 Embryo culture and tetraploid embryo complementation

The generation of mice by tetraploid embryo complementation was carried out as previously described [10]. Briefly, two-cell stage embryos were collected from oviducts of CD-1 females (white coat color), and electrofused to produce one-cell tetraploid embryos that were further cultured in CZB media. Ten to fifteen ES cells (originally with a B6D2F1 genetic background, or black coat colour) were injected into each tetraploid blastocyst. The constructed embryos were transferred to the uterus of CD-1 pseudopregnant recipient females.

3 Results and discussion

3.1 Mouse ES cells cultured with N2B27 complete medium produced fertile mice by tetraploid complementation

We first tested the developmental potential of the mES cells with B6D2F1 (C57BL/6×DBA/2) genetic background [11], these cells (including ESC1) was derived and cultured in conventional medium contain KOSR and LIF, and were gradually adapted with the N2B27 complete medium. The N2B27 complete medium composed of the basic medium supplemented with "2i" and LIF, which we

termed “2i” medium, the unadapted cells cultured with DMEM/F12 medium supplemented with 15 % KOSR and LIF are use as control. The ESC1 adapted to “2i” medium in five passages, and grew robustly with typical morphology of mESCs (Fig. 1a). Both groups of mESCs were alkaline phosphatase (AP) cells, expressed typical pluripotent markers (Oct4, Nanog and SSEA-1) (Fig. 1b), with no obvious difference between each other. Interestingly, fluorescence activated cell sorting (FACS) data showed a slightly higher percentage of Oct4 positive cells in N2B27 complete medium than that in the control medium (98.0 % vs 90.2 %), with no significant statistical difference (Fig. 1c).

To investigate the developmental potential of mESCs in N2B27 complete medium, we performed tetraploid complementation assays. From passage 20 to 23, 10 full-term pups (2.4 %) generated from ESC1 cultured in N2B27 complete medium, with similar efficiency as the control (KSOR medium) (2.2 %). The ESC1 mice survival to adulthood and gave birth to the next generation (Table 1). Taken together, the mES cells cultured with N2B27 complete medium maintained undifferentiated status and kept

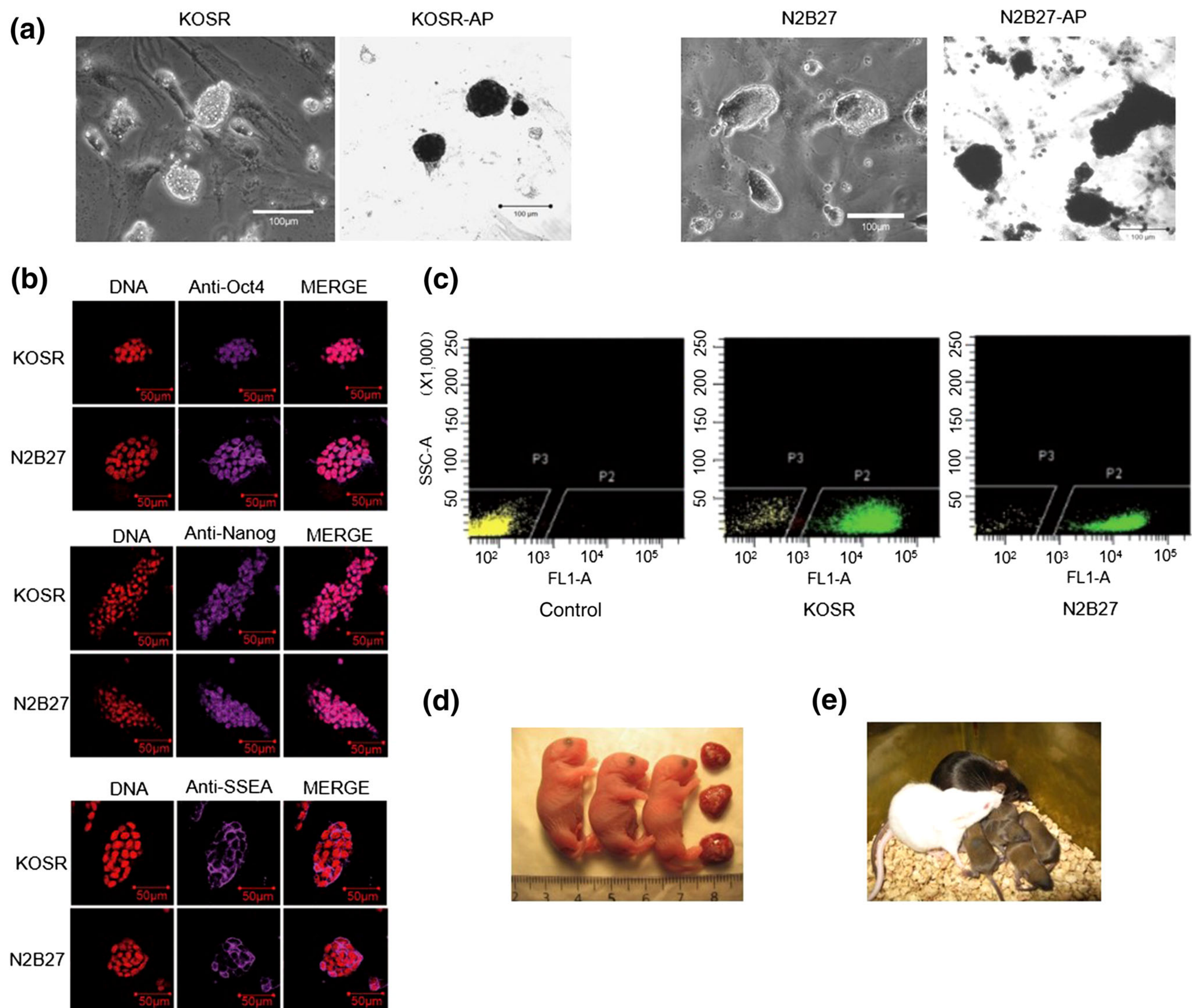

Fig. 1 ESC1 maintained an undifferentiated state in N2B27 culture system. **a** ESC1 clones or stained with an alkaline phosphatase kit. Scale Bars = 100 μm. **b** Immunostaining for pluripotency markers in ESC1 cells in different culture systems. Positive Oct4, Nanog and SSEA1 (purple) were observed. DNA was stained by propidium iodide (red). Scale Bars = 50 μm. **c** Oct4-GFP cells were analyzed by fluorescence-activated cell sorting. Two distinct populations were isolated. **d** Tetraploid-pups derived from ESC1 cultured in N2B27. **e** Adult-tetraploid male mouse derived from culturing in N2B27 and its pups, mated with a female CD-1 mouse (white color)

Table 1 Developmental efficiency of embryos produced by tetraploid complementation (4N)

Cell line	Derivation media	Culture media	Injected embryos	4N injection (%)		
				Embryos arrested at E10.5-E13.5	Embryos arrested at E13.5-E17.5	Live pups
ESC1	KOSR	KSOR	356	11 (3.1)	–	8 (2.2)
ESC1	KOSR	N2B27	423	15 (3.5)	–	10 (2.4)
GFP 2-1	N2B27	N2B27	130	2 (1.5)	1 (0.7)	1 (0.7)
GFP 1-2	N2B27	N2B27	285	–	–	14 (4.9)
GFP 1-3	N2B27	N2B27	312	–	–	17 (5.5)

The efficiencies (%) were calculated based on the total manipulated embryos. ESC1 has a genetic background of C57×DBA F1 (B6D2F1), the rest are from C57

the developmental potential to generate mESCs mice by tetraploid complementation.

3.2 Pluripotency of mESCs derived in N2B27 complete medium

We firstly derived mESCs in N2B27 complete medium to further test the pluripotency of mESCs. The E3.5 fertilized blastocysts were flushed out from uterus of inbred C57BL/6 pregnant mice, which carried an Actin-EGFP transgene in the genome. Eleven blastocysts were divided into two groups and implanted, six in the N2B27 complete medium, five in the control medium, respectively (Fig. 2a). Three cell lines were derived from the N2B27 complete medium (50 %), while only one cell line was derived from the control medium (20 %). We further performed serial assays to assess the pluripotency of the mESCs. Two cell lines (GFP 1-2, GFP 1-3) in the N2B27 complete medium and one cell line in the control medium (GFP 2-1) were selected. All three mES cell lines could expand for more than 30 passages with strong AP activity, and expressed key pluripotent markers such as *Oct4*, *Nanog* and *SSEA-1* (Fig. 2b). These data showed the mES cells cultured with N2B27 complete medium or control medium could maintain self-renewal for a long time with undifferentiated state.

We next performed tetraploid complementation assay to assess the developmental potential of the mES cells. One pup generated from GFP2-1 (0.7 %), but died in the next day after birth, which consistent with previous reports that the ES mice had poor survival potential with inbred background. By contrast, there were 14 and 17 mice generated from GFP 1-2 (4.9 %) and GFP 1-3 (5.5 %), respectively. Most of the mice were raised by their foster mother, survived to adulthood and gave birth to the next generation. It indicated that the N2B27 complete medium could improve the developmental potential of the mESCs of inbred genetic background, especially increase the survival rate of ES mice (Table 1).

4 Discussion

In this study, we described the N2B27 complete medium was a reliable medium for mESCs derivation and culture. During long-term cell culture in N2B27 complete medium, mESCs could maintain normal pluripotency and possess potential to produce fertile mice via tetraploid complementation. In addition, the N2B27 complete medium improved the survival efficiency of the ES mice with inbred genetic background.

Mouse embryonic stem cells serve as a critical tool to the develop novel biotechnologies [12], however, the precise mechanism is not clear yet, which hinders the application of mESCs. An chemical defined medium will facilitate the research of the self-renewal. Previous data showed that the N2B27 complete medium could support the mESCs for long term self-renewal. Our study here for the first time demonstrated that these cells were also pluripotent.

Most of what is known about mouse ES cells comes from studies in lines derived from the inbred 129 strain. Derivation from other mouse strains is much less efficient because other genetic backgrounds might have slightly different responses to MEK inhibition [13]. In addition, the efficiency of generation of tetraploid complementation mice is lower than that of outbred or hybrid mESCs [14], which may be caused by cryptic epigenetic and/or mutational changes arise during derivation or propagation of the ES cells [15]. Here, we show that the mESCs cultured in the N2B27 complete medium could generate fertile mice by tetraploid complementation, and improve the survival rate of the ES mice with inbred background. Our results suggested that mESCs could be derived from this N2B27 complete medium and cultured for a long time with typical mESCs features. This was the first time to show that the N2B27 system was an optimal system for mESCs derivation and culture. The N2B27 complete medium with clear

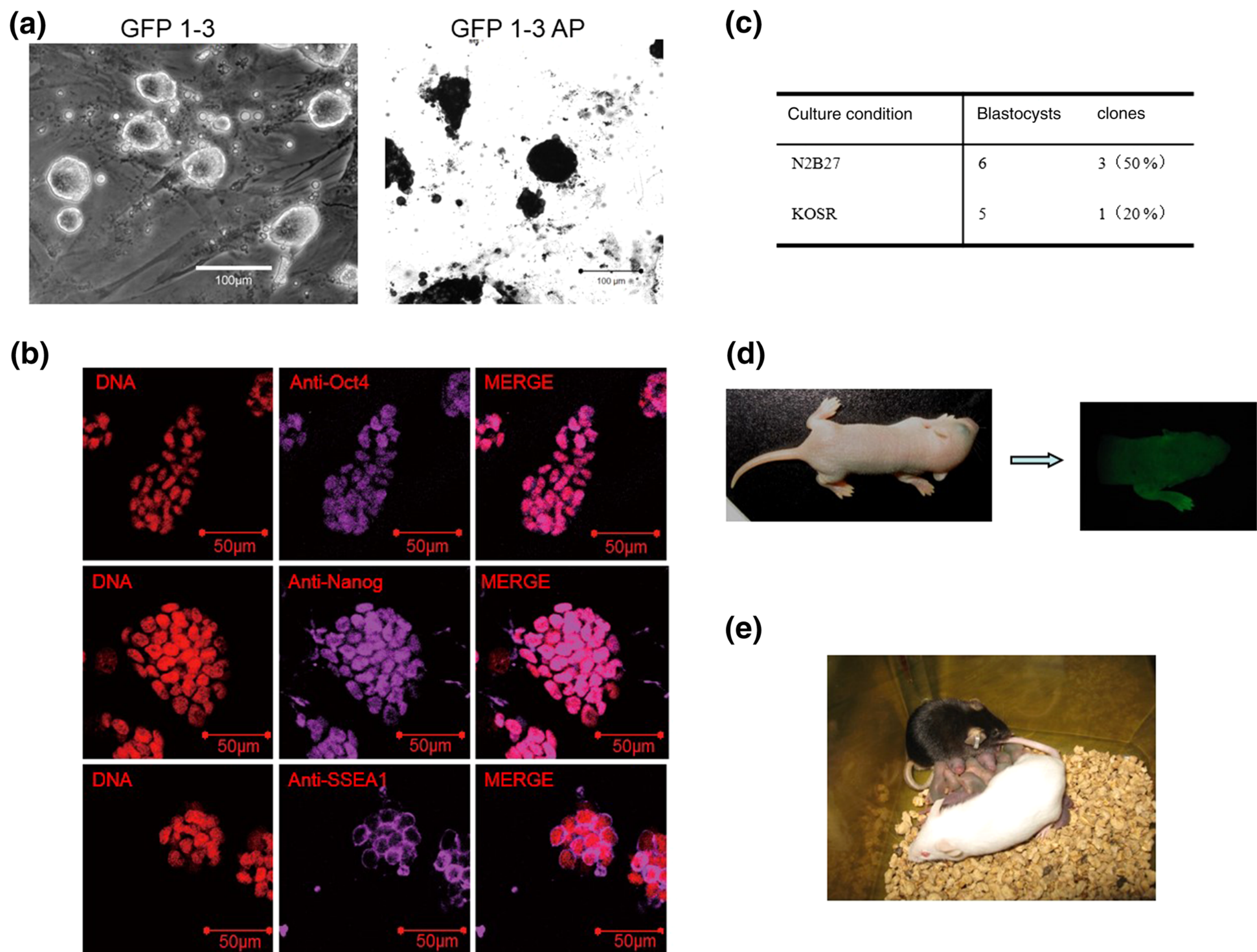

Fig. 2 Characterization of GFP 1-3 ESCs generated in N2B27 culture system. **a** GFP 1-3 clones and stained with an alkaline phosphatase kit. Scale Bars = 100 μM. **b** Immunostaining for pluripotency markers in GFP1-3 ES cells. Positive Oct4, Nanog and SSEA1 (purple) were observed. DNA was stained by propidium iodide (red). Scale Bars = 50 μm. **c** Efficiency of ESC established in N2B27 and KOSR culture sysytems. **d** Pups derived from GFP 1-3 generated in N2B27 with strong expression of GFP. **e** Adult tetraploid complementation male mouse derived from GFP 1-3 and its pups, mated with a female CD-1 mouse (white color)

components will facilitate elucidating the molecular mechanisms of self-renewal of mESCs.

5 Conclusions

In summary, mESCs could be derived in N2B27 medium supplemented with “2i” and LIF with defined components instead of conventional serum and LIF medium with higher efficiency. The N2B27 medium supplemented with “2i” and LIF could support the long-term expansion of mESCs; in addition, it could improve the survival ability of the ES mice with inbred genetic background. The N2B27 medium supplemented with “2i” and LIF would be a better medium to study the molecular mechanism of self-renewal and pluripotency.

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