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Non-viral reprogramming of fibroblasts into induced pluripotent stem cells by Sleeping Beauty and piggyBac transposons

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ABSTRACT

The generation of induced pluripotent stem (iPS) cells represents a promising approach for innovative cell therapies. The original method requires viral transduction of several reprogramming factors, which may be associated with an increased risk of tumorigenicity. Transposition of reprogramming cassettes represents a recent alternative to viral approaches. Since binary transposons can be produced as common plasmids they provide a safe and cost-efficient alternative to viral delivery methods. Here, we compared the efficiency of two different transposon systems, Sleeping Beauty (SB) and piggyBac (PB), for the generation of murine iPS. Murine fibroblasts derived from an inbred BL/6 mouse line carrying a pluripotency reporter, Oct4-EGFP, and fibroblasts derived from outbred NMRI mice were employed for reprogramming. Both transposon systems resulted in the successful isolation of murine iPS cell lines. The reduction of the core reprogramming factors to omit the proto-oncogene c-Myc was compatible with iPS cell line derivation, albeit with reduced reprogramming efficiencies. The transposon-derived iPS cells featured typical hallmarks of pluripotency, including teratoma growth in immunodeficient mice. Thus SB and PB transposons represent a promising non-viral approach for iPS cell derivation.

1. Introduction

Induced pluripotent stem (iPS) cells can be generated from somatic cells [1–3] by viral transduction of core reprogramming factors [4,5]. In proof of principle experiments in mice, the correction of genetic disease phenotypes by iPS-based cell therapy have been shown [6–10].

IPS cells have been achieved by forced expression of defined factors using multiple infections with dis-armed viral vectors [1,11–13]. Retro- and lentiviral vectors are the most commonly used gene delivery systems, because of their high transduction rates and their ability to integrate the reprogramming genes into the host genome [14,15]. A clear disadvantage of viral reprogramming is the risk of insertional mutagenesis [16]. Retro- and lentiviral vectors exhibit a strong preference for integrating in promoters and exonic region, which may cause unpredictable genetic dysfunction and may have genotoxic effects [17]. Consequently, alternatives for viral reprogramming are intensively studied [18–25]. Recently, transposons have been applied for reprogramming, where the PB [26] and the SB [27] systems gained considerable attention. Both the transposon and the transposase component can be delivered as easy-to-handle plasmid constructs. Once available, this allows the rapid and price-competitive production in large amounts in standard laboratories. Transposons have a high DNA cargo capacity of up to 150 kilo basepairs (kb) [28,29]. At the genomic scale the SB integration sites appear to be randomly distributed, whereas for PB a slight bias for exonic and promoter regions is described [30]. Data from transposon transgenic animals

Abbreviations: PB, piggy*Bac*; SB, Sleeping *Beauty*; iPS, induced pluripotent stem (cells); HMGB1, high-mobility group box1; Miz1, zinc finger and BTB domain containing 1; ITR, inverted terminal repeats; CAGGS, cytomegalovirus enhancer, chicken β actin promoter promoter; OG2, Oct4 promoter-EGFP (pluripotency reporter).

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suggest that SB and PB transposases seem to favor transcriptionally permissive loci for integration into the genome [31–35]. SB originates from salmonides, where it existed as inactive element, which was reawaken by an in vitro mutagenesis approach [36]. PB was identified as active element in the moth Trichoplusia ni [37]. Both elements show transposition activity in mammalian cells, and hyperactive transposase variants were recently generated by in vitro evolution approaches [38–40]. Both transposon systems are thought to be active without cellular co-factors in mammalian cells, but supportive effects of the DNA bending protein HMGB1 (high-mobility group box1) [41] and interaction of the cell cycle regulator Miz1 (also known as Zinc Finger and BTB Domain Containing 1) with SB have been shown [42]. These data suggest that the transposition activity of SB and PB in different mammalian cells may be influenced by cellular factors. Both transposon systems have been shown to be suitable for the derivation of iPS cells [26,27,43–45]. However, so far no direct comparison of SB and PB systems for reprogramming has been performed. Here we employed both transposon systems for the generation of murine iPS cells from different genetic backgrounds.

2. Materials and methods

2.1. Ethics statement

Experimental mice were maintained and handled according to international and national guidelines and laws for animal welfare and regarding genetically modified organisms.

2.2. Plasmid constructs

The PB reprogramming transposon (6F) contains a CAGGS promoter driven cassette containing SOX2, OCT4, KLF4, c-MYC, NANOG and LIN28, separated by sequences coding for self-cleaving 2A peptides, and flanked by PB-inverted terminal repeats (ITR). A cDNA coding for a hyperactive PB is driven by the CMV promoter on a helper plasmid. From the PB reprogramming cassette carrying 6 transcription factors, deletions clones were generated to produce PB reprogramming cassettes carrying 4 (SOX2, OCT4, KLF4, c-MYC) and 3 (SOX2, OCT4, KLF4) reprogramming factors. The Sleeping Beauty plasmids were described before [27,43].

2.3. Isolation of murine embryonic fibroblasts and iPS generation

Outbred NMRI mice were bought from Harlan (Netherlands). The inbred BL/6 mice carrying the Oct4-EGFP (OG2) pluripotency reporter were gifted by Hans Schöler (MPI Münster) and maintained by homozygous breeding.

Primary mouse embryonic fibroblasts (MEFs) were prepared from fetuses at 13.5 days post conception. Murine fetal fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (PAA), 2 mM L-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 0.05 mM mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were passaged with 0.25% trypsin/0.1% EDTA. Cells at passage 2 were used for electroporation with both SB and PB transposon plasmids, respectively. An electroporator with square wave function (BioRad Gene Pulser Xcell) was used

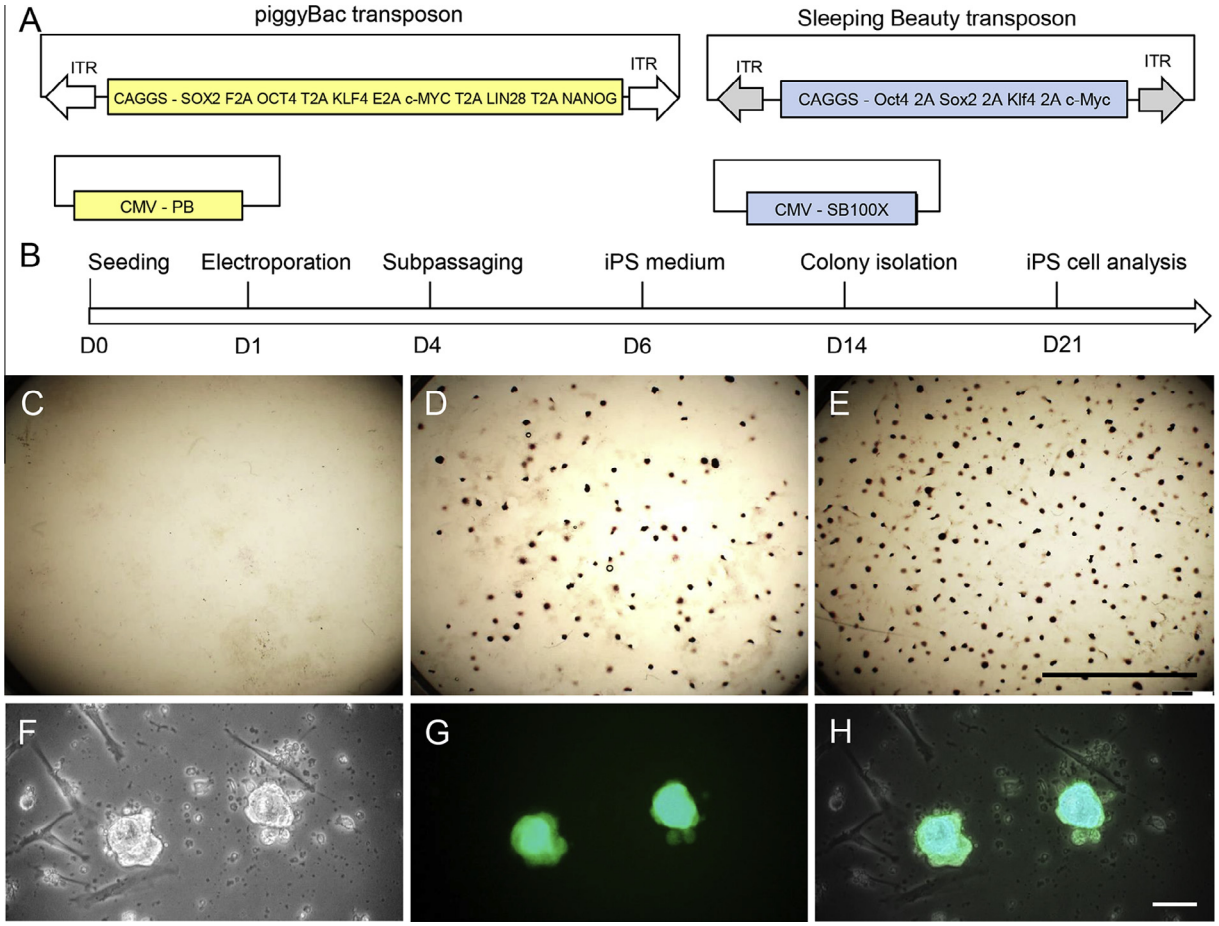

Fig. 1. Reprogramming transposons and experimental design. (A) Schematic depiction of PB and SB transposons. (B) Time course of experimental steps. (C) Colony assay of control cells (AP stained). (D) Colony assay of SB-4F treated fibroblasts (AP). (E) Colony assay of PB-6F treated cells. Bar = 1 cm. (F) Bright field image of forming colony (SB-4F, day 8). (G) Specific excitation of pluripotency reporter (OG2). (H) Overlay of A and B. Note that only the iPS colonies are EGFP-positive, bar = 10 μm.

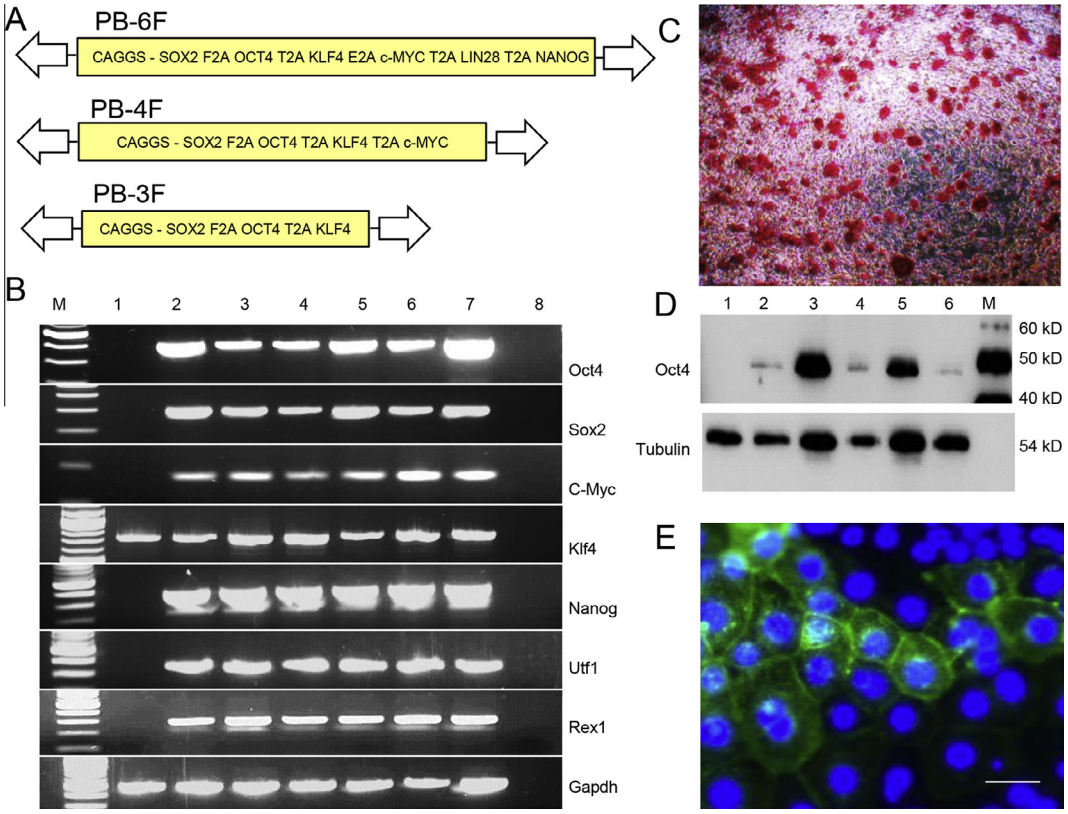

Fig. 2. Characterization of iPS clones. (A) Schematic diagram displaying PB reprogramming transposons with 6 factors, 4 factors and 3 factors. (B) Expression of stemness genes in PB and SB transposon-derived murine iPS cells. M, DNA ladder. 1, Murine fetal fibroblasts; 2–4, iPS cells (NMRI) generated by PB-3F, PB-4F, PB-6F, respectively; 5, iPS cells (NMRI) generated by SB-4F; 6 and 7, iPS cells (OG2) generated by using PB-6F and PB-4F. 8, no RT control. (C) AP expression in iPS cells, D21. (D) Oct4 expression in transposon-derived iPS cells.1, Fibroblasts; 2–4, iPS cells (NMRI) generated by PB-3F, PB-4F, PB-6F, respectively, 5, iPS cells (NMRI) generated by SB-4F; 6, iPS cells (OG2) generated by PB-6F; M, size ladder. (E) SSEA1 expression in PB-6F-derived iPS cells (green). Nuclei are counterstained with Hoechst 33342 (blue). Bar = 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Murine iPS cell derivation by SB and PB reprogramming.

Mouse strain	BL/6 (OG2)		NMRI				BL/6 (OG2)		NMRI	
Transposase	SB	PB	SB	PB	PB	PB	–	–	–	–
Transposon	4F	6F	4F	6F	4F	3F	4F	6F	4F	6F
No. of replications	4	4	4	4	4	2	8	8	12	12
No. of cells used for electroporation	1×10^5	1×10^5	1×10^5	1×10^5	1×10^5	1×10^5	1×10^5	1×10^5	1×10^5	1×10^5
Reprogramming efficiency[a]	0.009%	0.027%	0.011%	0.021%	0.019%	0.004%	0%	0%	0%	0%
No. of colonies picked	2	9	5	4	4	2	0	0	0	0
Stable iPS cell lines	2	9	5	4	4	2	n.a	n.a	n.a	n.a
OG2 expression	+	+	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a
AP expression	Yes	Yes	Yes	Yes	Yes	Yes	n.a	n.a	n.a	n.a
Teratoma formation	1/1	8/9	2/2	3/3	2/3	2/2	n.a	n.a	n.a	n.a

[a] Number of colonies obtained/number of cells, n.a = not applicable.

for electroporation (250 V, single pulse/10 ms). Electroporated MEFs were cultured in fibroblast medium, which was later replaced by DMEM/nutrient mixture F-12 supplemented with 20% knockout serum replacement (Millipore), 1 mM L-glutamine, 0.1 mM nonessential amino acids, 0.1 mM mercaptoethanol, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1000 U/ml human leukemia inhibitory factor (hLIF). Presumptive iPS colonies were picked by a pipette under microscopic control, and plated into individual wells of 96-well tissue culture plates containing trypsin. Trypsin was neutralized with DMEM and 10% FBS, and the cells within each well were then transferred to individual wells of 96-well tissue culture plates. Murine fibroblasts and iPS cells were cultured in a humidified atmosphere consisting of 5% CO_2 in air at 37 °C. Alkaline phosphatase (AP) staining was done as described [27]. For fluorescence microscopy, an Olympus BX60 (Olympus) microscope equipped with epifluorescence and a 12-bit digital camera (Olympus DP 71) was used.

2.4. Reverse transcription PCR (RT-PCR)

Total RNA was prepared from iPS cell lines, tissues and tumors with TriReagent (Ambion) according to the manufacturer's instructions. Isolated total RNA was treated with RNase-free DNase (1U/μg RNA) (Epicentre Biotechnologies) and 0.5 μg RNA was used for cDNA synthesis. PCR details were described before [27]. The cDNA samples were subjected to PCR amplification with the primer pairs mentioned in Supplementary Table 1. The PCR reaction included an initial denaturation at 94 °C for 5 min followed by 30

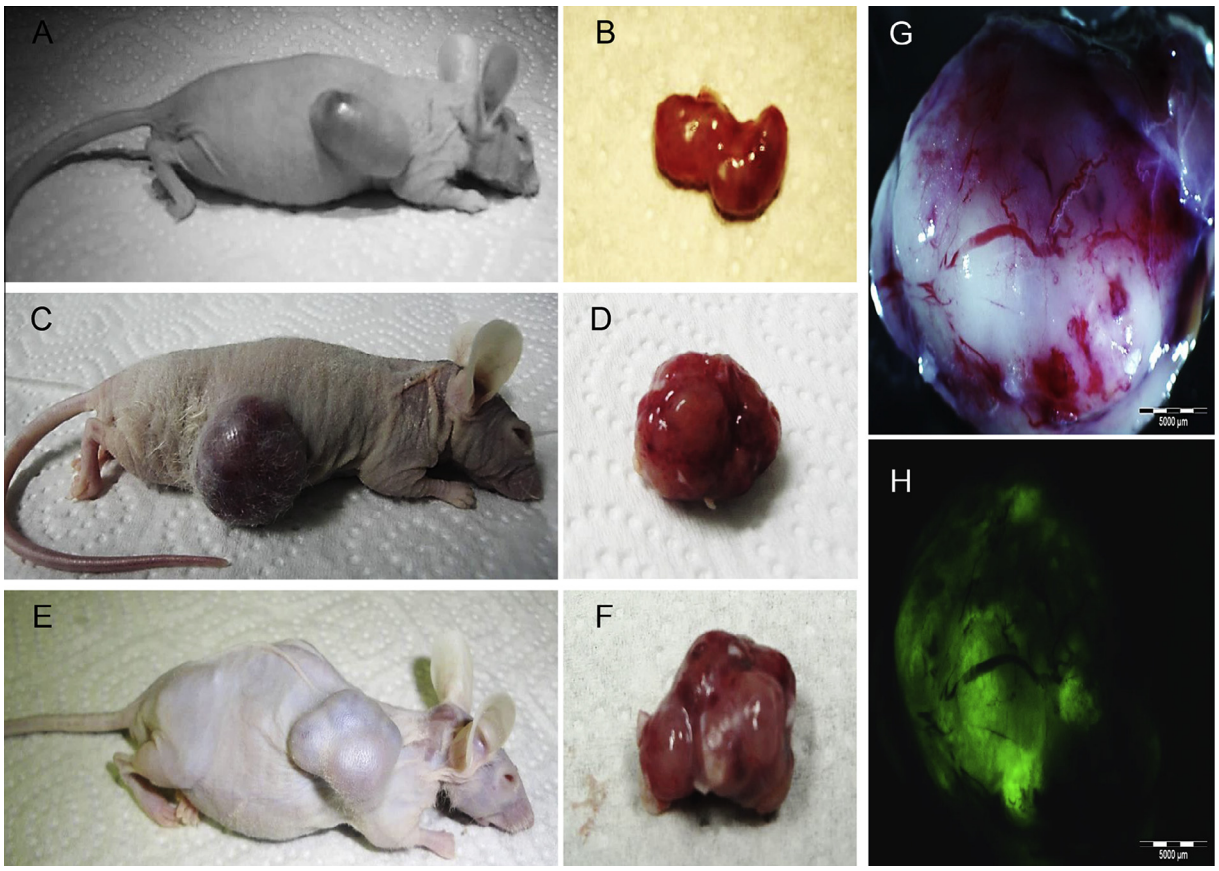

Fig. 3. Teratoma assay in nude mice. (A, C and E) Immunodeficient nude mice with teratoma development after iPS cell injection (PB-3F, 4F and 6F, respectively). (B, D and F) Isolated teratomas. (G) Teratoma derived from OG2 iPS cells (SB-4F), shown at white light conditions. (H) Specific excitation of the EGFP reporter. The EGFP fluorescent areas depict undifferentiated OG2 cells, the non-fluorescent areas depict differentiated cell types with turned off the OG2 reporter.

cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 45 s, and extension at 72 °C for 30 s. For normalization, the transcript of the housekeeping glycerinaldehyd-3-phosphat dehydrogenase (GAPDH) was used.

2.5. Western blotting

Total protein isolation and SDS–PAGE were described before [27]. The primary antibodies anti-Oct4 (Santa Cruz, sc-5279 (1:500)) and anti-tubulin (Developmental Studies Hybridoma Bank, E7 (1:500)) were used, respectively and detected with horseradish peroxidase-coupled secondary antibodies (1:10 000; Sigma) and an enhanced chemoluminescence reagent (ECL plus; GE Healthcare).

2.6. Immunohistochemistry

Cell cultures were grown on gelatinized coverslips and fixed in 80% methanol. The coverslips were washed 3 times with PBS/0.01% Triton-X100, and were then incubated with the anti-SSEA-1 (1:200) antibody (DSHB), subsequently followed by incubation with an anti-IgM secondary antibody conjugated with Alexa 488. Fibroblasts were used as controls, and samples without the first or without the secondary antibody were run in parallel.

2.7. Teratoma assay

To assess the teratoma potential, 1×10^6 cells of a clonal iPS culture were subcutaneously injected into the flank of a CD-1 nude mouse. Mice were analyzed for signs of tumor formation twice a week, and sacrificed when tumor size exceeded a diameter of 1 cm. Tumors were fixed in 4% formaldehyde and sections were stained with hematoxylin/eosin for histological analyses.

3. Results

3.1. Reprogramming of mouse fibroblasts into iPS cells by SB and PB transposons

Fetal fibroblasts were derived from BL/6 mice carrying a pluripotency reporter, OG2, and from outbred NMRI mice. Fibroblasts were derived from fetuses at day 13.5 p.c., and passage 2 cells were electroporated with the reprogramming transposons (Fig. 1). For electroporation, the transposons encoding multi-gene constructs for reprogramming and the respective helper plasmids were mixed in a 5:1 molar ratio and applied to murine fibroblasts by square wave pluses. Histochemical staining showed that the forming colonies were strongly positive for alkaline phosphatase (Figs. 1, 2C). The iPS cells derived after electroporation of the PB and SB transposon systems displayed typical embryonic stem cell-like colonies with distinct round clear cut borders. In case of the OG2 cells, expression of the EGFP reporter started 7–9 days post electroporation and EGFP expression was strictly confined to colonies with rounded cells (Fig. 1F). The initial iPS colonies were picked with a pipette and clonally expanded. The SB and PB-derived iPS lines could be maintained in an undifferentiated stage for more than 20 passages (Table 1).

The SB reprogramming carried a construct for expression of 4 reprogramming factors (SB-4F) (Fig. 1). The original PB transposon carried a construct for expression of six reprogramming factors (PB-6F), OCT4, SOX2, KLF4, c-MYC, NANOG and LIN28. To allow

for a better comparison between the two transposon systems, deletion clones of the original PB transposon carrying only 4 and 3 reprogramming factors were generated (Fig. 2A). The PB-3F transposon carried OCT4, SOX2 and KLF4 cDNAs and omitted the proto-oncogenic c-MYC.

In summary, reprogramming efficiencies of 0.004–0.027% could be achieved (Table 1). The SB-4F transposon resulted in an efficiency of 0.009% for BL/6 cells and 0.011% for NMRI cells. The PB-6F transposon reprogrammed BL/6 fibroblasts with 0.027% efficiency and NMRI fibroblasts with 0.021%. The PB-4F and PB-3F transposon were only tested with NMRI fibroblasts, where the BP-3F transposon resulted in the lowest reprogramming efficiency (0.004%). The iPS cell lines obtained showed the normal karyotype of mouse with 40 chromosomes (Supplementary Fig. S1). To determine the copy number and integration of the transposons, Southern blot analysis was done and one to three integrations were found in individual iPS cell clones (Supplementary Fig. S2).

3.2. Comparative characterization of SB and PB-mediated iPS cells

Gene expression analysis by RT-PCR showed that the iPS cells expressed the endogenous pluripotency related genes Oct4, Sox2, c-Myc, Klf4, Nanog, Rex1 and Utf1 (Fig. 2B). Total protein from the iPS cell lines was used for immunodetection of the Oct4 protein (Fig. 2D). Interestingly, the iPS cell lines derived from different transposon showed highly variable Oct4 expression, however the antibody detection cannot discriminate between endogenous and exogenous Oct4 expression. Expression of the cell surface marker SSEA1 was confirmed by immuno-fluorescence (Fig. 2), the SSEA1 intensity was relative similar between the different iPS cell lines.

To test the in vivo differentiation potential of the transposon-mediated iPS lines, iPS cells from SB and PB iPS lines were injected subcutaneously into the flank of immunodeficient nude mice. After 4 week, the majority of mice were found to have developed solid tumors (Fig. 3, Table 1). In case of the BL/6 derived iPS cells, which carry the OG2 reporter, the recovered tumors were partially EGFP-positive indicating pluripotent cells, and partly EGFP-negative, indicating differentiated cells of the tumor (Fig. 3H). Histological examination revealed that the tumor tissues contained cell types indicative of the 3 germ lineages including muscle and adipose tissue (mesoderm), neural rosettes (ectoderm), and ciliated epithelium (endoderm) (Fig. 4A–F). Gene expression analysis of teratoma samples revealed expression of differentiation markers of endoderm (α-Fetoprotein, Sox17), ectoderm (Nestin, Pax6) and mesoderm (Gata4, Gata6, cardiac troponin and Brachury) (Fig. 4G).

To assess the in vitro differentiation potential of SB-4F transposon-derived iPS cells, embryoid bodies were derived by the hanging drop method. When the embryoid bodies reached a diameter of >300 μm, they were seeded on gelatinized culture plates and differentiated in iPS medium without LIF supplementation. Spontaneously contraction areas, indicative for cardiac differentiation, developed 10–14 days after seeding (Supplementary Video 1).

4. Discussion

In the present study, we compared the efficacy of two different transposon systems, PB and SB, for reprogramming of murine

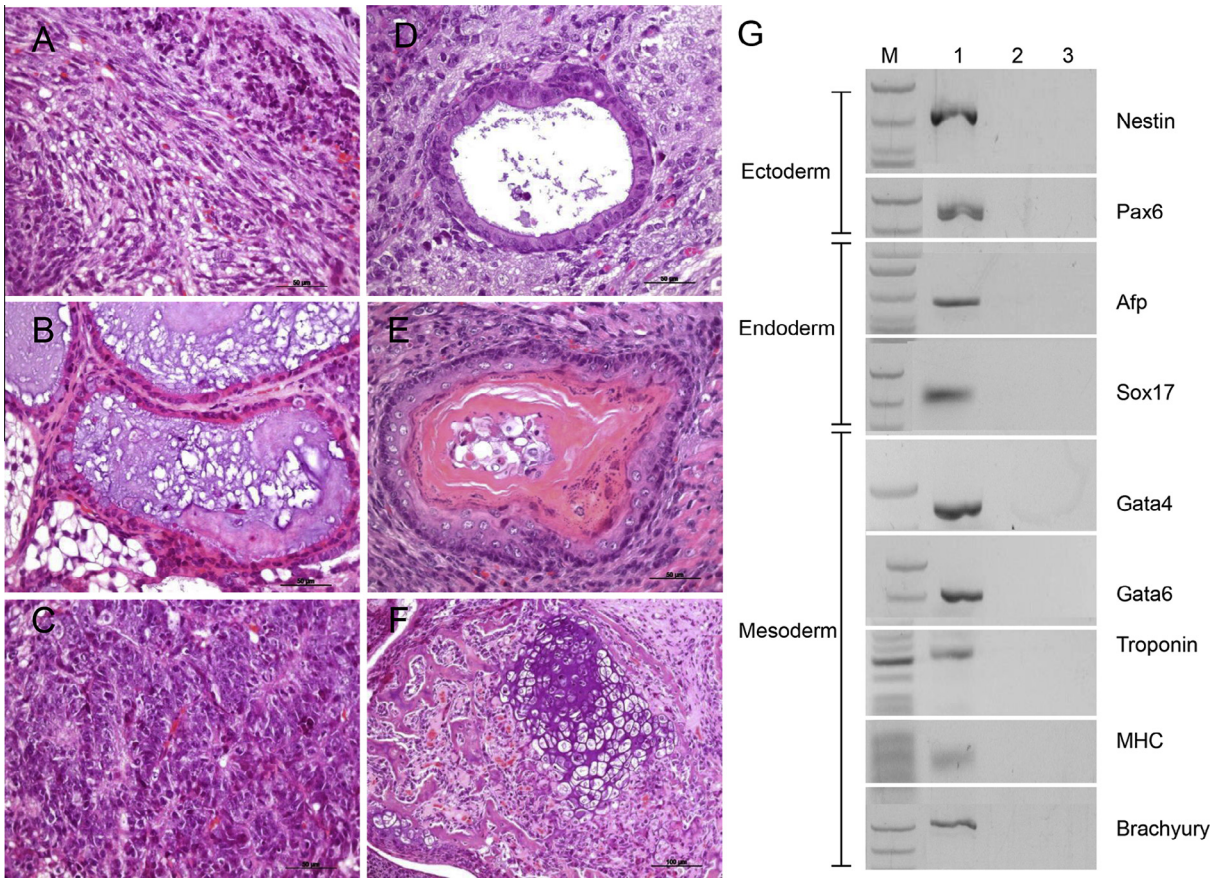

Fig. 4. Characterization of teratomas. Sections of teratomas displaying differentiated cell types of the three germ layers. (A) connective tissue; (B) mucosa producing ciliated epithelium; (C) neuronal rosettes; (D) ciliated epithelium; (E) keratinized epithelium; (F) bone and cartilage. (G) Gene expression profile of teratoma for differentiation markers. 1, Teratoma tissue; 2, murine iPS cells and 3, no RT.

fibroblasts to iPS cells. Fibroblasts were obtained from two different genetic backgrounds, i.e. inbred BL/6 and outbred NMRI mice. The primary fibroblasts were co-transfected with the transposon systems carrying variable numbers of reprogramming factors, respectively. Both non-viral transposon systems were equally efficient for the derivation of murine iPS cells, and stable iPS cell lines could be isolated from outbred and inbred mice strains. The murine iPS cells expressed typical features of pluripotent stem cells, including in vitro differentiation and formation of teratomas.

Transposon-mediated reprogramming is an attractive alternative to viral methods. Both SB and PB transposons have been shown to have a huge cargo capacity [29]. Previously, transposon reprogramming employing either PB or SB systems have been shown for human, murine, porcine and equine cells [43,45–48]. However, no direct comparisons between SB and PB reprogramming systems have been done so far. PB and SB transposon system provide stable transgene expression after a single treatment by transposition of the transgene into the genome. Genome wide targeting profiles of PB and SB in various human cell lines and primary cells demonstrate that the transposon systems display a lower frequency in targeting near cancer related genes and less bias in targeting active genes compared to viral-based vectors [49–53] and thus provide a relative safe approach for gene transfer.

In this study a direct comparison was made between PB and SB transposon systems in terms of their reprogramming efficiency of murine fibroblasts. High reprogramming efficiency of up to 0.027% of the treated fibroblasts was achieved, which comes close to the initially obtained reprogramming efficiencies by viral vectors. PB transposons with a reduced number of 4 and 3 reprogramming factors resulted in reduced reprogramming efficiencies, but even the PB-3F transposon, which did not include the proto-oncogenic c-MYC, allowed the derivation of stable iPS lines. Seamless removal of PB transposons by remobilization has been shown before [54,55]. No effects were observed regarding the genetic background of the used mouse lines in terms of reprogramming [56]. The genetic backgrounds of mice may result in crucial differences [57]. Here, we showed that transposon technology is suitable for reprogramming fibroblasts from two different genetic backgrounds, and even from outbred backgrounds, like NMRI, where it is difficult to generate ES cell lines. In conclusion, the present study shows that the transposon mediated approach is suitable for generating iPS cells from different genetic backgrounds of mice. Both PB and SB transposons allowed the derivation of murine iPS lines in a safe and straightforward manner.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.014.

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