

Immortalization of pig fibroblast cells by transposon-mediated ectopic expression of porcine telomerase reverse transcriptase

Shan He · Yangyang Li · Yang Chen · Yue Zhu ·
Xinyu Zhang · Xiaoli Xia · Huaichang Sun

Received: 24 August 2014 / Accepted: 20 July 2015 / Published online: 4 September 2015
© Springer Science+Business Media Dordrecht 2015

Abstract Pigs are the most economically important livestock, but pig cell lines useful for physiological studies and/or vaccine development are limited. Although several pig cell lines have been generated by oncogene transformation or human telomerase reverse transcriptase (TERT) immortalization, these cell lines contain viral sequences and/or antibiotic resistance genes. In this study, we established a new method for generating pig cell lines using the Sleeping Beauty (SB) transposon-mediated ectopic expression of porcine telomerase reverse transcriptase (pTERT). The performance of the new method was confirmed by generating a pig fibroblast cell (PFC) line. After transfection of primary PFCs with the SB transposon system, one cell clone containing the pTERT expression cassette was selected by dilution cloning and passed for different generations. After passage for more than 40 generations, the cell line retained stable expression of ectopic pTERT and continuous growth potential. Further characterization showed that the cell line kept the fibroblast morphology, growth curve,

population doubling time, cloning efficiency, marker gene expression pattern, cell cycle distribution and anchorage-dependent growth property of the primary cells. These data suggest that the new method established is useful for generating pig cell lines without viral sequence and antibiotic resistant gene.

Keywords Porcine telomerase reverse transcriptase · Transposon vector · Pig fibroblast cell line

Introduction

Pigs are the most economically important livestock, but pig cell lines useful for physiological studies, virus isolation and/or vaccine development are limited in cell banks. One of the many reasons for the limited number of pig cell lines is that it is difficult to establish such cell lines (Donai et al. 2014). The most common method for generating animal cell lines is to transform the primary cells with viral or cellular oncogenes. However, the cell lines generated with these approaches are invariably transformed, genomically unstable and/or phenotypically different from their normal counterparts (Ouellette et al. 2000). More recently, human telomerase reverse transcriptase (hTERT) has been used to immortalize animal cells. Unlike oncogene transformation, TERT-mediated immortalization does not cause cellular transformation and genomic instability, which represents a major

S. He · Y. Li · Y. Chen · Y. Zhu · X. Zhang ·
X. Xia · H. Sun (✉)
College of Veterinary Medicine, Jiangsu Co-Innovation
Center for Prevention and Control of Important Animal
Infectious Diseases and Zoonoses, Yangzhou University,
Yangzhou 225009, People's Republic of China
e-mail: sunh@yzu.edu.cn

improvement over the use of oncogenes for generating animal cell lines (Morales et al. 1999; Jiang et al. 1999; Ouellette et al. 2000).

Several pig cell lines have been generated by hTERT-mediated immortalization using plasmid- or viral vector-based methods (Cao et al. 2011; Hong et al. 2007; Ibi et al. 2007; Kuruvilla et al. 2007; KwaK et al. 2006; Pan et al. 2010; Sagong et al. 2012; Uebing-Czipura et al. 2008). The plasmid-mediated gene transfer is inefficient and thus requires the use of cytotoxic drugs for the selection of desired cell clones with long culture times. Viral vectors (e.g., lentiviral vectors) have the advantages of high gene transfer efficiency and stable gene expression (O'Reilly et al. 2012; Mátrai et al. 2010). However, the genomic integration of lentiviral DNA is strongly biased toward transcriptionally active loci, leading to an increased risk of insertional mutagenesis and/or biosafety concerns (Staunstrup et al. 2009). On the other hand, transposons have been considered as natural, non-viral gene delivery vehicles capable of efficient genomic insertion (Izsvák et al. 2009). In addition, transposons use a precise recombinase-mediated mechanism to mediate gene integration, providing long-term gene expression with improved simplicity and safety (Izsvák et al. 2009; Belay et al. 2011). In this report, we present a new method for immortalization of pig cells by using Sleeping Beauty (SB) transposon system-mediated expression of porcine TERT (pTERT).

Materials and methods

Preparation of primary pig fibroblast cells

A 2-week-old Chinese local breed Jangquhai pig was provided by the National Pig Breed Conservation Center (Taizhou, China). Primary pig fibroblast cells (PFCs) were prepared from the pig kidney as precisely described (Onishi et al. 2000) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA). Cells were incubated in a 37 °C incubator supplemented with 5 % CO₂ and non-PFC cells were removed by 3 cycles of incubation for 20 min according to their slower adhesion onto flask surface.

PCR for cloning porcine elongation factor-1a promoter

Genomic DNA was extracted from PK-15 cells (ATCC, Manassas, VA, USA) using Genomic DNA Extraction Kit (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's protocol. Porcine elongation factor 1a (pEF-1 α) promoter was cloned by PCR using Ex TaqTM Polymerase (TaKaRa Biotechnology, Dalian, China) and the primer pair in Table 1. The PCR was carried out at 95 °C for 5 min, 30 cycles of 94 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 10 min. The PCR product was subcloned into pMD18-T vector (TaKaRa Biotechnology, Dalian, China) for sequencing.

RT-PCR for pTERT cDNA cloning

Cellular RNA was extracted from PK-15 cells using RNAiso Plus (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's manual. pTERT cDNA was cloned by RT-PCR using PrimeScriptTM RT-PCR Kit (TaKaRa Biotechnology, Dalian, China) and the primer pair in Table 1. The PCR was carried out at 95 °C for 5 min; 2 cycles of 94 °C for 30 s, 64 °C for 30 s and 72 °C for 3 min; 2 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 3 min; 2 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 3 min; 26 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 3 min, and a final extension at 72 °C for 10 min. The PCR product was subcloned into pMD18-T vector for sequencing.

SB transposon vector construction

The poly(A) signal of bovine growth hormone (BGH) gene was excised from pcDNA3 vector (Invitrogen Life Technologies, Carlsbad, CA, USA) by restriction digestion and inserted into SB transposon vector pT2/ HB (Addgene, Cambridge, MA, USA) as a *Xba*I/*Bgl*II segment. The pEF-1 α promoter was excised from pMD18-T vector and cloned into the transposon vector as an *Eco*RV/*Not*I segment. The modified SB transposon vector was called pTEG (Fig. 1). The coding sequence for green fluorescent protein (GFP) was amplified from pEGFP-N1 vector using the primer pair in Table 1. The PCR product was inserted into the pTEG vector and the resultant vector pTEG-GFP was

Table 1 The PCR primers used in this study

Gene	Primer pair	Sequence (5' → 3')	Amplicon (bp)	GenBank Accession
pEF-1 α promoter	Sense	CAAGGGCGGTGGAGAAGCCC	1255	FM995601
	Anti-sense	TCACGACACCTAAGACGACA		
gfp	Sense	ATGGTGAGCAAGGGCGAG	720	LC008492
	Anti-sense	TTACCTGTACAGCTCGTCC		
pTERT	Sense	ATGCCGCGCGCCCCGGTG	3399	NM_001244300
	Anti-sense	TCAGTCCAGGATGGTCCGGA		
ampR	Sense	TCCGTGTCGCCCTTATTC	411	KJ146689.1
	Anti-sense	AAGCGGTTAGTCCTTCG		
pTERT segment	Sense	AAAGCCAGAAACGCAGGGAT	236	NM_001244300
	Anti-sense	GTCTGTAGTCAGGGCTGGGC		
CD29	Sense	TTGGCTGGAGGAATGTTACAC	423	NM_213968.1
	Anti-sense	TCACCCATTTTTGCAGTAAG		
Vimentin	Sense	CGCCAGCAGTATGAGAGTGT	344	AK400847.1
	Anti-sense	AGCAGGTCTTGGTATTCACGA		
CK18	Sense	CGTCTTGCTGCTGATGACTTC	385	EU131884.1
	Anti-sense	ACTGTGAGGTGACCACTGAGG		
p53	Sense	ACAGCGTGGTGGTGCCCTAC	370	AF098067
	Anti-sense	CGTTCACGCCACGGATCTG		

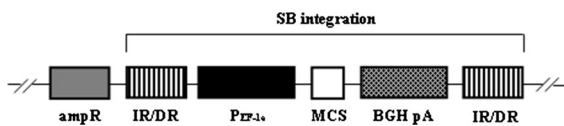


Fig. 1 The schematic structure of modified SB transposon vector pTEG. IR/DR, left and right terminal inverse repeats of SB transposon; PEF-1 α , porcine elongation factor-1 α promoter; MCS, multiple cloning sites; BGH pA, poly(A) signal of bovine growth hormone; SB integration; SB transposase-integrated expression cassette

used for validation purpose. The pTERT cDNA was subcloned into the pTEG vector and the resultant vector pTEG-TERT was used for generating pig cell lines.

Cell transfection

The SB transposon vectors pTEG-GFP and pTEG-TERT, as well as the transposase vector pSB16 which encodes SB transposase (Addgene, Cambridge, MA, USA), were prepared from the transformed DH5 α *E. coli* cultures using EndoFree Plasmid Maxi Kit (Qiagen, Shanghai, China). Primary PFCs were seeded

at 10⁵ cells/well on 6-well plates, grown to 80 % confluency, and transfected with the transposase vector (0.6 μ g/well) and/or transposon vector (3.4 μ g/well) using LipofectamineTM 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction.

Validation of SB transposon system

Primary PFCs were transfected with the transposase vector and/or transposon vector pTEG-GFP, and passed for 7 generations before fluorescent microscopy for GFP-positive cells. The integration efficiencies for random (without transposase) and recombinase-mediated (with transposase) gene transfer were calculated according to the percentages of GFP-positive cell numbers.

Cell cloning and identification

Primary PFCs were transfected with the transposase vector and transposon vector pTEG-TERT as described. On day 2 after transfection, the confluent cells were passed for 1 generation and cloned by 3 cycles of dilution cloning as previously described

(Freshney 2010). The genomic DNA was extracted from each cell clone using Genomic DNA Extraction Kit (TaKaRa Biotechnology, Dalian, China) and amplified for pTERT expression cassette or ampicillin-resistant gene (*ampR*) on the vector by PCR using the primer pairs in Table 1. The PCR was carried out at 95 °C for 5 min, 25 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The PCR products were analyzed by 1.2 % agarose gel electrophoresis. One cell clone containing pTERT expression cassette (*ampR*⁻), but not intact transposon vector (*ampR*⁺), was passed for different generations and further characterization.

Telomerase assay

Both PFC cell line and the primary cells at different generations were seeded on 6-well plates and grown to 80 % confluency. The cells were trypsinized, diluted to 10⁶/ml with PBS (pH7.4) and lysed by 3 cycles of freeze/thawing. The telomerase activities in the centrifuged cell extracts were measured using Porcine Telomerase ELISA Kit (Jianglaibio, Shanghai, China) according to the manufacturer's instruction. The primary PFC (passage 3) lysate was heated for 15 min at 85 °C and used as the negative control.

Growth curve plotting, population doubling time and cloning efficiency calculation

Both PFC cell line and the primary cells at different generations were seeded in triplicates at 10⁴ cells/well on 4-well plates. On day 2 after plating, the cells were trypsinized daily for growth curve plotting and population doubling time calculation as previously described (Hong et al. 2007). At the same times, the cells were seeded in triplicates at 1000, 500, 200, 100 or 50 cells/well on 6-well plates and grown for 2 weeks for cloning efficiency calculation as previously described (Hong et al. 2007).

RT-PCR for detection of marker gene expression

The cellular RNA was extracted from PFC cell line (passage 40), primary PFCs (passage 3) or PK-15 cells using RNAiso Plus and the cDNAs were generated using PrimeScriptTM RT-PCR Kit (TaKaRa Biotechnology, Dalian, China). The primer pairs for PCR amplification of porcine CD29, vimentin (Vim), CK18

and p53 transcripts are listed in Table 1. The PCR was carried out at 95 °C for 5 min, 25 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The PCR products were analyzed by 1.2 % agarose gel electrophoresis.

Cell cycle analysis

Both PFC cell line (passage 40) and the primary cells (passage 3) were seeded at 10⁵ cells/well on 6-well plates and grown to 80 % confluency. The cells were trypsinized, fixed overnight at 4 °C with 70 % ethanol and stained with 50 µg/ml propidium iodide for DNA content measurement on BD FACSARIA III (BD Biosciences, Franklin Lakes, NJ, USA) as previously described (Hang and Fox 2004).

Soft agar clone formation assay

The trypsinized PFC cell line (passage 40) and primary PFCs (passage 3), as well as mouse myeloma cell line Sp2/0 (Santa Cruz Biotechnology, Paso Robles, CA, USA), were diluted to 10⁶ cells/mL with 2 × DMEM containing 20 % FBS, mixed with equal volumes of 0.7 % low melting agarose solution (40 °C) and incubated in 35-mm dishes. After incubation for 2 weeks, the cell colonies were observed under light microscope as previously described (Akagi et al. 2003).

Statistical analysis

The data of telomerase activities and cell numbers of PFC cell line and primary cells were expressed as mean ± SD (n = 3) and statistically analyzed using Student's *t* test.

Results

Modification of SB transposon vector

Eukaryotic EF-1α promoters have a robust transcriptional activity in a variety of cell types (Norrman et al. 2010). However, the transcriptional activity of pEF-1α promoter has not been tested. To modify the SB transposon vector for stable pTERT expression in pig cells, we cloned pEF-1α promoter from PK-15 cells by PCR. Sequence analysis showed that the cloned

promoter was almost identical to the published sequence (GenBank: FM995601) with only one C to T transition at position 20. The pEF-1 α promoter, as well as the poly(A) signal of BGH gene, was subcloned into the SB transposon vector and the modified vector was called pTEG (Fig. 1). To validate the SB transposon system in pig cells, primary PFCs were transfected with the transposon vector pTEG-GFP alone or co-transfected with the transposase vector pSB16. At 24 h after transfection, similar numbers of GFP-positive cells were revealed in the cell cultures transfected with pTEG-GFP with or without pSB16. After passage for 7 generations (P7), however, much more GFP-positive cells remained in the cell culture co-transfected with pTEG-GFP and pSB16, but not in the cell culture transfected with pTEG-GFP alone (Fig. 2).

Cloning and expression of pTERT cDNA

Several pig cell lines have been generated by hTERT-mediated immortalization. Although the cDNA sequence for pTERT is available in GenBank, it has

not been tested experimentally whether the cDNA can encode a functional enzyme. Therefore, we cloned pTERT cDNA from PK-15 cells by RT-PCR. Sequence analysis showed that the cloned cDNA was 99.6 % identical to the published sequence (GenBank: NM-001244300) with only two G to A transitions at positions 548 and 582, and a TGCT to GCTG change from positions 2169 to 2172. The cDNA was subcloned into the pTEG vector and transfected into primary PFCs. Enzymatic assay showed that more than 200 ng/L telomerase activity was over expressed in the vector-transfected cells.

Selection and identification of PFC clones

To obtain the cell clones containing pTERT expression cassette only, primary PFCs were transfected with the SB transposon system and cloned by three cycles of dilution cloning. After one generation passage, the genomic DNA was extracted from each cell clone and analyzed for pTERT expression cassette or *ampR* gene on the vector by PCR. Among ten cell clones identified, two clones harbored the pTERT expression

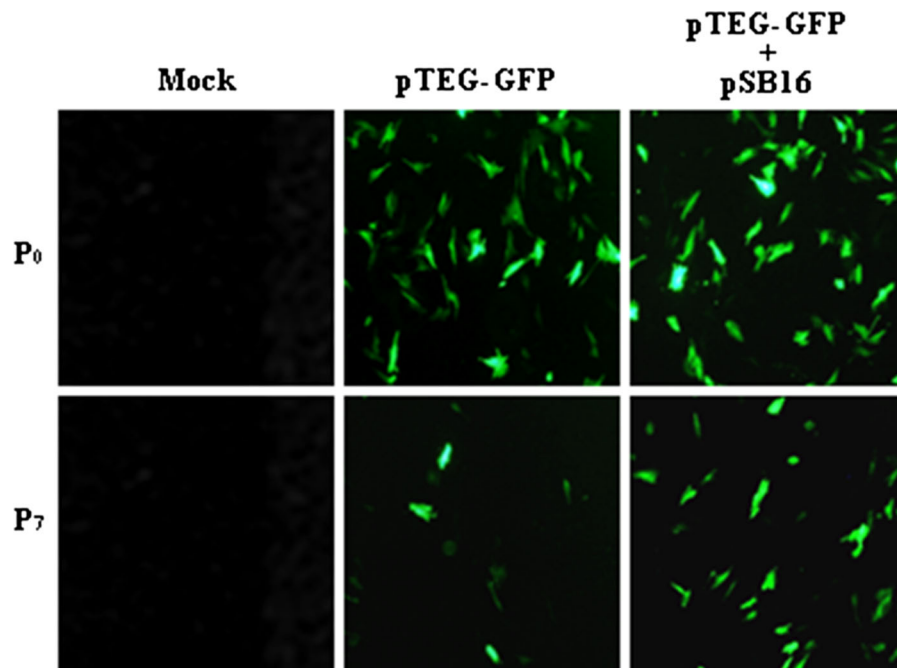


Fig. 2 Validation of the SB transposon system in pig cells. Primary PFCs were transfected with the transposon vector pTEG-GFP or co-transfected with the transposon vector pTEG-GFP and the transposase vector pSB16 which encodes SB

transposase. The cell cultures were observed under fluorescent microscope for GFP-positive cells before (P0) and after passage for 7 generations (P7)

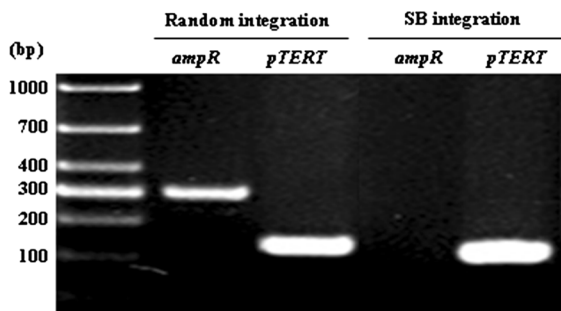


Fig. 3 Identification of cell clones by PCR. Primary PFCs were co-transfected with transposon vector pTEG-TERT and the transposase vector pSB16. After 1 generation passage, the cells were cloned by three cycles of dilution cloning and the genomic DNA was extracted from cell clones for detection of *pTERT* expression cassette or *ampR* on the transposon vector. Random integration indicates the cell clone with randomly integrated transposon vector and SB integration indicates another cell clone with transposase-integrated TERT expression cassette

cassette only (Fig. 3) and one of them was selected for generation of a PFC cell line.

Ectopic pTERT expression in PFC cell line

Two different methods were used to test the ectopic pTERT expression in PFC cell line using the primary cells as the control. First, cellular RNA was isolated at different passages and analyzed for pTERT transcription by RT-PCR. An expected 236-nt pTERT transcript segment was detected in the cell line at passage 0, 10, 20, 30 or 40 (Fig. 4a), whereas the same transcript was detected in the primary cells before passage 5 (Fig. 4b). Then, cell extracts were prepared at different passages and analyzed for telomerase activity using telomerase-inactivated PFC lysate as the negative control. An averaged 410 ng/L telomerase activity was detected in PFC cell line before passage, which was decreased slightly by passage 10 and remained at the similar level by passage 40 (Fig. 4a). In contrast, only 200 ng/L telomerase activity was detected in the primary cells before passage 5, which was undetectable by passage 10 (Fig. 4b).

Growth properties of PFC cell line

The PFC cell line kept active proliferation by passage 40, whereas the primary cells have undergone senescence as early as passage 10. Light microscopy showed that the PFC cell line at different passages

kept the fibroblast morphology of primary cells (Fig. 5a). The cell line at different passages exhibited contact inhibition in FBS-containing medium on day 5 after cultivation. Growth curve plotting showed that the PFC cell line at passage 10, 20, 30 or 40 had a growth curve similar to the primary cells, reaching the stationary phase by day 6 after plating (Fig. 5b). However, the cell numbers of PFC cell line at stationary phase were significantly higher than those of the primary cells, especially after passage 20 (Fig. 5b). The PFC cell line had a slightly shorter population doubling time and higher cloning efficiency than the primary cells, especially after passage 20 (Table 2).

Marker gene expression of PFC cell line

To confirm the identity of PFC cell line, cellular RNA was extracted and analyzed for marker gene expression using primary and PK-15 cells as the controls. As expected, the transcript for porcine mesenchymal stromal cell marker CD29 was detected in all of the three cell types tested (Fig. 6), whereas the transcript for porcine mesenchymal cell marker vimentin (Vim) was detected in PFC cell line and the primary cells (Fig. 6a, b), but not in PK-15 cells (Fig. 6c). On the other hand, the transcript for porcine epithelial cell marker CK18 was detected in PK-15 cells (Fig. 6c), but not in PFC cell line and the primary cells (Fig. 6a, b). Additionally, the transcript for porcine p53 was also detected in the three cell types (Fig. 6).

Cell cycle distribution of PFC cell line

Flow cytometry was used to analyze the cell cycle distribution of PFC line using the primary cells as the control. Histogram plots showed no significant difference in cell cycle distribution between PFC line and the primary cells under normal culture conditions (Fig. 7).

Clone formation of PFC cell line

PFC cell line was plated on soft agar plates using the primary cells and mouse myeloma cell line Sp2/0 as the controls. After incubation for 2 weeks, no cell colony was observed on the agar surface plated with the PFC cell line or primary cells. In contrast, small cell colonies were visible on Sp2/0 cell-plated agar

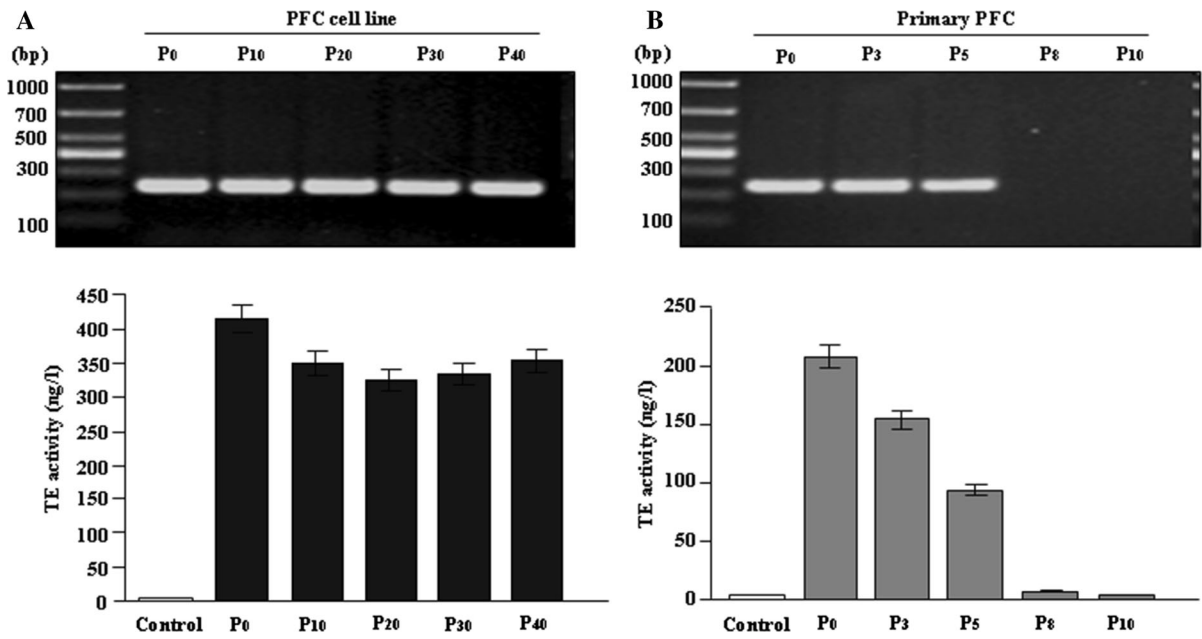


Fig. 4 Detection of ectopic pTERT expression in PFCs. Top panels, cellular RNA was extracted from PFC cell line (a) or the primary cells (b) at different passages (P) and amplified for pTERT transcript by RT-PCR. Bottom panels, cell extracts were

prepared at different passages and assayed for telomerase activity by ELISA using heat-activated cell extract as the negative control

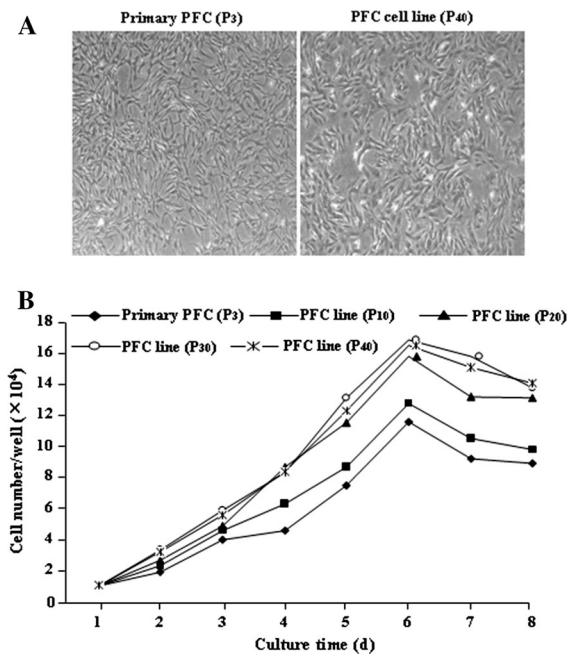


Fig. 5 Morphology and growth curve of PFC cell line. a PFC cell line and the primary cells were observed under light microscope (x45). b Cell numbers of PFC cell line or the primary cells at different passages were plotted against the culture times

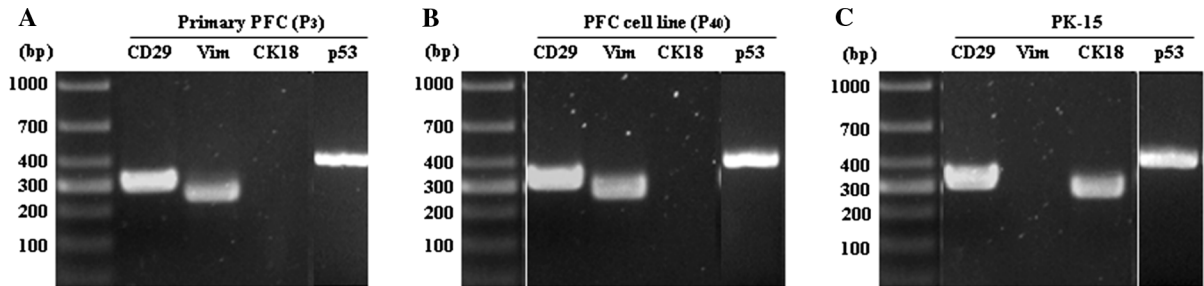
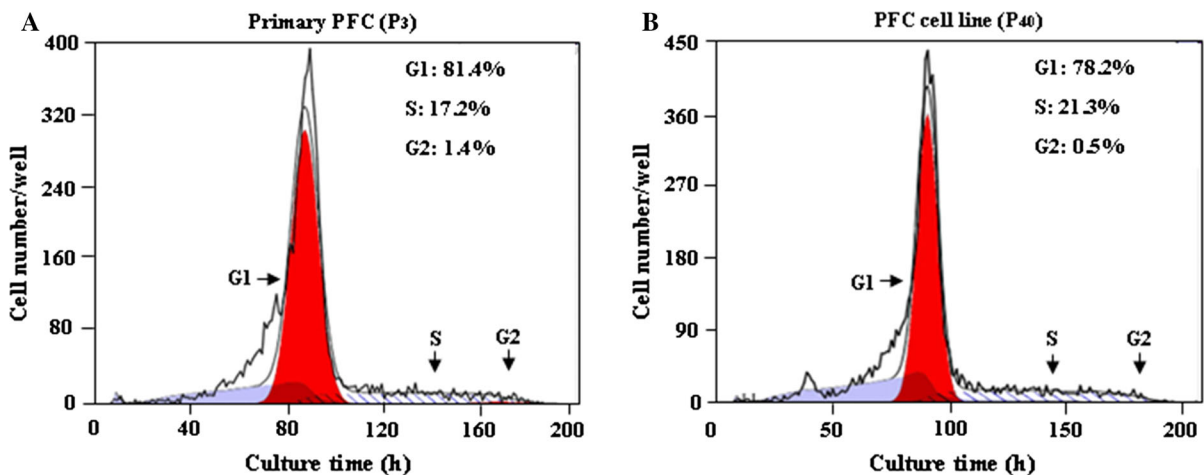
plate as early as day 6, which became much larger by day 10 after plating (Fig. 8).

Discussion

The main methods for producing genetically engineered cells use viral vectors, of which safety issues and manufacturing costs remain a concern. In addition, selection of desired cells typically relies on the use of cytotoxic drugs with long culture times (Kachervsky et al. 2012). Furthermore, the transcriptional activities of viral promoters are prone to inactivation few weeks after modification (Qin et al. 2010; Scharfmann et al. 1991), which is detrimental for generating cell lines. In recent years, the SB transposon has been emerging as the favorable gene delivery system because of its random integration profile and the lack of similar transposon-like elements in animal genomes, which significantly minimizes the risk often presented by viral-based methods (Kolacsek et al. 2011). Compared to the previous methods for generating pig cell lines, the new method established in this study had the following improvements. First, for the first time

Table 2 Population doubling times and cloning efficiencies of PFCs

Item	Primary PFC	PFC cell line			
		P10	P20	P30	P40
Doubling time (h)	40.3	40.2	37.0	35.5	35.7
Cloning efficiency (%)	6.1	7.8	10.1	10.9	10.4

**Fig. 6** Marker gene expression of PFC cell line. Cellular RNA was extracted from primary PFCs (a), PFC cell line (b) or PK-15 cells (c), and amplified for the transcript of porcine CD29, vimentin (Vim) or CK18 by RT-PCR**Fig. 7** Cell cycle distribution of PFC cell line. Primary PFCs (a) or PFC cell line (b) were grown to 80 % confluent growth and stained with propidium iodide for DNA content measurement by flow cytometry

pTERT cDNA was used as the immortalization gene of pig cells which should express a more authentic telomerase enzyme than hTERT cDNA. Second, the pEF-1 α promoter was used to drive ectopic pTERT expression and the stable expression was maintained after repeated passages. Finally, by combining the SB transposon system with dilution cell cloning, the PFC cell line generated had no antibiotic resistance gene or viral sequence which minimized the safety concern associated with plasmid- or viral-based methods.

Our pilot experiment confirmed that the SB transposon system is a highly efficient gene delivery vehicle (Izsvák et al. 2009). After passages for 7 generations, for example, much more GFP-positive cells remained in the PFC culture transfected with the transposon system than for those transfected with the transposon vector alone (Fig. 2). The same conditions were used to transfect primary PFCs with the SB transposon system. Among ten cell clones identified, however, only two contained a pTERT expression cassette and the remaining contained randomly

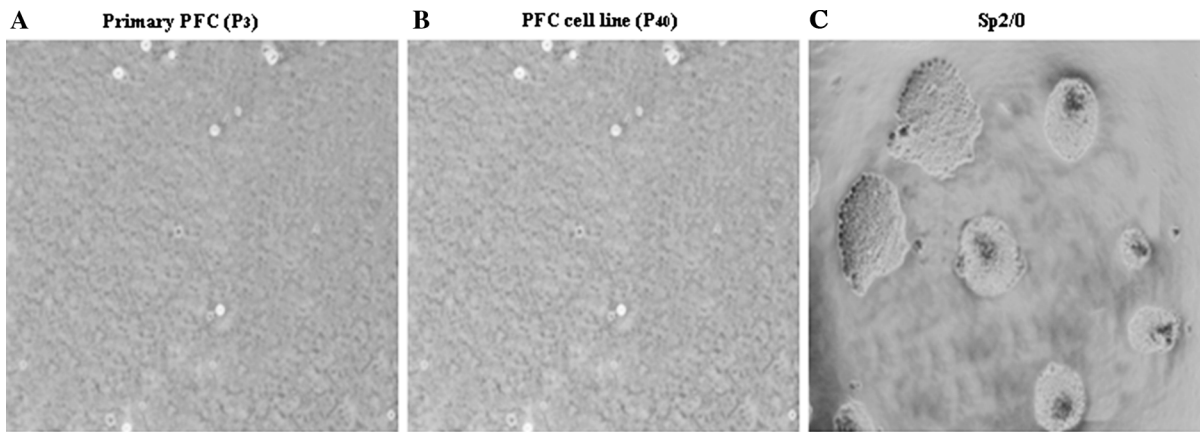


Fig. 8 Clone formation of PFC cell line. Primary PFCs (a), PFC cell line (b) or mouse myeloma cell line Sp2/0 (c) were plated on 0.7 % soft agar plates and cell colonies were observed under light microscope ($\times 25$) after incubation for 2 weeks

integrated intact vector. This could be due to the use of early time for cell cloning (after 1 passage) at which time residual free pTEG-TERT vector may be still present in some cells. Although the exact reason remains to be defined, it is worth to consideration for generation of animal cell lines without an antibiotic resistant gene and/or viral sequence.

Both RT-PCR and ELISA assays showed that the pTERT cDNA cloned from pig kidney cell line encoded a functional telomerase enzyme (Fig. 4a). Furthermore, the ectopic expression of pTERT in PFC cell line was stable during different passages, indicating the pEF-1 α promoter was not prone to inactivation by repeated passage. It should be noted that the endogenous telomerase activity was also expressed in primary PFCs before passage 5 (Fig. 4b). This may be the reason for active proliferation of primary PFCs before passage 5 and reaching senescence by passage 10. The expression of endogenous telomerase in primary pig cells has been reported previously (Gomes et al. 2011).

Our cell characterization studies showed that the pTERT-immortalized PFC cell line kept the growth properties of primary cells, including contact growth inhibition in FBS-containing medium, population doubling time and cloning efficiency (Table 2). The cell cycle analysis showed the cell cycle distribution of PFC cell line was similar to the primary cells with a limited number of apoptotic cells detected (Fig. 7). Furthermore, the growth of PFC cell line was anchorage-dependent (Fig. 8), indicating the lack of anchorage-independent growth which is one of the hallmarks of cell transformation (Akagi et al. 2003).

In some cases, hTERT alone is sufficient for immortalization of some cell types such as foreskin fibroblasts (Bodnar et al. 1998) and endothelial cells (Chang et al. 2005). However, this is not the case for other cell types such as human epithelial cells (Kiyono et al. 1998). Even fibroblasts derived from lung can not be immortalized with hTERT alone (Itahana et al. 2003), and additional inactivation of the p16INK4a/Rb pathway is required for efficient immortalization (Kiyono et al. 1998; Dickson et al. 2000; Taylor et al. 2004). More recent studies have also shown that the efficient immortalization of human cells requires the combination of p16^{INK4a} or p53 suppression with hTERT overexpression (Haga et al. 2007; Liu et al. 2013). However, contradictory evidence has also been found in other studies. For example, it has been shown that p16^{INK4a} inactivation is not required to immortalize human mammary epithelial cells. The premature growth arrest and loss of p16 in several epithelial cell types are due to inadequate growth conditions that can be overcome by the use of feeder layers (Herbert et al. 2002). A more recent study has also shown that hTERT alone can immortalize epithelial cells of renal proximal tubules without changing their functional characteristics (Wieser et al. 2008). Therefore, whether hTERT alone is sufficient to immortalize human cells is still a matter of controversy, which may be cell type- and/or culture condition-dependent (Liu et al. 2013). In this study, we characterized only one PFC cell line, which was just a tiny fraction of the number of transfected cells. Further studies are needed to clarify whether the cell line was immortalized by

pTERT alone or by a second event such as loss of p16 function.

Conclusions

The new method established herein is useful for generating pig cell lines without an antibiotic resistant gene and viral sequence. The PFC cell line generated may be more suitable for pig physiological analyses and/or vaccine development.

Acknowledgments This work was supported by R & D Special Fund (1251012923) from the Ministry of Agriculture, China, and the Priority Academic Program Development (PAPD) of Jiangsu Higher Education Institutions.

Compliance with ethical standards

Conflict of interest There is no conflict interest in this manuscript.

References

- Akagi T, Sasai K, Hanafusa H (2003) Refractory nature of normal human diploid fibroblasts with respect to oncogene-mediated transformation. *Proc Natl Acad Sci USA* 100:13567–13572
- Belay E, Dastidar S, Vanden-Driessche T, Chuah MK (2011) Transposon-mediated gene transfer into adult and induced pluripotent stem cells. *Curr Gene Ther* 11:406–413
- Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE (1998) Extension of life-span by introduction of telomerase into normal human cells. *Science* 279:349–352
- Cao H, Chu Y, Zhu H, Sun J, Pu Y, Gao Z, Yang C, Peng S, Dou Z, Hua J (2011) Characterization of immortalized mesenchymal stem cells derived from foetal porcine pancreas. *Cell Prolif* 44:19–32
- Chang MW, Grillari J, Mayrhofer C, Fortschegger K, Allmaier G, Marzban G, Katinger H, Voglauer R (2005) Comparison of early passage, senescent and hTERT immortalized endothelial cells. *Exp Cell Res* 309:121–136
- Dickson MA, Hahn WC, Ino Y, Ronfard V, Wu JY, Weinberg RA, Louis DN, Li FP, Rheinwald JG (2000) Human keratinocytes that express hTERT and also bypass a p16(INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. *Mol Cell Biol* 20:1436–1447
- Donai K, Kiyono T, Eitsuka T, Guo Y, Kuroda K, Sone H, Isogai E, Fukuda T (2014) Bovine and porcine fibroblasts can be immortalized with intact karyotype by the expression of mutant cyclin dependent kinase 4, cyclin D, and telomerase. *J Biotechnol* 176:50–57
- Freshney RI (2010) Culture of animal cells: a manual of basic technique and specialized applications, 6th edn. Wiley-Blackwell, New Jersey, pp 208–211
- Gomes NM, Ryder OA, Houck ML, Charter SJ, Walker W, Forsyth NR, Austad SN, Venditti C, Pagel M, Shay JW, Wright WE (2011) Comparative biology of mammalian telomeres: hypotheses on ancestral states and the roles of telomeres in longevity determination. *Aging Cell* 10:761–768
- Haga K, Ohno S, Yugawa T, Narisawa-Saito M, Fujita M, Sakamoto M, Galloway DA, Kiyono T (2007) Efficient immortalization of primary human cells by p16INK4a-specific short hairpin RNA or Bmi-1, combined with introduction of hTERT. *Cancer Sci* 98:147–154
- Hang H, Fox MH (2004) Analysis of the mammalian cell cycle by flow cytometry. *Methods Mol Biol* 241:23–35
- Herbert BS, Wright WE, Shay JW (2002) p16(INK4a) inactivation is not required to immortalize human mammary epithelial cells. *Oncogene* 21:7897–7900
- Hong HX, Zhang YM, Xu H, Su ZY, Sun P (2007) Immortalization of swine umbilical vein endothelial cells with human telomerase reverse transcriptase. *Mol Cells* 24:358–363
- Ibi M, Ishisaki A, Yamamoto M, Wada S, Kozakai T, Nakashima A, Iida J, Takao S, Izumi Y, Yokoyama A, Tamura M (2007) Establishment of cell lines that exhibit pluripotency from miniature swine periodontal ligaments. *Arch Oral Biol* 52:1002–1008
- Itahana K, Zou Y, Itahana Y, Martinez JL, Beausejour C, Jacobs JJ, Van Lohuizen M, Band V, Campisi J, Dimri GP (2003) Control of the replicative life span of human fibroblasts by p16 and the polycomb protein Bmi-1. *Mol Cell Biol* 23:389–401
- Izsvák Z, Chuah MK, Vandendriessche T, Ivics Z (2009) Efficient stable gene transfer into human cells by the Sleeping Beauty transposon vectors. *Methods* 49:287–297
- Jiang XR, Jimenez G, Chang E, Frolkis M, Kusler B, Sage M, Beeche M, Bodnar AG, Wahl GM, Tlsty TD, Chiu CP (1999) Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype. *Nat Genet* 21:111–114
- Kacherovsky N, Harkey MA, Blau CA, Giachelli CM, Pun SH (2012) Combination of Sleeping Beauty transposition and chemically induced dimerization selection for robust production of engineered cells. *Nucleic Acids Res* 40:e85
- Kiyono T, Foster SA, Koop JI, McDougall JK, Galloway DA, Klingelutz AJ (1998) Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. *Nature* 396:84–88
- Kolacsek O, Krízšik V, Schamberger A, Erdei Z, Apáti A, Várady G, Mátés L, Izsvák Z, Ivics Z, Sarkadi B, Orbán TI (2011) Reliable transgene-independent method for determining Sleeping Beauty transposon copy numbers. *Mob DNA* 2:5
- Kuruvilla L, Santhoshkumar TR, Kartha CC (2007) Immortalization and characterization of porcine ventricular endothelial cells. *Endothelium* 14:35–43
- Kwak S, Jung JE, Jin X, Kim SM, Kim TK, Lee JS, Lee SY, Pian X, You S, Kim H, Choi YJ (2006) Establishment of immortal swine kidney epithelial cells. *Anim Biotechnol* 17:51–58
- Liu TM, Ng WM, Tan HS, Vinitha D, Yang Z, Fan JB, Zou Y, Hui JH, Lee EH, Lim B (2013) Molecular basis of immortalization of human mesenchymal stem cells by

- combination of p53 knockdown and human telomerase reverse transcriptase overexpression. *Stem Cells Dev* 22:268–278
- Mátrai J, Chuah MK, Vanden-Driessche T (2010) Recent advances in lentiviral vector development and applications. *Mol Ther* 18:477–490
- Morales CP, Holt SE, Ouellette M, Kaur KJ, Yan Y, Wilson KS, White MA, Wright WE, Shay JW (1999) Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. *Nat Genet* 21:115–118
- Norrmann K, Fischer Y, Bonnamy B, Wolfhagen Sand F, Ravassard P, Semb H (2010) Quantitative comparison of constitutive promoters in human ES cells. *PLoS One* 5:e12413
- Onishi A, Iwamoto M, Akita T, Mikawa S, Takeda K, Awata T, Hanada H, Perry AC (2000) Pig cloning by microinjection of fetal fibroblast nuclei. *Science* 289:1188–1190
- O'Reilly M, Shipp A, Rosenthal E, Jambou R, Shih T, Montgomery M, Gargiulo L, Patterson A, Corrigan-Curay J (2012) NIH oversight of human gene transfer research involving retroviral, lentiviral, and adeno-associated virus vectors and the role of the NIH recombinant DNA advisory committee. *Methods Enzymol* 507:313–335
- Ouellette MM, McDaniel LD, Wright WE, Shay JW, Schultz RA (2000) The establishment of telomerase-immortalized cell lines representing human chromosome instability syndromes. *Hum Mol Genet* 9:403–411
- Pan X, Du W, Yu X, Sheng G, Cao H, Yu C, Lv G, Huang H, Chen Y, Li J, Li LJ (2010) Establishment and characterization of immortalized porcine hepatocytes for the study of hepatocyte xenotransplantation. *Transplant Proc* 42:1899–1906
- Qin JY, Zhang L, Clift KL, Hultur I, Xiang AP, Ren BZ, Lahn BT (2010) Systematic comparison of constitutive promoters and the doxycycline-induced promoter. *PLoS One* 5:e10611
- Sagong M, Park CK, Kim SH, Lee KK, Lee OS, du Lee S, Cha SY, Lee C (2012) Human telomerase reverse transcriptase-immortalized porcine monomyeloid cell lines for the production of porcine reproductive and respiratory syndrome virus. *J Virol Methods* 179:26–32
- Scharfmann R, Axelrod JH, Verma IM (1991) Long-term in vivo expression of retrovirus-mediated gene transfer in mouse fibroblast implants. *Proc Natl Acad Sci USA* 88:4626–4630
- Staunstrup NH, Moldt B, Mátés L, Villesen P, Jakobsen M, Ivics Z, Izsvák Z, Mikkelsen JG (2009) Hybrid lentivirus-transposon vectors with a random integration profile in human cells. *Mol Ther* 17:1205–1214
- Taylor LM, James A, Schuller CE, Brce J, Lock RB, Mackenzie KL (2004) Inactivation of p16INK4a, with retention of pRB and p53/p21cip1 function, in human MRC5 fibroblasts that overcome a telomere-independent crisis during immortalization. *J Biol Chem* 279:43634–43645
- Uebing-Czipura AU, Dawson HD, Scherba G (2008) Immortalization and characterization of lineage-restricted neuronal progenitor cells derived from the porcine olfactory bulb. *J Neurosci Methods* 170:262–276
- Wieser M, Stadler G, Jennings P, Streubel B, Pfaller W, Ambros P, Riedl C, Katinger H, Grillari J, Grillari-Voglauer R (2008) hTERT alone immortalizes epithelial cells of renal proximal tubules without changing their functional characteristics. *Am J Physiol Renal Physiol* 295:F1365–F1375