The proliferative lifespan of adult sheep fibroblasts can be conditionally extended by tetracycline-inducible expression of human telomerase reverse transcriptase

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Received: 14 June 2018 / Accepted: 20 October 2018 / Published online: 2 January 2019
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Abstract Forced expression of human telomerase reverse transcriptase (hTERT) has been used to immortalize mammalian cells. Here, we report conditional extension of proliferative lifespan of adult sheep somatic cells by introducing tetracycline-inducible (Tet-on) expression of hTERT. After transfecting adult sheep fibroblasts with the vector for Tet-on induced conditional expression of hTERT, we obtained several hTERT-positive clones that exhibited extended-lifespan under the induction of tetracycline analogue, doxycycline. Further assays for a representative cell clone A3h38 indicated that the cells had a much stronger proliferative ability than control primary cells, as assessed by population doubling levels and single-cell cloning efficiency. A3h38 cells could maintain vigorous growth in culture for more than 150 days but they became senescent when hTERT expression was abrogated by withdrawal of doxycycline. Although having undergone long-term culture, the nuclei of A3h38 cells could support higher preimplantation development of somatic cell nuclear transfer embryos than control primary cells. These results demonstrated that conditional expression of hTERT could reversibly extend the lifespan of adult

sheep fibroblasts, enhance their proliferation and maintain their ability to be reprogrammed after nuclear transfer. This strategy would also be applicable to many other somatic cell types in more species.

Keywords Human telomerase reverse transcriptase (hTERT) · Somatic cell · Somatic cell nuclear transfer (SCNT) · Sheep · Tet-on

Introduction

Animal cells have important utilities in biological research and biotechnological applications, such as cell models, tissue engineering, genetic modification and somatic cell nuclear transfer (SCNT). However, the finite lifespan of primary somatic cells constraints their wide applications in these areas. For example, the limited proliferative capacity of cultured somatic cells is considered to be a major obstacle to SCNT-assisted gene targeting in livestock, as the cells often senesce during the prolonged culture for expansion of single-cell clones with genetic modification (Denning et al. 2001). This cellular senescence is partially attributed to telomere shortening due to the lack of sufficient telomerase activity in cells (Allsopp et al. 1992). It has been demonstrated that reconstitution of telomerase activity by introducing exogenous human telomerase reverse transcriptase (hTERT) is an effective approach

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for extending the lifespan of primary somatic cells or even immortalizing the cells without causing transformation (Bodnar et al. 1998; Jiang et al. 1999; Morales et al. 1999).

hTERT-induced cellular immortalization has been applied in a variety of cell types in many species other than human (Cui et al. 2003; He et al. 2016, 2009; Hong et al. 2007; Xiang et al. 2000; Xie et al. 2013; Zhang et al. 2016b; Zhao et al. 2010). However, most of these studies used constitutive hTERT expression and the cells were thus irreversibly immortalized. To achieve a controllable extension of cellular lifespan, we previously established a reversible immortalization method by adopting a tetracycline-inducible (Tet-on) hTERT expression system (Zhang et al. 2016a). The effectiveness of this method has been confirmed in sheep fetal fibroblasts but needs to be tested in adult somatic cells.

Compared to fetal fibroblasts, adult fibroblasts are more readily available and their derivation has less animal ethical concerns. More importantly, adult fibroblasts can be used for propagation of individual animals with excellent traits and also for protection of the rare and endangered animals by virtue of SCNT. However, adult fibroblasts have much poorer proliferative capacity and lower nuclear transfer efficiency than fetal fibroblasts. If ectopic hTERT expression is also capable of immortalizing adult fibroblasts or extending their lifespan, it will provide important cellular material for genetic conservation and genetic modification of valuable animals. In this study, we attempted to extend the lifespan of adult fibroblasts from Australian Merino sheep with a superfine wool phenotype, by introducing Tet-on mediated conditional hTERT expression. This work would supply cellular materials for research and breeding of wool sheep breed, and may also provide new information for further extending the conditional hTERT expression system to more somatic cell types in more species.

Materials and methods

Cell culture and transfection

Adult sheep fibroblast cells (aSFCs) were derived from the ear skin of two 6-year-old male Australian Merino sheep numbered 6256 and 6830, respectively.

The procedure of animal experiments was in accordance with the animal care policies of China Agricultural University and was approved by the Animal Ethics Committee at the university. A small piece of ear skin tissue was cut, rinsed and minced into pieces (1 mm³). Minced pieces were digested with 0.25% trypsin in phosphate-buffered saline (PBS) for about 50 min and cells were collected by filtration and centrifugation. Cells were cultured in DMEM/F12 (Gibco, Invitrogen Corporation, Grand Island, NY, USA) supplemented with 10% (v/v) FBS (HyClone, Logan, UT, USA) at 37 °C in 5% CO₂.

After 4 passages, aSFCs were transfected by electroporation with the plasmids, pTet-on hTERT or pTet-on EGFP, as described previously (Zhang et al. 2016a). After limiting dilution, the electroporated cells were seeded in several 10-cm dishes and cultured in medium supplemented with 1 µg/ml doxycycline (Dox; Clontech, Mountain View, CA, USA). Twenty-four hours later, the cells were selected with 150 µg/ml hygromycin B (Sigma-Aldrich, St. Louis, MO, USA) for about 2 wk and hygromycin B-resistant colonies were isolated using the cloning cylinders. The isolated clones were cultured, passaged and expanded in a serial of plates or dishes.

Genomic PCR detection

Total cellular DNA was extracted from cells according to the standard procedure and the genomic integration of plasmid vector was detected by PCR. The primers (forward: 5'-CGTCAGATCGCCTGGAGAAT-3'; reverse: 5'-ACAGAAACCACGGTCACTCG-3') were designed to amplify a fragment across the P_{tight} promoter and the upstream region of hTERT gene in the plasmid pTet-on hTERT. The expected size of the amplification fragment was 870 bp.

Reverse transcription PCR (RT-PCR)

Total RNA was isolated from cells using Trizol. RT-PCR was performed using the Superscript II kit (Tiangen Biotech, Beijing, China) in accordance with the manufacturer's protocol. The specific primers used were 5'-CCGTACATGCGACAGTTC-3' (forward) and 5'-GCAGAGCAGCGTGGAGAG-3' (reverse) for hTERT, and 5'-TGATGACATCAAGAAGGTGGTGAAG-3' (forward) and 5'-TCCTTGAGGCCATGTAGGCCAT-3' (reverse) for GAPDH as internal

reference. The expected sizes for hTERT and GAPDH PCR products were 200 bp and 275 bp, respectively.

Proliferation assay

Once confluent, the cells were passaged and the total cell number was counted using a blood count plate. The cumulative population doubling levels (PDLs) of cells during growth were calculated as follows: $PDLs = \log_2(a/b) + PDL_{s0}$, where “a” is the number of cells counted in the passage, “b” represents the number of seeded cells and “PDL_{s0}” means the original population doubling levels before the passage. The cumulative number of PDLs over time was derived from these calculations.

Western blotting

Cells were lysed with lysis buffer (Beyotime, Beijing, China). Cell lysates were separated by 10% (v/v) SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were probed with primary antibodies against hTERT (Abcam, Cambridge, UK; 1:2000 dilution) or β -actin (Abcam; 1:2000 dilution), followed by incubation with secondary antibodies against rabbit or mouse IgG (HRP conjugate, Abcam; 1:2000 dilution). Membranes were treated with super ECL Plus and the western blot signals were detected by exposure on films.

Senescence-associated β -galactosidase (SA- β -gal) staining

Cells were washed in PBS and fixed in 2% formaldehyde/0.2% glutaraldehyde for 2–4 min at room temperature. Cells were incubated in SA- β -gal staining solution (Beyotime) overnight at 37 °C in accordance with the manufacturer’s protocol.

Soft agar assay

Soft agar assay was performed as described previously (Zhang et al. 2016a). A3h38(+) cells continuously cultured for 150 d were tested. Primary 6830 cells and Hela cells were used as a negative and positive control, respectively.

Focus forming assay

Cells in exponential growth phase were collected by digestion, diluted with culture medium and seeded into 100-mm dishes, with 100 cells in each dish. After cultured for 10 d, the cells were washed once in PBS and then fixed in absolute ethanol for 1 min. After that, the cells were washed twice in PBS and then incubated in 10 mM borate buffer for 10 min. Finally, the cells were stained with 1% methylene blue for 30 min to detect the number of colony forming units-fibroblastic (CFU-Fs).

SCNT

The procedure of SCNT was described previously (Fu et al. 2012). Briefly, matured oocytes were enucleated in manipulation medium. Hepes-buffered TCM199 supplemented with 10% FBS and 5 μ g/ml cytochalasin B. A donor cell was injected into the perivitelline space of enucleated oocyte and the couplets were induced to fuse on a cell fusion apparatus (BLS CF-150/B, Budapest, Hungary). Fused embryos were activated by 5 μ M ionomycin for 5 min, followed by incubation in 2 mM 6-dimethylaminopurine for 4 h. Finally, the cloned embryos were cultured in synthetic oviduct fluid (SOF) medium supplemented with 8 mg BSA/ml, 2% (v/v) essential amino acids and 1% (v/v) non-essential amino acids, at 38.5 °C in 5% CO₂, 7% O₂ and 88% N₂. Cleavage and blastocyst development were recorded 2 d and 8 d after culture, respectively. The total cell number in blastocysts was counted by staining the nuclei of embryos with 5 μ g/ml 4, 6-diamidino-2-phenylindole (DAPI).

Statistical analysis

Data were statistically analyzed using GraphPad Prism 5 Software. Student’s *t* test was used to compare the number of CFU-Fs and the passage number between A3h38(+) and primary 6830 cells. The comparison of embryonic development between the two groups was made using one-way ANOVA. Difference at *p* < 0.05 was considered significant.

Results and discussion

Selection and characterization of hTERT-positive clones

aSFCs were transfected with pTet-on hTERT or pTet-on EGFP (as a control) vectors and cultured in Dox-supplemented medium to induce the expression of hTERT and hygromycin B resistant gene. Single-cell clones were isolated after hygromycin selection for 2 wk and subjected to a serial expanding culture as illustrated in Fig. 1a. The number of isolated clones in each case and the survival of the clones are shown in Table 1. A total of 56 clones transfected with pTet-on hTERT were isolated, of which 16 clones were able to extend in subsequent culture after isolation, with a survival probability of 28.6%. While, under the same conditions, a total of 50 clones were obtained from transfection with pTet-on EGFP, of which only 5 clones survived in the extending culture with a

Table 1 The survival rate of isolated clones

Plasmids	pTet-on hTERT			pTet-on EGFP		
	6830	6256	Total	6830	6256	Total
Cells						
Isolated (N) ^a	38	18	56	32	18	50
Survival (N) ^b	14	2	16	4	1	5
Probability (%) ^c			28.6			10

^aThe number of isolated clones in each case; ^bThe number of survived clones that were able to expand to 60-mm dishes after serial culture; ^c% total survival/total isolated

probability of 10% (Table 1). The higher survival rate of clones derived from pTet-on hTERT-transfected cells compared to those from pTet-on EGFP transfection (28.6% vs 10%) suggested that ectopic expression of hTERT at early cell growth stages might have improved the proliferation of cells and thus rescued some clones from suffering senescence during expanding culture.

Fig. 1 Selection and characterization of hTERT-positive cell clones. **a** Diagrammatic illustration for the expanding culture of single-cell clones. The clones were isolated and propagated in a serial of plates or dishes (one clone in one well). **b** PCR detection of the genomic integration of hTERT gene in cells. “6256” and “6830” denote genomic DNA from the non-transfected primary 6256 cells and 6830 cells, respectively, and serve as negative controls. “P” denotes the plasmid pTet-on hTERT as a positive control. **c** RT-PCR analysis of the hTERT expression. “6256” and “6830” denote mRNA from the non-transfected primary 6256 cells and 6830 cells, respectively, serving as negative controls. “HeLa” denotes mRNA from

HeLa cells as a positive control. h, hTERT clones. E, EGFP clones. **d** Growth curve of cells from hTERT-positive clones and EGFP control clones. The population doubling levels (PDLs) of 7 clones were evaluated at every passage during continuous culture for around 70 d when EGFP clones stopped proliferation. Alh8 (+) and Alh10 (+) are the number 1 and 2 clones, respectively, from 6256-hTERT group; A2h3 (+) and A3h38 (+) are the number 3 and 14 clones, respectively, from 6830-hTERT group. AIE14 is an EGFP clone from 6256 cells, while A2E19 and A2E22 are the number 2 and 4 clones, respectively, from 6830-EGFP group

PCR detection indicated that 9 of 14 clones derived from pTet-on hTERT-transfected 6830 cells and both of the 2 clones derived from pTet-on hTERT-transfected 6256 cells had integrated the hTERT gene in their genomes (Fig. 1b). As the hygromycin B resistant gene and hTERT gene were controlled by the same promoter in our pTet-on hTERT plasmids, clones survived the hygromycin selection were expected to be all hTERT-positive. However, there were 5 of 16 survived clones in the pTet-on hTERT group having no hTERT integration (Fig. 1b). These false-positive clones might result from the high inoculation density of cells or incomplete integration of the plasmid sequence. Nevertheless, RT-PCR showed that almost all of the hTERT-positive clones, except the number 6 in 6830 group, expressed hTERT significantly (Fig. 1c), proving that the hTERT expression was efficiently induced by Dox.

After identification of integration and expression of hTERT gene, 4 hTERT-positive clones and 3 EGFP group clones were selected randomly. All of these 7 clones were maintained in culture for around 70 d. We monitored the proliferation of clones and evaluated their proliferative capacity by estimating the PDLs. As shown in Fig. 1d, the 4 hTERT-positive clones could continuously proliferate and displayed vigorous growth throughout the test period. In contrast, the growth rate of the 3 EGFP-transfected clones gradually decreased beyond 60 d and all of them ceased division ultimately. These results indicated that the proliferative lifespan of adult sheep fibroblasts had been greatly extended by induced hTERT expression.

The extended lifespan of hTERT clones could be reversibly regulated

In the design of pTet-on hTERT plasmid, we introduced the Tet-on system to achieve conditional hTERT expression (Zhang et al. 2016a). Therefore, in theory, the expression of hTERT can be controlled in hTERT-positive clones, which allows the proliferative lifespan of cells to be reversed when Dox is withdrawn. In order to verify this, we chose a representative hTERT clone A3h38 and subdivided it into two groups. One group was remained in Dox-supplemented medium referred to A3h38(+) and the other group was cultured in medium without Dox referred to A3h38(−). A2E19, a clone from EGFP group, was cultured as a control.

When the cells were cultured for around 30 d after grouping, we detected the mRNA and protein of hTERT in these cells. As shown in Fig. 2a, b, the hTERT expression was maintained at high levels in A3h38(+) cells, but it decreased dramatically in A3h38(−) cells, confirming that the expression of hTERT was tightly controlled by Dox. After grouping, A3h38(+), A3h38(−) and A2E19 cells were passaged serially and their proliferation were monitored (Fig. 2c). A2E19 cells tended to growth arrest and eventually died after 50 d of continuous culture. When cultured for 80 d, A3h38(−) cells exhibited a slower growth and suffered growth arrest around 110 d. By contrast, A3h38(+) cells maintained vigorous growth throughout the 150 d of continuous culture. A3h38(+) cells cultured for 120 d exhibited no signs of senescence when detected by SA- β -gal staining, and morphologically, they maintained normal fibroblast shape; while, A3h38(−) cells cultured for 120 d displayed a typical morphology of senescence when stained for SA- β -gal (Fig. 2d). These results demonstrated that the extended lifespan of cells is controllable; the cells became senescence when the hTERT expression was turned off by withdrawal of Dox. We also found that the lifespan-extended cells showed no transformed phenotypes as assessed by soft agar assay (Fig. 2e).

Above results are consistent with our previous study on fetal fibroblasts showing that the lifespan of cells could be reversibly regulated by Tet-on mediated conditional hTERT expression (Zhang et al. 2016a). However, we noted that the growth of the lifespan-extended adult sheep fibroblasts was much slower than that of the immortalized fetal sheep fibroblasts established in our previous study (Zhang et al. 2016a). In the present study, the average PDLs of hTERT-positive adult cell clones cultured for 60 d was 30, compared with an average of 70 PDLs for hTERT-positive fetal cell clones in the previous study (Zhang et al. 2016a). Also, the PDLs of A3h38(+), the representative clone of adult fibroblasts, was 100, while for h2(+), a representative clone of fetal fibroblasts (Zhang et al. 2016a), was 180, when both of them were cultured continuously for 150 d. In a word, adult fibroblasts had an approximately twice longer doubling time than fetal fibroblasts even though they were both induced by hTERT. These data seem to suggest that it might be more difficult to immortalize adult fibroblasts than fetal fibroblasts by ectopic

Fig. 2 The lifespan of hTERT clone could be reversibly regulated. **a** RT-PCR analysis of the hTERT expression. GAPDH was used as an internal control. **b** Western blotting analysis of hTERT protein. β -actin was used as an internal control. **c** Growth curves of cells. The PDLs were evaluated at every passage for the whole test period. **d** Senescence-associated β -galactosidase (SA- β -gal) staining detection of senescence. The senescent cells were dyed blue. Scale bar, 50 μ m. A3h38(+) means the cells that were continuously induced by

doxycycline, while A3h38(-) represents the cells deprived of doxycycline induction. A2E19 cells or Primary 6830 cells served as negative controls, and HeLa cells were used as a positive control. **e** Soft agar assay. HeLa cells formed colonies after grown in soft agar media for 2 weeks (left). A representative colony indicated by white arrow was magnified ten-fold and is shown in the lower right corner. A3h38(+) cells cultured for 150 d continuously failed to form any colonies in soft agar (right). Scale bar, 200 μ m. (Color figure online)

expression of hTERT. We did not track the proliferation of hTERT-positive adult cell clones beyond 150 d of culture and thus could not confirm whether ectopic expression of hTERT in adult sheep fibroblasts would result in an absolute immortalization, however, the current assays indicated that the lifespan of adult sheep cells was indeed greatly extended by hTERT.

Lifespan-extended cells had high single-cell cloning efficiency

One of major utilities of the lifespan-extended cells is to be used in genetic modification in animals, for which there usually needs a selection and propagation of single-cell clones. Therefore, we detected the growth and proliferation ability of A3h38(+) cells under the condition of extremely low inoculation density, namely the single-cell cloning efficiency.

A3h38(+) cells continuously cultured for 136 d were inoculated in five 10-cm dishes, with 100 cells in each dish. Primary 6830 cells were subjected to the

same treatment as a control. After 10 d of culture, 3 of 5 dishes were stained with 1% methylene blue. Colony forming units-fibroblastic (CFU-Fs) was dyed blue and the number of CFU-Fs in each dish was counted by visualizing (Fig. 3a). The statistical analysis showed that the single-cell colony formation ability of A3h38(+) cells was very significantly stronger than that of primary 6830 cells (Fig. 3b).

The two additional dishes for A3h38(+) or control 6830 cells were used to test the proliferation ability of the single-cell clones. A total of 20 CFU-Fs from either A3h38(+) or 6830 groups were picked and seeded into 48-well plates (one clone in one well). The clones were subsequently passaged and propagated in serial as described in Fig. 1a. The survival rates of clones during the expanding culture are shown in Fig. 3c. A total of 16 clones from A3h38(+) cells were able to expand to 60-mm dishes, while only 3 clones derived from primary 6830 cells had such ability, indicating that the propagative generations of A3h38(+) clones were much higher than that of the

Fig. 3 The single-cell cloning efficiency of the lifespan-extended cells.
a Examination of colony forming units-fibroblastic (CFU-Fs) by methylene blue staining. The blue dots were colonies stained with methylene blue. **b** Statistical analysis of CFU-Fs number. The data are shown as mean $\pm$ SEM. ***Represents significant difference between two groups ($p < 0.001$). **c** The numbers of survived single-cell clones during 16 d of expanding culture in serials of plates and dishes. **d** The morphology of cells from representative clones after 16 d of subculture. Scale bar, 50 μ m. (Color figure online)

control 6830 clones (Fig. 3c). When cultured for 16 d, A3h38(+)-derived clones maintained a normal morphology, whereas the cell morphology of 6830 clones became much worse (Fig. 3d). Therefore, we concluded that the expansion and proliferative ability of A3h38(+)-derived single-cell clones was much stronger than that of the control 6830 clones when they were cultivated initially at extremely low densities. The performance of lifespan-extended cells capable of undergoing a new round of single cell propagation would be particularly useful for genetic modification or other relevant applications.

Lifespan-extended cells supported higher preimplantation development of SCNT embryos

When somatic cells transfected with foreign genes undergo longer drug screening and expanded culture, even if the cells are still alive, some features of cells may have changed, which may impair the development of cloned embryos after SCNT. In order to test the development of SCNT embryos produced from long-term cultured cells, A3h38(+) cells that had been continuously cultured for more than 80 d were used as donor cells to generate SCNT embryos. The primary

6830 cells, as control nuclear donors, were also used for SCNT.

As shown in Table 2, the blastocyst percentage of SCNT embryos derived from A3h38(+) cells was significantly higher than that of the embryos produced from 6830 cells. This result indicated that the extended culture of A3h38(+) cells did not compromise their ability to support the early development of embryos after SCNT. Previous studies suggested that long-term cultured primary donor cells yielded lower blastocyst formation, probably due to the senescence of cells (Lan et al. 2006; Liu et al. 2016). Although the lifespan of cells could be extended by simian virus 40 large T antigen (SV40LT), the SCNT embryos derived from those cells exhibited a very restricted development (Eun et al. 2017). In the present study, the enhanced blastocyst development of A3h38(+)-derived cloned embryos implied that extended lifespan of cells induced by hTERT may have a beneficial role on the SCNT embryo development. This potency needs to be tested in more clones. Previous study showed that hTERT-immortalized sheep fetal fibroblasts could be reprogrammed by SCNT to support early development, but constitutive expression of hTERT might hamper the later fetal development of SCNT embryos (Cui et al. 2003). By contrast, our conditional hTERT

Table 2 Number and mean $\pm$ SEM percentage of in vitro development of sheep SCNT embryos derived from A3h38 and 6830 cells

Donor cells in blastocyst	No. of reconstructed oocytes	No. of fused (%)	No. of cleaved (%)	No. of blastocysts (%)	Total cell number
A3h38	167	128 (77.0 $\pm$ 1.2)	104 (80.3 $\pm$ 3.5)	20 (19.2 $\pm$ 0.6) ^a	89.3 $\pm$ 4.2
6830	155	123 (79.6 $\pm$ 4.0)	97 (77.2 $\pm$ 4.7)	12 (12.3 $\pm$ 1.0) ^b	77.9 $\pm$ 8.0

A3h38, a representative hTERT-positive clone. 6830, primary cells as a control

Fusion percentage: No. of fused/No. of reconstituted embryos; Cleavage percentage: No. of cleaved/No. of fused; Blastocyst percentage: No. of blastocyst/No. of cleaved

^a, ^bRepresent significant difference between the two groups

expression strategy would be a possible solution to such problem, as the hTERT expression could be terminated in the absence of the inducer Dox. Future investigation is needed to determine the in vivo development of SCNT embryos derived from the lifespan-extended somatic cells that we have established.

Conclusions

Our results showed that the lifespan of adult sheep fibroblasts could be greatly extended by introducing hTERT. The cells exhibited strong proliferative capacity, particularly with the competence of growing as single-cell clones. The lifespan of cells could be reversely controlled by Dox-induced conditional hTERT expression and the nuclei of cells were able to support early embryonic development after SCNT. The established adult fibroblast cell line with extended-lifespan is expected to be a good cell material for genetic conversation and genetic modification in animals and this strategy would also be applicable to other cell types for relevant researches and biotechnological applications.

Acknowledgements This work was supported by the National Natural Science Foundation of China (Grant No. 31072030) and China Agriculture Research System (Grant No. CARS-39-04).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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