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RESEARCH ARTICLE

Assessment of tumourigenic potential in long-term cryopreserved human adipose-derived stem cells

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Abstract

Cryopreservation represents an efficient way to preserve human mesenchymal stem cells (hMSCs) at early culture/passage, and allows pooling of cells to achieve sufficient cells required for off-the-shelf use in clinical applications, e.g. cell-based therapies and regenerative medicine. To fully apply cryopreserved hMSCs in a clinical setting, it is necessary to evaluate their biosafety, e.g. chromosomal abnormality and tumourigenic potential. To date, many studies have demonstrated that cryopreserved hMSCs display no chromosomal abnormalities. However, the tumourigenic potential of cryopreserved hMSCs has not yet been evaluated. In the present study, we cryopreserved human adipose-derived mesenchymal stem cells (hASCs) for 3 months, using a slow freezing method with various cryoprotective agents (CPAs), followed by assessment of the tumourigenic potential of the cryopreserved hASCs after thawing and subculture. We found that long-term cryopreserved hASCs maintained normal levels of the tumour suppressor markers p53, p21, p16 and pRb, hTERT, telomerase activity and telomere length. Further, we did not observe significant DNA damage or signs of *p53* mutation in cryopreserved hASCs. Our findings suggest that long-term cryopreserved hASCs are at low risk of tumourigenesis. These findings aid in establishing the biosafety profile of cryopreserved hASCs, and thus establishing low hazardous risk perception with the use of long-term cryopreserved hASCs for future clinical applications. Copyright © 2016 John Wiley & Sons, Ltd.

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Keywords **long-term cryopreservation; human adipose-derived stem cells; tumourigenic potential assessment; low risk; biosafety; clinical applications**

1. Introduction

Human mesenchymal stem cells (hMSCs) hold great promise in many clinical applications, e.g. cell-based therapies and regenerative medicine, due to their immunomodulation and multilineage differentiation ability (Doulatov and Daley, 2013; Nauta and Fibbe, 2007). For instance, hMSCs have been successfully applied in clinical trials for graft-versus-host disease (GvHD) treatment and articular cartilage regeneration (Jo *et al*., 2014; Le Blanc *et al*., 2008). To fully apply hMSCs in a clinical setting, it is necessary to know the biosafety of these stem cells, e.g. chromosomal abnormalities and tumourigenic potential, in addition to their therapeutic efficacy (Fink, 2009; Goldring *et al*., 2011).

In general, hMSCs need to be expanded *in vitro* for a long duration to obtain sufficient cells required for clinical applications. During cell expansion, hMSCs may be at risk of chromosomal aberrations and tumourigenesis

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(Roemeling-van Rhijn *et al*., 2013; Rosland *et al*., 2009) and thus may raise safety issues in clinical application (Sharpe *et al*., 2012). For instance, it has been reported that hMSCs showed chromosomal aberrations and spontaneous malignant transformation after culture beyond 10 passages (Froelich *et al*., 2013; Roemeling-van Rhijn *et al*., 2013; Rosland *et al*., 2009), which may have been due to stressful culture conditions, e.g. long-term culture and frequent enzymatic cell dissociation, and potential contamination with an external cell source, such as tumour cells (Barkholt *et al*., 2013; de la Fuente *et al*., 2010). These concerns restrict the wide use of long-term cultured hMSCs for clinical applications. In fact, hMSCs at early passage (within passage 4) are safe for clinical use (Barkholt *et al*., 2013). Therefore, hMSCs at early passage should be preserved and stored in the long term for off-the-shelf clinical use. To date, cryopreservation, an efficient method of preserving the viability and functional properties (e.g. proliferation and differentiation potential) of hMSCs in the long term (Gonda *et al*., 2008; Yong *et al*., 2015), is an alternative method that allows pooling of hMSCs to obtain sufficient cells required for clinical applications. However, the biosafety profile of cryopreserved hMSCs has not yet been established.

Recently, biosafety concerns relating to cryopreserved cells have been raised. For instance, it has been reported that using dimethylsulphoxide (DMSO) as a CPA may modify chromosome stability and cause changes in the telomere length of cells, including embryonic stem cells, which may in turn lead to tumour formation (Diaferia *et al*., 2008; Jenkins *et al*., 2012; Yong *et al*., 2015). These concerns have established the need for assessments of chromosomal abnormality and the tumourigenic potential of cryopreserved hMSCs prior to clinical application. To date, chromosomal abnormality has been evaluated in cryopreserved hMSCs via karyotyping, which reported no alteration in chromosome numbers and structures in hMSCs (Angelo *et al*., 2012; de Lima Prata *et al*., 2012; Luetzkendorf *et al*., 2015; Miranda-Sayago *et al*., 2012). However, the tumourigenic potential of long-term cryopreserved hMSCs has not been evaluated.

In this study we evaluated the effects of cryopreservation on the tumourigenic potential of human adipose-derived mesenchymal stem cells (hASCs), in terms of expression of tumour suppressor markers and hTERT, changes in telomerase activity and telomere length, as well as analysis of *p53* mutations and DNA damage. The findings from this study would impact the establishment of the therapeutic use of long-term cryopreserved hASCs in the future.

2. Materials and methods

2.1. hASCs isolation and culture

This study was carried out in accordance with the guidelines and experimental protocols approved by the Medical Ethics Committee of the University of Malaya Medical Centre (UMMC). Human adipose tissues were collected from six different female donors aged 25–35 years undergoing Caesarean section, with prior informed written consent. All experimental protocols were approved by the Medical Ethics Committee of UMMC (Reference No. 996.46). Isolation of hASCs was performed using methods previously described (Choi *et al*., 2014; Yong *et al*., 2015). hASCs were cultured in a medium composed of Dulbecco's modified Eagle's medium (DMEM)/Ham F-12, 10% fetal bovine serum (FBS), 1% antibiotic–antimycotic, 1% glutamax (Gibco, New York, USA) and 1% vitamin C (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C and 5% CO_2.

2.2. Cryopreservation

About 1×10^6 hASCs at passage 2 suspended in each of the generally used CPAs were loaded into a cryovial. We tested six generally used CPAs, including: 1, 0.25 M trehalose; 2, 5% DMSO; 3, 10% DMSO; 4, 5% DMSO + 20% FBS; 5, 10% DMSO + 20% FBS; and 6, 10% DMSO + 90% FBS. All cryovials were first stored at –80 °C overnight and then transferred to liquid nitrogen (–196 °C) the next day. After 3 months of cryopreservation, the frozen hASCs were thawed rapidly in a water bath at 37 °C and subcultured to passage 3 prior to tumourigenic potential assessment. Population doubling (PD) and population doubling time (PDT) were calculated using the formulae $PD = \log_{10}[(a)/(b)]/\log_{10} 2$ and $PDT = t/PD$, where a represents the number of cells harvested, b represents the number of cells plated and t represents the time between plating at the previous passage and harvest at the subsequent passage (Luetzkendorf *et al*., 2015). The PD level which represents the sum of PDs from passage 1 to passage 3, was calculated. Cryopreserved and fresh hASCs (non-cryopreserved hASCs at passage 3) in this study have been characterized previously (Yong *et al*., 2015), based on the minimal criteria of hMSCs (Choi *et al*., 2014; Dominici *et al*., 2006).

2.3. RNA extraction, cDNA synthesis and real-time polymerase chain reaction (real-time PCR)

Extraction of RNA was performed using TRI reagent (Ambion, Austin, TX, USA), followed by phase separation with chloroform (Fisher Scientific) and subsequent to precipitation of RNA with isopropanol (Sigma). A high capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, USA) was used to convert RNA to cDNA. Gene expression analyses were conducted using TaqMan gene expression assays (Applied Biosystems) and a real-time PCR system (StepOnePlus, Applied Biosystems). The genes included tumour suppressor markers, such as *TP53/p53* (Hs00153349_m1), *CDKN1A/p21* (Hs00355782_m1), *CDKN2A/p16* (Hs00923894_ml), *RB1/pRb* (Hs01078066_ml)

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and *hTERT* (Hs00972656_ml); the housekeeping gene used for normalization was *GAPDH* (Hs99999905_m1). The gene expression level of the control group (fresh hASCs) was normalized to 1. Data were expressed as fold change in gene expression relative to the control.

2.4. Telomerase assay

The telomerase activity of hASCs was determined using the TeloTAGGG PCR ELISA PLUS kit (Roche Applied Science, Indianapolis, IN, USA), based on a telomere repeat amplification protocol (TRAP) according to the manufacturer's recommendations, and relative telomerase activity was calculated using the formula provided by the manufacturer.

2.5. Telomere length analysis

Genomic DNA was extracted from hASCs using the PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, USA), followed by telomere length analysis, which was performed using TeloTAGGG Telomere Length Assay kit (Roche), according to the manufacturer's instructions. Chemiluminescence produced in this assay was detected using a gel documentation imaging system (Vilber Lourmat, Marne la Vallee, France). By comparing the signal relative to a molecular weight standard, the terminal restriction fragment (TRF) length was determined.

2.6. *p53* nucleotide sequence mutation detection

Initially, genomic DNA of hASCs was extracted using PureLink Genomic DNA Mini Kit (Invitrogen). PCR was conducted using Platinum® Taq DNA Polymerase (Invitrogen) according to the manufacturer's recommendations. The details of p53 primers used are described in Table 1. The PCR profile was 94 °C for 3 min (initial denaturation), followed by 35 cycles of 94 °C for 30 s (denaturation), 55 °C for 30 s (annealing) and 72 °C for 1 min (extension). The PCR products were subjected to 4% gel agarose electrophoresis for 45 min, followed by purification using Purelink® PCR Purification Kit (Invitrogen). The purified PCR products were cloned using TOPcloner™ TA core kit (Enzynomics, Daejeon, Korea). The vectors were transformed into DH5α chemically competent *Escherichia coli* (Enzynomics) and then inoculated on LB agar (Sigma) premixed with 50 mg/ml ampicillin (Sigma), 500 mM IPTG (Corning Cellgro, Manassas, USA) and 20 mg/ml X-gal (Corning Cellgro). After being kept overnight, positive clones (white colonies) were picked and inoculated in LB broth (Sigma) premixed with 100 μg/ml ampicillin, followed by incubation overnight at 37 °C. The positive clones were confirmed with PCR, using specific primers for *p53* exons (Table 1), followed by plasmid DNA extraction using Purelink® Quick Plasmid Miniprep Kit (Invitrogen). Finally, the purified plasmid DNA containing PCR product was sequenced. To detect mutation in the *p53* nucleotide sequence, the sequences of plasmid DNA were aligned with the wild-type *p53* DNA sequence, using Basic Local Alignment Search Tool (BLAST) software.

2.7. Comet assay

A Comet assay was carried out in alkaline conditions using Comet Assay Kit (Trevigen, Gaithersburg, MD, USA), according to the manufacturer's instructions. DNA of hASCs was eventually stained with ethidium bromide (Invitrogen) and visualized using a fluorescence microscope. The images were then captured by a digital camera connected to an inverted fluorescence microscope (Nikon ECLIPSE TI-S, Tokyo, Japan). Open Comet software (Gyori *et al*., 2014) was used to analyse 100 nuclei for each sample. The parameters tested include tail length, percentage tail DNA and tail moment.

2.8. Statistical analysis

Statistical analysis was conducted using one-way ANOVA and a *post hoc* Tukey test to compare data among fresh and cryopreserved hASC groups. Each datum was expressed as mean ± standard error of the mean (SE). Statistical significance was accepted at $p < 0.05$. SPSS 18.0 software was used to perform all data analyses.

Table 1. *p53* primer sequences, primer annealing positions and expected length of PCR products

p53 primer	Sequence*	Annealing position	Product length (bp)
Exon 5 forward	5′-gctgccgtgttccagttgct-3′	12980–12999	294
Exon 5 reverse	5′-ccagccctgtcgtctctcca-3′	13254–13273	
Exon 6 forward	5′-ggcctctgattcctcactga-3′	13290–13309	199
Exon 6 reverse	5′-gccactgacaaccaccctta-3′	13469–13488	
Exon 7 forward	5′-tgccacaggtctccccaagg-3′	13943–13962	196
Exon 7 reverse	5′-agtgtgcagggtggcaagtg-3′	14119–14138	
Exon 8 forward	5′-ccttactgcctcttgcttct-3′	14413–14432	225
Exon 8 reverse	5′-ataactgcacccttggtctc-3′	14618–14637	

*European Molecular Biology Laboratory (EMBL) Accession No. X54156.1 (*p53* gene sequence).

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3. Results and discussion

Among MSCs derived from various sources in the human body, hASCs have attracted special attention because of the abundance and ready accessibility of adipose tissues (Kolle *et al*., 2013; Mizuno *et al*., 2012). For instance, the frequency of hMSCs that can be isolated from the stromal vascular fraction of adipose tissues (2%) is much higher than that from bone marrow tissues (0.01%) (Strem and Hedrick, 2005). In the present study, hASCs (passage 3) at a PD level of 5–7 were analysed. hASCs were cryopreserved using a slow freezing/cooling method for 3 months with various well-known CPAs, including one intracellular compound (DMSO) and two extracellular agents (trehalose and FBS). Among various CPAs, DMSO is the most widely used for cell cryopreservation (Berz *et al*., 2007), while FBS is routinely added to cryopreservation media as a source of nutrients and for prevention of loss of cell viability during thawing (Gonda *et al*., 2008; Jochems *et al*., 2002). In this study, 10% DMSO + 90% FBS acted as a standard CPA; this has been used to preserve many types of cells in large volume and maintain high cell viability (Li and Ma, 2012; Zeisberger *et al*., 2011). However, the clinical use of cells preserved with 10% DMSO have caused many adverse effects, such as neurotoxicity and respiratory depression (Benekli *et al*., 2000; Windrum and Morris, 2003). To reduce the risk of such adverse effects, the concentration of DMSO in the cryomedium is reduced to 5% for the preservation of clinically used cells (Windrum *et al*., 2005; Yong *et al*., 2015). On the other hand, regulatory guidelines aim to minimize or avoid the use of FBS as part of cryopreservation media (Balci and Can, 2013) to reduce the risk of xenogeneic immune response to recipients after implantation of cryopreserved cells. Therefore, the concentration of FBS in the cryomedium is reduced to 20% (a general concentration of FBS used for cryopreservation of hMSCs) (Liu *et al*., 2008, 2011; Zhang *et al*., 2011) or 0% (xeno-free cryomedium). Meanwhile, trehalose, a non-permeating or extracellular CPA, at a concentration of 0.25 M, was effective in preserving viability and functional properties of adipocytes from human adipose tissues (Pu *et al*., 2005), suggesting its potential as an alternative to DMSO and FBS to preserve hASCs isolated from human adipose tissues.

Our previous findings showed that these CPAs can maintain the phenotype and functional properties of hASCs. hASCs preserved in 5% DMSO without FBS displayed a viability rate similar to those preserved in 10% + 90% FBS (standard CPA), and higher than those preserved in 0.25 M trehalose. These results indicate that a reduced concentration of DMSO in cryomedium with the exclusion of FBS is also efficient in maintaining a high viability rate of hASCs. Taken together, 5% DMSO without FBS is an ideal CPA for the cryopreservation of hASCs, as it maintains a high rate of cell viability and induces a low risk of xenogeneic immune response. Further, it is less cytotoxic than 10% DMSO, reducing the risk of adverse effects in recipients after implantation of cryopreserved hASCs (Windrum *et al*., 2005; Yong *et al*., 2015). To date, the tumourigenic potential of hASCs preserved in these six groups of CPAs is unknown.

We are aware that tumourigenic transformation of cells may not happen immediately but requires a multi-step process (e.g. telomere dysfunction, chromosomal instability and inactivation of tumour suppressors) (Deng *et al*., 2008; Hackett and Greider, 2002). However, it has been shown that data collected at our time point is useful to evaluate the risk of tumourigenesis in cryopreserved cells (de Lima Prata *et al*., 2012; Jenkins *et al*., 2012; Luetzkendorf *et al*., 2015). For instance, it has been reported that the telomere length of lymphocytes appeared to be significantly reduced immediately after cryopreservation (Jenkins *et al*., 2012), which might increase the risk of chromosomal instability that could potentially lead to tumourigenesis. Therefore, in the present study, observing the early changes in terms of tumour suppressor expression, telomere length, telomerase activity and DNA damage of hASCs in response to cryopreservation is significant to evaluate their risk for tumourigenesis.

3.1. Effects of cryopreservation on tumour suppressor markers and hTERT expression of hASCs

Tumour suppressor markers (including *p53*, *p21*, *p16* and *pRb*) play an important role in suppressing uncontrolled cell proliferation, one of the hallmarks of human cancer (Hanahan and Weinberg, 2011), by regulating cell cycle and cellular senescence via two main tumour suppressor pathways, i.e.the *p53–p21–pRb* and *p16–pRb* pathways (Pelicci, 2004). To evaluate tumour suppressor activity in the cryopreserved hASCs, we determined the expression levels of the tumour suppressors *p16*, *p21*, *p53* and *pRb* using the real-time PCR method. We found that hASCs preserved in various CPAs expressed similar levels of the tumour suppressor markers *p53*, *p21*, *p16* and *pRb* compared to those of fresh hASCs (Figure 1a), suggesting that there is no sign of an uncontrolled proliferation rate. This result was supported by our previous study (Yong *et al*., 2015), in which we observed a similar proliferation rate in cryopreserved and fresh hASCs. In addition, we also determined the PDT of fresh hASCs and hASCs preserved in various CPAs. We found that there was no significant difference ($p > 0.05$) in terms of PDT among fresh and cryopreserved hASCs (see supporting information, Figure S1), suggesting that cryopreserved and fresh hASCs have a similar proliferation rate. Generally, upon encounter with oncogenic stimuli which induce uncontrolled proliferation of cells, tumour suppressor markers such as *p53*, *p21* and *p16* will be highly expressed, which in turn activates their downstream target, *pRb*. Upregulation of these tumour suppressor markers maintains pRb in its hypophosphorylated state, which results in cell cycle arrest and apoptosis to reduce cell proliferation, thus preventing the formation of tumours (Chuaire-Noack *et al*., 2010; Pelicci, 2004).

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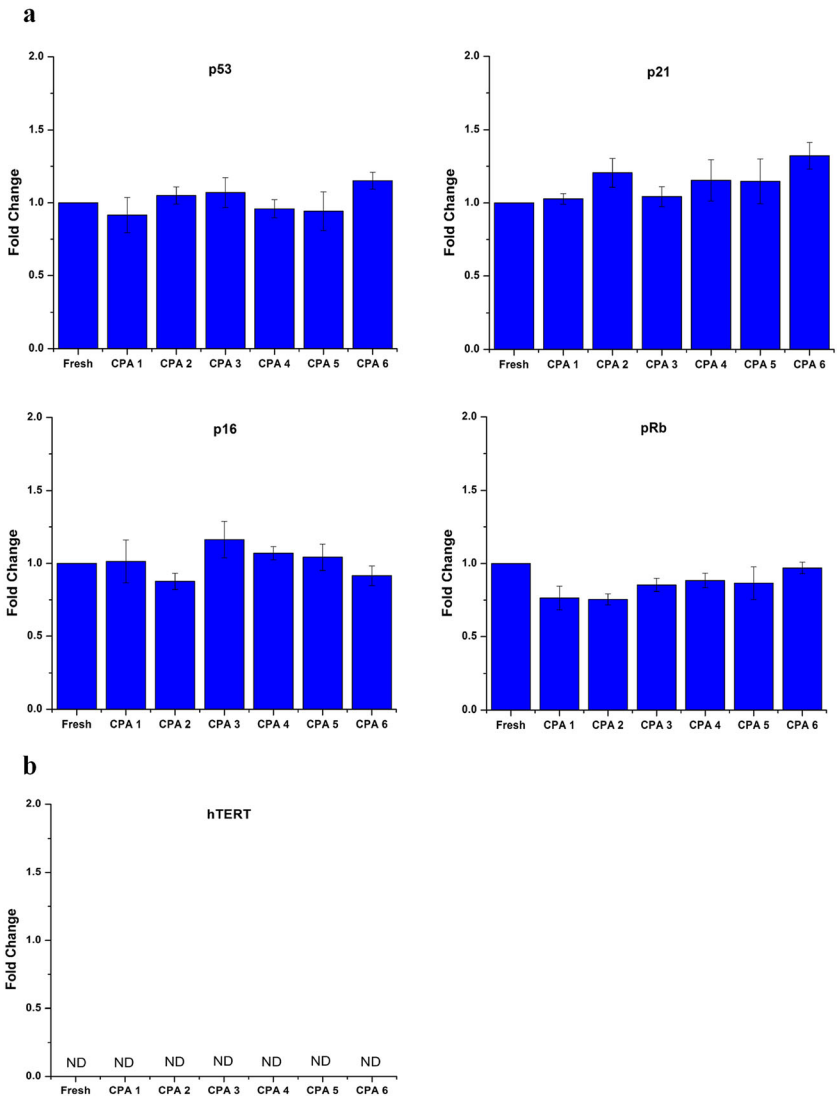

Figure 1. Cryopreservation maintained normal expressions level of (a) tumour suppressor markers, e.g. p53, p21, p16 and pRb, and (b) hTERT in hASCs. CPAs: 1, 0.25 M trehalose; 2, 5% DMSO; 3, 10% DMSO; 4, 5% DMSO + 20% FBS; 5, 10% DMSO + 20% FBS; 6, 10% DMSO + 90% FBS; ND, not detectable

On the other hand, there was no detectable expression of human telomerase reverse transcriptase (*hTERT*; a catalytic subunit of telomerase for protecting the telomeres) in hASCs preserved with various CPAs and fresh hASCs (Figure 1b). These results indicate the extremely low levels of hTERT in the cells (Choi *et al*., 2015). It has been reported that hTERT is easily detected in tumour cells but is undetectable in normal human cells (Elenitoba-Johnson, 2001; Murofushi *et al*., 2006). In general, the upregulation of hTERT favours an immortal/tumour phenotype (e.g. increased telomerase activity and telomere length) by blocking programmed cell death (apoptosis) (Lamy *et al*., 2013). For instance, hTERT overexpressed in MSCs derived from human bone marrow increased telomerase activity and thus resulted in spontaneous malignant transformation after long-term culture (Rosland *et al*., 2009). Taken together, cryopreservation does not affect the expression of tumour suppressor markers and hTERT in hASCs.

3.2. Effects of cryopreservation on telomerase activity and telomere length of hASCs

In general, the transformation from normal cells (including hASCs) to tumour cells usually engages a telomere-maintenance mechanism, which occurs through the upregulation of telomerase (Xu *et al*., 2013). Telomerase is an enzyme involved in the maintenance of the length of telomeres, specialized structures at the chromosome ends, that are essential for genome stability and the regulation of cell proliferation (Blackburn, 1991; Gomez *et al*., 2012). It has been reported that telomere dysfunction drives tumour initiation, while the subsequent activation of telomerase and restoration of telomeres promote tumour progression (Ding *et al*., 2012; Hu *et al*., 2012; Xu *et al*., 2013). For instance, in a prostate cancer model in *p53/Pten*-null and telomerase-deficient mice, telomere dysfunction drove tumour initiation, but progression of the resulting tumours was inhibited by the ongoing

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DNA-damage response induced by dysfunctional telomeres. However, once the telomerase had been reactivated, DNA-damage signalling was abolished and this enabled the progression of aggressive tumours (Ding *et al.*, 2012). Another study also showed that the combination of initial telomere dysfunction followed by telomerase reactivation enables the development of aggressive T cell lymphomas in a T cell lymphoma model in mice deficient of ataxia telangiectasia mutated protein (ATM), a kinase that regulates DNA damage in response to short telomeres (Hu *et al.*, 2012). The findings from both studies suggest that telomerase activation is one of the important tumour escape mechanisms to evade the telomere-dependent pathways of cell mortality for driving tumour progression (Hanahan and Weinberg, 2011). Telomerase activity is enhanced and telomere length is not shortened, resulting in indefinite cell proliferation, which is implicated in human tumour progression (Artandi and DePinho, 2010). Therefore, telomerase activity would not be an early event in transformation but rather a consequence of tumourigenic transformation, as indicated by its important role in tumour immortality and progression. Increased telomerase activity is present in 85–90% of all human tumours (Elenitoba-Johnson, 2001; Kim *et al.*, 1994).

To determine the telomerase activity and telomere length of cryopreserved hASCs, we conducted an enzyme-linked immunosorbent assay (ELISA) and Southern blotting, respectively. We observed that there was no significant ($p > 0.5$) change in relative telomerase activity (Figure 2a) and TRF length (Figure 2b) of hASCs preserved in various CPAs and fresh hASCs, suggesting that cryopreserved and fresh hASCs have a similar telomerase activity and telomere shortening rate. In conjunction with the findings indicating low expression of hTERT, we suggest that cryopreserved hASCs are at a low risk of tumourigenesis caused by telomere dysfunction, which is essential for tumour initiation (Raynaud *et al.*, 2008).

3.3. Effects of cryopreservation on *p53* nucleotide sequence mutation in hASCs

It is essential to evaluate the *p53* gene in relation to tumour formation, as the mutation of *p53* nucleotide sequences can be observed in 60% of human malignancies (Kusser *et al.*, 1993). To detect *p53* mutation in cryopreserved hASCs, we performed DNA sequence analysis. Exons 5–8 of *p53* were analysed, as 95–98% of *p53* mutations exist mainly in this region (Berloco *et al.*, 2003; Lleonart *et al.*, 1998). Through DNA sequence analysis using BLAST software, we found that exons 5–8 of *p53* nucleotide sequences of hASCs preserved in various CPAs and fresh hASCs are 100% matched with those of wild-type *p53* (NCBI Accession No. X54156.1) (Figure 3), indicating no occurrence of *p53* mutation. If mutations occur in the genomic structure of *p53*, its role in regulating cell growth may be altered, thus causing tumour formation (Muller and Vousden, 2013). For instance, bone-marrow derived MSCs showed accelerated proliferation and spontaneous malignant transformation in the absence of *p53* (Armesilla-Diaz *et al.*, 2009). Our findings suggest that long-term cryopreserved hASCs are at a low risk of malignant transformation, as they maintain normal nucleotide sequences of *p53*.

3.4. Effects of cryopreservation on DNA damage in hASCs

In general, DNA damage response (DDR; a guardian of genomic integrity) is activated following the upregulation of tumour suppressor markers, e.g. *p53* and *p21*, in response to oncogenic stresses, which in turn induces apoptosis of tumour cells to prevent uncontrolled cell replication (Bartek *et al.*, 2007). Further, DNA damage in cells could be induced by numerous factors in the cryopreservation processes, such as CPA toxicity, osmotic shock or reactive oxygen species (Kopeika *et al.*, 2015;

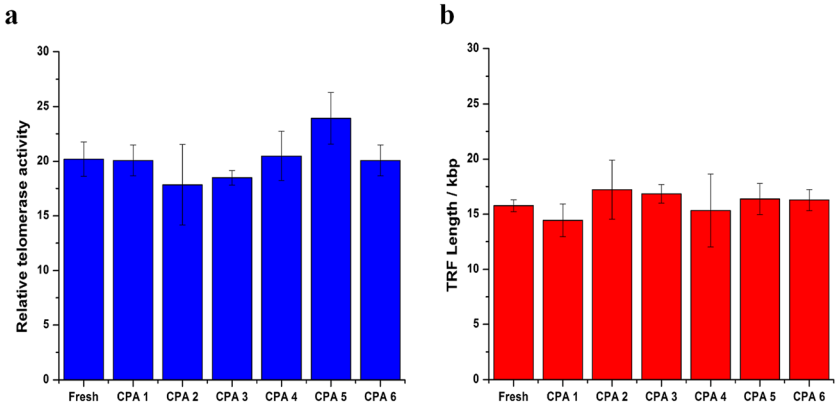

Figure 2. Telomerase activity (a) and telomere length (b) of hASCs was maintained following long-term cryopreservation. CPAs: 1, 0.25 M trehalose; 2, 5% DMSO; 3, 10% DMSO; 4, 5% DMSO + 20% FBS; 5, 10% DMSO + 20% FBS; 6, 10% DMSO + 90% FBS; TRF, terminal restriction fragment

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a. p53 exon 5

Homo sapiens p53 gene for transformation related protein p53 (also called transformation-associated protein p53, cellular tumor antigen p53, and non-viral tumour antigen p53)
Sequence ID: emblX54156.1| Length: 20303 Number of Matches: 1

Range 1: 12980 to 13273 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
544 bits(294)	3e-157	294/294(100%)	0/294(0%)	Plus/Plus

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Query  697    TTCCTCTTCCTACAGTACTCCCCTGCCCTCAACAAGATGTTTTGCCAACTGGCCAAGACC  756
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Query  757    TGCCCTGTGCAGCTGTGGGTTGATTCCACACCCCCGCCCGGCACCCGCGTCCGCGCCATG  816
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Sbjct  13220  CGCTGCTCAGATAGCGATGGTGAGCAGCTGGGGCTGGAGAGACGACAGGGCTGG  13273
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b. p53 exon 6

Homo sapiens p53 gene for transformation related protein p53 (also called transformation-associated protein p53, cellular tumor antigen p53, and non-viral tumour antigen p53)
Sequence ID: emblX54156.1| Length: 20303 Number of Matches: 2

Range 1: 13290 to 13489 GenBank Graphics ▼ Next Match ▲ Previous Match

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Query  1066   AAGGGTGGTTGTCAGTGGCC  1085
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Sbjct  13470  AAGGGTGGTTGTCAGTGGCC  13489
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c. p53 exon 7

Homo sapiens p53 gene for transformation related protein p53 (also called transformation-associated protein p53, cellular tumor antigen p53, and non-viral tumour antigen p53)
Sequence ID: emblX54156.1| Length: 20303 Number of Matches: 1

Range 1: 13943 to 14138 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
363 bits(196)	1e-102	196/196(100%)	0/196(0%)	Plus/Plus

```
Query  1070   TGCCACAGGTCTCCCCAAGGCGCACTGGCCTCATCTTGGGCCTGTGTTATCTCCTAGGTT  1129
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  13943  TGCCACAGGTCTCCCCAAGGCGCACTGGCCTCATCTTGGGCCTGTGTTATCTCCTAGGTT  14002

Query  1130   GGCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGGCGGC  1189
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  14003  GGCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGGCGGC  14062

Query  1190   ATGAACCGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAGGTCAGGAGCCACT  1249
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  14063  ATGAACCGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAGGTCAGGAGCCACT  14122

Query  1250   TGCCACCCTGCACACT  1265
              ||||||||||||||||
Sbjct  14123  TGCCACCCTGCACACT  14138
```

d. p53 exon 8

Homo sapiens p53 gene for transformation related protein p53 (also called transformation-associated protein p53, cellular tumor antigen p53, and non-viral tumour antigen p53)
Sequence ID: emblX54156.1| Length: 20303 Number of Matches: 1

Range 1: 14413 to 14637 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
416 bits(225)	1e-118	225/225(100%)	0/225(0%)	Plus/Plus

```
Query  1037   CCTTACTGCCTCTTGCTTCTCTTTTCCTATCCTGAGTAGTGGTAATCTACTGGGACGGAA  1096
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  14413  CCTTACTGCCTCTTGCTTCTCTTTTCCTATCCTGAGTAGTGGTAATCTACTGGGACGGAA  14472

Query  1097   CAGCTTTGAGGTGCGTGTTTGTGCCTGTCCTGGGAGAGACCGGCGCACAGAGGAAGAGAA  1156
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  14473  CAGCTTTGAGGTGCGTGTTTGTGCCTGTCCTGGGAGAGACCGGCGCACAGAGGAAGAGAA  14532

Query  1157   TCTCCGCAAGAAAGGGGAGCCTCACCACGAGCTGCCCCCAGGGAGCACTAAGCGAGGTAA  1216
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  14533  TCTCCGCAAGAAAGGGGAGCCTCACCACGAGCTGCCCCCAGGGAGCACTAAGCGAGGTAA  14592

Query  1217   GCAAGCAGGACAAGAAGCGGTGGAGGAGACCAAGGGTGCAGTTAT  1261
              ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  14593  GCAAGCAGGACAAGAAGCGGTGGAGGAGACCAAGGGTGCAGTTAT  14637
```

Figure 3. Cryopreserved hASCs demonstrated no mutations in *p53* nucleotide sequences. Representative BLAST data showed that exons 5–8 (a–d) of *p53* nucleotide sequences of fresh and cryopreserved hASCs were 100% matched with those of wild-type *p53* (NCBI Accession No. X54156.1); Query, p53 nucleotide sequence of hASCs; subject (sbjet), wild-type p53 nucleotide sequence

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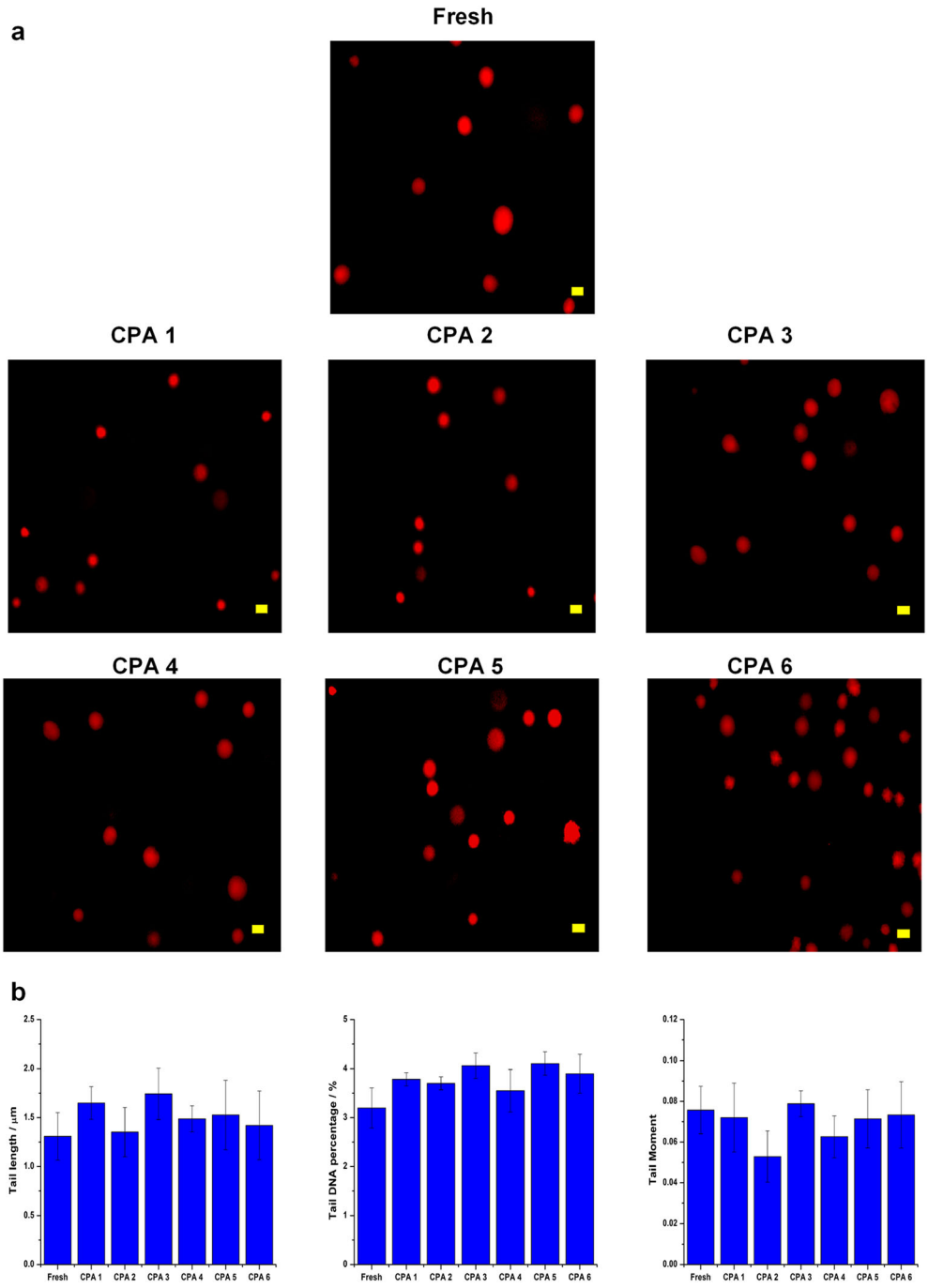

Figure 4. Cryopreserved hASCs displayed low levels of DNA damage. (a) A representative figure of DNA demonstrated that more DNA with short or without comet tails were observed in fresh and cryopreserved hASCs; magnification = ×100; scale bars = 100 μm. (b) Comet tail length, tail DNA % and tail moment was similar in both fresh and cryopreserved hASCs. CPAs: 1, 0.25 M trehalose; 2, 5% DMSO; 3, 10% DMSO; 4, 5% DMSO + 20% FBS; 5, 10% DMSO + 20% FBS; 6, 10% DMSO + 90% FBS

Stachowiak *et al.*, 2009), potentially causing chromosomal aberrations that may in turn lead to tumour formation (van Gent *et al.*, 2001). To evaluate the DNA damage patterns in cryopreserved hASCs, we performed a Comet assay. We found that comet tail length, percentage of DNA and moment in hASCs preserved with various CPAs were similar to those in fresh hASCs (Figure 4a, b), indicating low levels of DNA damage in long-term cryopreserved hASCs. In conjunction with the findings indicating normal expression of tumour suppressor markers in cryopreserved hASCs, the data further indicate that there is no sign of oncogenic stress, suggesting a low risk of tumourigenesis in long-term cryopreserved hASCs.

4. Conclusions

In summary, long-term cryopreserved hASCs showed a low risk of tumourigenicity, as they maintained normal expression of tumour suppressor markers and *hTERT*, telomerase activity, telomere length and *p53* nucleotide

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sequences without significant DNA damage. In conjunction with our previous findings (Yong *et al*., 2015), we suggest that hASCs preserved in 5% DMSO without FBS offer great potential in clinical application, due to their low risk of cytotoxicity, xenograft rejection and tumour formation, as well as their high cell viability, intact phenotype and functional properties. Further investigation is needed to assess the immunomodulatory properties of cryopreserved hASCs and to evaluate their therapeutic efficacy (including delivery mode) and biosafety *in vivo*.

Conflict of interest

The authors declare no conflicts of interest.

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Author contributions

K.W.Y. and W.K.Z.W.S. designed the experiments; K.W.Y. and J.R.C. performed the experiments and analysed the data; W.A.B.W.A. and B.P.M. contributed materials, reagents and instruments; M.A.N.A. and S.Z.O. performed surgeries to provide us with adipose tissue samples; and K.W.Y. wrote the manuscript, while F.X., X.Z., K.H.C. and W.K.Z.W.S. revised it. All authors reviewed the manuscript and approved the final version for submission.

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Supporting information on the internet

The following supporting information may be found in the online version of this article:

Figure S1. Population doubling time of fresh hASCs and hASCs preserved in various CPAs at passage 3

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