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# Immortalization of ameloblastoma cells via reactivation of telomerase function: Phenotypic and molecular characteristics

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SUMMARY

Ameloblastoma (AM) is recognized as a benign tumour but locally invasive with a high risk of recurrence. *In vitro* model systems for studying AM are limited due to the fact that AM cells grow poorly and begin to senesce early. Japanese researchers have reported the construction of an AM cell line, AM-1, by exposing cells to human papillomavirus 16 (HPV16) but retaining the potential of transformation. In this study, we used a retroviral infection method to over-express the human telomerase reverse transcriptase (hTERT) gene to acquire immortality of hTERT$^+$-AM cells. Furthermore, it was revealed both by reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot that the pathway of immortalization was loss of p16, not p53 or p21. Also, there was no evidence indicating that the hTERT$^+$-AM cells underwent malignant transformation by the nude mouse tumorigenicity assay. Taken together, this hTERT-immortalized cell line may be a potentially valuable and reliable cell model for further study of the invasive properties of AM *in vitro*.



## Introduction

As we know, replicative senescence occurs when a telomere, which is regulated by telomerase, is shortened to a critical minimal length.[1,2] Human telomerase has two major components: the protein component, human telomerase reverse transcriptase catalytic subunit (hTERT) and the genetic component, human telomerase RNA (hTR).[3,4] Usually, telomerase is undetectable in normal somatic cells, except in proliferative cells of renewal tissue and germline cells.[5,6] Activated telomerase can take advantage of its integral RNA as a template to synthesize telomeric repeats (TTAGGG) directly onto the ends of a chromosome. Interestingly, the hTR component of telomerase is expressed constitutively in normal cells, but the hTERT gene undergoes silencing that result in repression of the enzyme activity.[7] So, to some extent, it is implied that hTERT is the rate-limiting factor for telomerase activity.

Ameloblastoma (AM) is a benign but locally invasive odontogenic neoplasm. Compared with other benign odontogenic tumours, AM tends to be more invasive and has a higher risk of recurrence even after aggressive treatment. In order to improve the prognosis, there has been a great deal of clinical and basic research into AM for many years. However, the molecular mechanism underlying the development and invasion of AM is unclear owing to the few reliable cell models *in vitro*. Like human somatic cells, AM cells have a fairly short lifespan and senescence-associated pathways, such as telomere dynamics, to regulate their growth and proliferation. To overcome the challenges, immortalization with hTERT seems to be a successful technique.[8] In this study, the hTERT gene was inserted into AM cells by using a retrovirus vector. Furthermore, we determined the morphologic, cellular and biochemical phenotypic characteristics of these cells after immortalization.

## Materials and methods

### Cell culture

The AM specimen used in this study was obtained from a 57-year old Chinese male patient (pathological number 2008-691) at the Department of Oral and Maxillofacial Surgery, Guanghua School of Stomatology, Sun Yat-sen University. The subtype was determined as a plexiform by three pathologists. After removing extraneous soft connective tissues, the solid part of the specimen was dissected into pieces with an approximate size of 1 $mm^3$, and placed into 6-well plates coated with collagen I (Invitrogen, USA), incubating at 37 °C in a 5% (v/v) $CO_2$ atmosphere. Minimum essential medium with α modification (α-MEM, Invitrogen, USA)

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containing 15% (v/v) fetal bovine serum (FBS) was added after incubation for 5 h and used for subculture of the proliferative cells.

*Vector and transfection*

The retroviral vector pLXSN-hTERT was constructed previously by us. After activating by the PA317 packaging cells, the positive clones were selected with G418 (800 μg/ml) for 2 weeks and transferred to other flasks. The supernatant of the cell clones was harvested, passed through a 0.45 μm pore size filter and used to infect AM cells. The empty vector pLSXN was used as the negative control.

**Table 1**
Primers for RT-PCR.

| Specificity (length) | Primers |
|---|---|
| hTERT (145 bp) | F:5′-CGGAAGAGTGTCTGGAGCAA-3′<br>R:5′-GGATGAAGCGGAGTCTGGA-3′ |
| p16 (198 bp) | F:5′-GACATCCCCGATTGAAAGAA-3′<br>R:5′-TTTACGGTAGTGGGGGAAGG-3′ |
| p21 (172 bp) | F:5′-GACACCACTGGAGGGTGACT-3′<br>R:5′-CAGGTCCACATGGTCTTCCT-3′ |
| p53 (156 bp) | F:5′-GGCCCACTTCACCGTACTAA-3′<br>R:5′-GTGGTTTCAAGGCCAGATGT-3′ |
| β-Actin (205 bp) | F:5′-CCTTCCTGGGCATGGAGTCCT-3′<br>R:5′-GGAGCAATGATCTTGATCTTC-3′ |

F, forward primer sequence; R, reverse primer sequence.

AM cells at passage 2 (P2) were cultured in 6-well plates with α-MEM containing 15% FBS. The supernatant of the virus was added when the cells reached 70% confluence. Then the infected cells were selected in medium containing G418 (1000 μg/ml) for 1 week, and maintained in medium containing G418 (800 μg/ml) for the next week. The cells transfected with hTERT and empty vector were named hTERT$^{+}$-AM and pLXSN-AM, respectively.

*Immunocytochemistry*

Cells that reached 70–80% confluence in 24-well plates were fixed with 10% (v/v) formaldehyde for 15 min and washed twice with PBS. Endogenous peroxidase activity was blocked by 0.3% (v/v) hydrogen peroxide for 30 min. Non-specific binding of the antibodies was avoided by using of 5% bovine serum albumin (BSA) for 30 min. The cells were incubated for 12 h at 4 °C with primary antibodies, pan-cytokeratin (1:100) and vimentin (1:100) (Boster, China), then treated with the secondary antibody for 1 h at 37 °C. Next, the cells were dyed for 5 min with diaminobenzidine (DAB) and then counterstained with hematoxylin.

*Proliferation assay*

Briefly, the triplicate $10^4$ cells were added into 24-well plates and incubated at 37 °C. Cells were harvested and counted every 24 h. As for MTT assay, the triplicate $10^3$ cells were seeded into 96-well plates, 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytet-

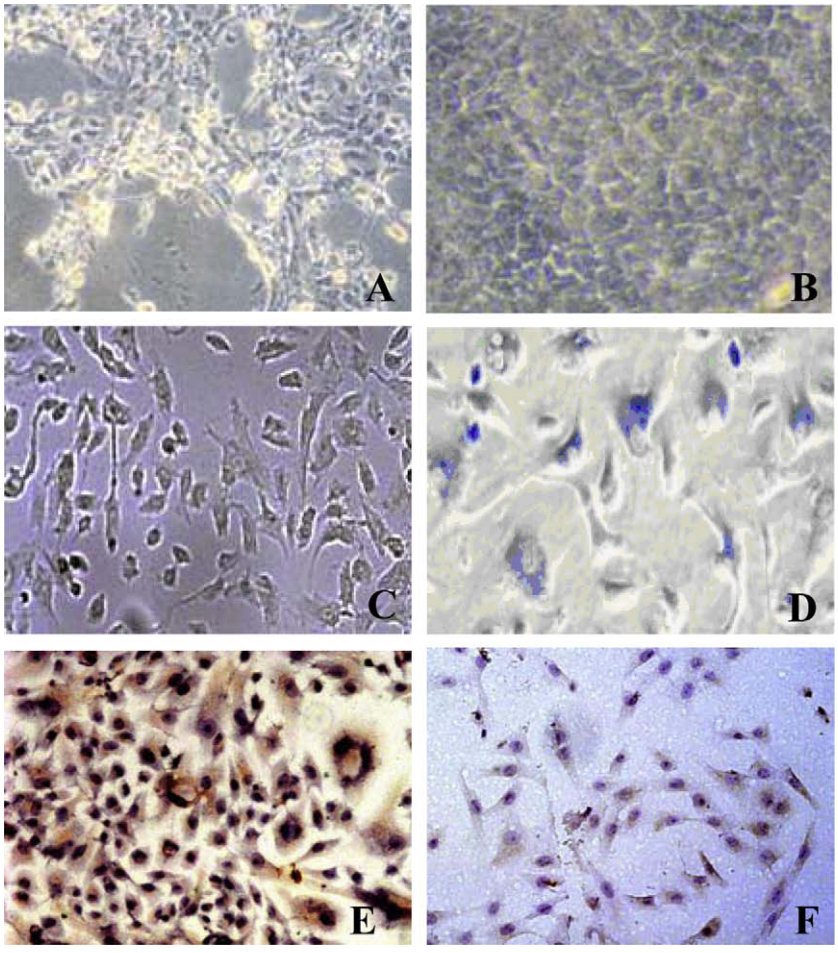


**Figure 1** hTERT-AM cells. (A) Clone formations of primary AM cells during G418 selection (×200). (B) The cells showed polygonal morphology and a flagstone arrangement (×400). Senescence-associated β-galaciosidase staining showed hTERT$^{+}$-AM cells were negative for β-gal (C), while untransfected AM cells were positive for senescent signals (D) (×200). Immunocytochemistry detection showed hTERT$^{+}$-AM cells were positive for pan-cytokeratin (E) and weakly positive for vimentin (F) (×200).

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razoliumromide (MTT) (100 μg) was added to wells at different time-points and incubated for 4 h. The medium was removed and 150 μl of dimethyl sulfoxide (DMSO) was added to dissolve the purple formazan formed by the reduction of MTT. The absorbance at a wavelength of 490 nm was measured with a microplate reader.

During the subculture, the average population doubling time (PDT) was calculated as $\log_2 t/(\log_2 N_h - \log_2 N_p)$, where $t$ is the incubation time, while $N_h$ and $N_p$ represent the numbers of cells harvested and the number of plates, respectively. Population doubling (PDL)/passage was calculated as $\log_2 (N_h/N_p)$. Cumulative PDLs were plotted against total time in culture to determine the lifespan and the onset of proliferation arrest.

*Telomerase assay*

Telomerase activity was determined with a TRAPeze kit (Oncor, USA). Briefly, the protein from about $10^6$ hTERT$^+$-AM cells that reached PDLs of 5, 15, 45, 65, and untransfected AM cells that reached 6 PDLs were extracted. Lysis buffer was used as the negative control, while a human cancer cell line known to express telomerase was used as the positive control.

*Senescence-associated β-galactosidase staining*

β-Galactosidase staining was done with a senescence-associated β-Galactosidase Staining Kit (Beyotime, China). Cells were washed three times with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Next, the cells were incubated overnight at 37 °C in darkness with the working solution containing 0.05 mg/ml 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal)).

*Reverse transcription-polymerase chain reaction analysis (RT-PCR)*

Total RNA was extracted using TRIZOL Reagent (Gibco, USA). The cDNA was synthesized from 1 μg of RNA using a Fermentas RT kit (MBI, Lithuania). The reaction conditions used for PCR were:

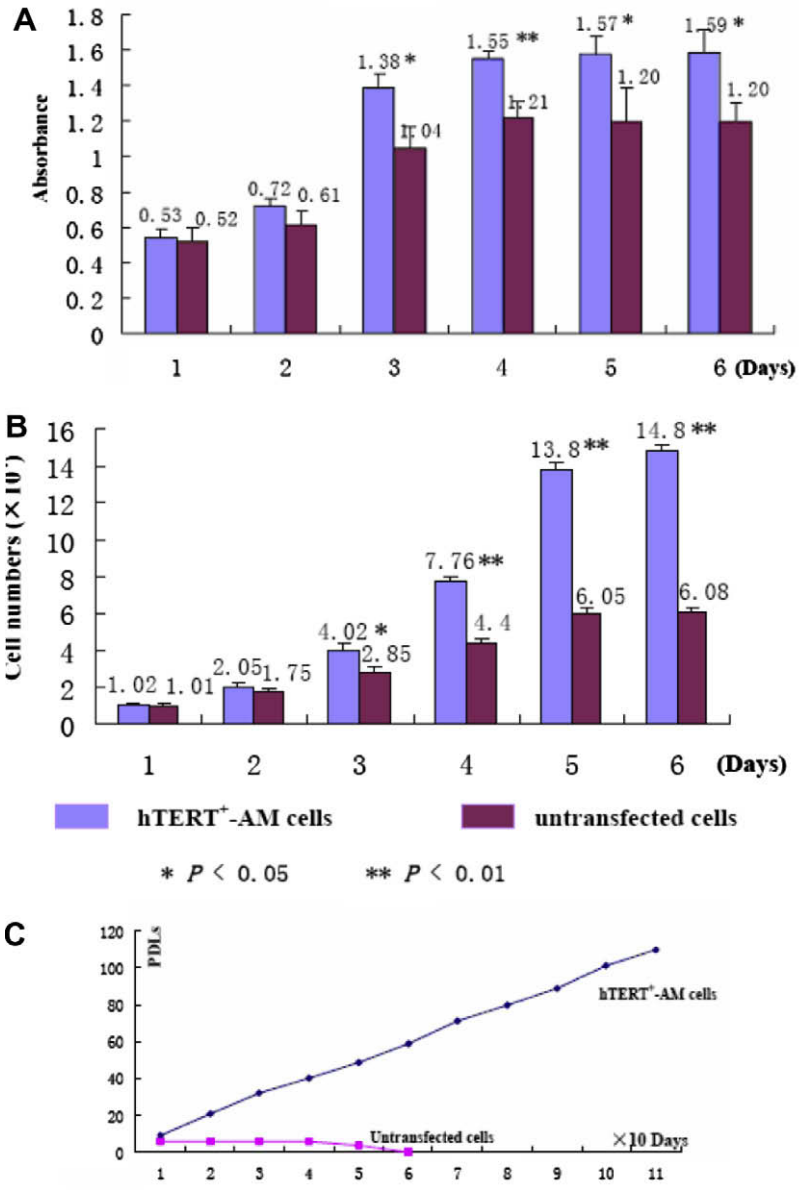


**Figure 2** Cell kinetics curves. The growth trends obtained by both MTT (A) and cell counting (B) assays revealed a distinct proliferative state between hTERT$^+$-AM cells and untransfected AM cells from 3-day culture ($p < 0.05$). (C) A more significant difference between the two cells in long-term culture assay were indicated on the PDL curves ($p < 0.01$).

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hTERT (31 cycles); p53, p21 and p16 (35 cycles) all at annealing temperature of 60 °C. The sequences of the primers are given in Table 1.[9,10] The PCR products were subjected to electrophoresis in 1.5% (w/v) agarose gels then stained with ethidium bromide (EB).

*Western blot analysis*

Total protein was extracted as described.[11] Briefly, 40 μg of protein was subjected to SDS–PAGE with 6% polyacrylamide gels for hTERT, and 12% polyacrylamide gels for p53, p21 and p16. The gels were transferred to PVDF membranes, then blocked with 5% (w/v) non-fat dry milk in Tris-buffered saline (TBS) for 2 h at room temperature. The membranes were then incubated with each primary antibody (1:1000) overnight at 4 °C, washed twice and incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. Subsequently, the protein bands were detected by enhanced chemiluminescence (ECL).

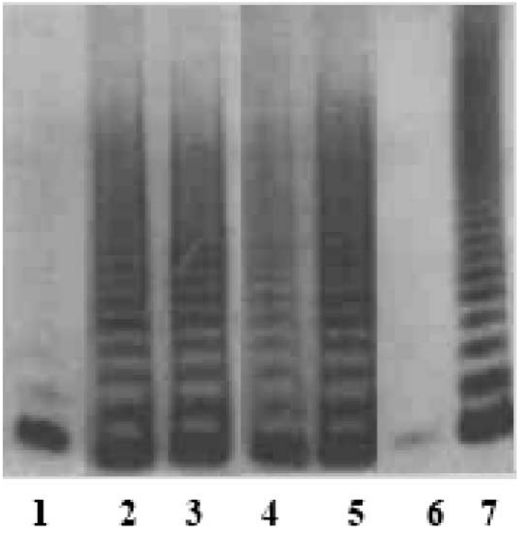


**Figure 3** Telomerase activity assay. Line **1**, untransfected AM cells at 6 PDLs showed negative expression of telomerase. Lines **2**–**5**, hTERT$^+$-AM cells at 5, 15, 45, 65 PDLs showed positive telomerase activities. Line **6**, lysis buffer as negative control. Line **7**, a cancer cell line (Hela) expressing telomerase.

*Statistical analysis*

The data from the proliferation assay were analyzed by Wilcoxon's rank-sum-test (PDL) and paired Student's *t*-test (MTT and cell counting) for comparison between the untransfected and transfected cells. All statistical tests were done with the Statistical Analysis System 8.0 (SAS 8.0). A *P*-value of $<0.05$ was set as statistically significant.

## Results

*Immortalization*

During selection, both hTERT$^+$-AM cells and pLXSN-AM cells resisted G418, while untransfected AM cells died within 5 days (Fig. 1A). There was no significant morphological alteration between untransfected AM cells and hTERT$^+$-AM cells, which were polygonal and arranged like flagstones (Fig. 1B). The senescence-associated β-galactosidase staining showed a dramatic difference between the senescent signals of hTERT$^+$-AM cells and untransfected AM cells (Fig. 1C and D). However, the pLXSN-AM cells showed instability of both morphology and proliferation; they were spindle-shaped and died at about 2.5 PDLs (data not shown). Immunocytochemistry showed that hTERT$^+$-AM cells expressed pancytokeratin normally and vimentin weakly (Fig. 1E and F).

*Cell kinetics*

It was observed that hTERT$^+$-AM cells proliferated quickly from 3-day culture in *vitro*. (Fig. 2A and B). During lengthy periods of culture, the average PDT in the logarithmic growth phase of hTERT$^+$-AM cells was 23.5 h. Cumulative PDLs of hTERT$^+$-AM cells were up to 100, while untransfected cells died at 6 PDLs (Fig. 2C).

*Telomerase activity*

In this study, telomerase activity was assessed at different passages of hTERT$^+$-AM cells that corresponded to 5–65 PDLs. We used untransfected AM cells that reached 6 PDLs as a control. The results showed that hTERT$^+$-AM cells were positive for telomerase activity at every passage, while the untransfected AM cells and the lysis buffer were negative (Fig. 3).

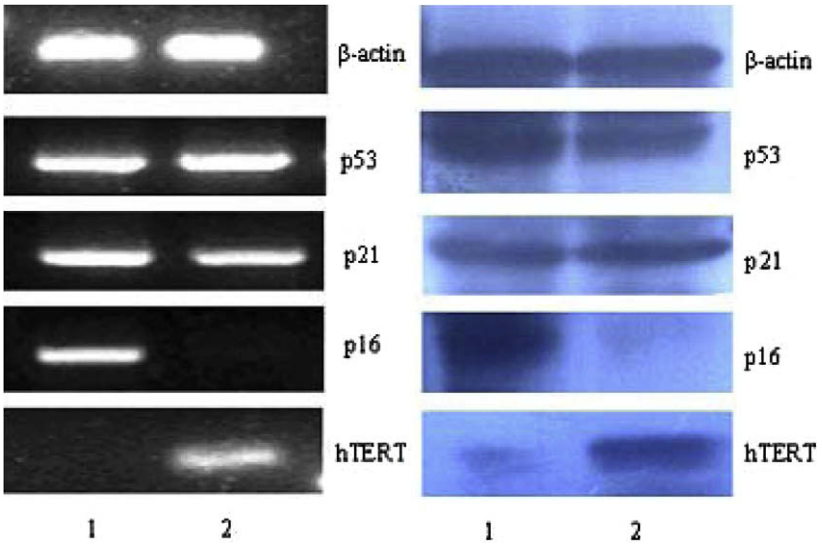


**Figure 4** Expressions of hTERT, p53, p21 and p16. When induced by hTERT, hTERT$^+$-AM cells lacked expression of p16 at the mRNA and protein levels, while p53 and p21 were still positive compared to untransfected AM cells. (**1**: untransfected cell, **2**: hTERT$^+$-AM cell).

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*Expressions of hTERT, p53, p21 and p16 at mRNA and protein levels*

We tested the activity of the three cell growth-dependent factors p53, p21 and p16 by RT-PCR and Western blot (Fig. 4), which revealed that when hTERT was over-expressed in AM cells, there was a significant down-regulation of p16 at both the mRNA and protein levels. However, the activities of another two proteins, p53 and p21, were still high in both transfected and untransfected AM cells.

## Discussion

Many studies have indicated that normal human cells stably expressing hTERT present a proliferative signal of immortalization without changing the phenotype to that of malignant cells.[8] The aim of this study was to establish a cell line from AM that could survive by bypassing normal programmed senescence and thus extend the usefulness of cells for exploring cellular mechanisms involved in the generation and development of this tumour. It was reported that immortalization was defined as at least 50 PDLs of cell growth,[12] while our hTERT$^+$-AM cells proliferated over 100 PDLs within three months. Besides, the mRNA and protein levels of hTERT as well as the telomerase activity were detected in hTERT$^+$-AM cells but not in untransfected cells or pLSXN-AM cells. All these characteristics together indicated that a stable expression of hTERT in AM cells had been constructed.

As described, infinite cell growth without altering phenotypic or differentiated characteristics was required by a meaningful and successful immortalization.[13–15] It has been reported that at least two immortalized cell lines (uterine leiomyoma and pleomorphic adenoma) have been constructed by being transfected with hTERT in human benign tumours.[16,17] Discounting human uterine leiomyoma cell lines and human pleomorphic adenoma cell lines, the characteristics of normal phenotypes or differentiations were unchanged by insertion of the hTERT gene into the parental cells. As for AM, Harada et al.[18] tried to immortalize the cells by liposome-mediated transfection with 22.4 kb gene fragments of human papillomavirus 16 (HPV16). Even though these immortalized cells maintained the epithelial origins regardless of morphological or immunological detections, more detailed studies were limited because of the transient transfection with such a long fragment. Moreover, HPV16 was not considered to be involved in the origin, proliferation or recurrence of AM, unlike its role in squamous cell carcinomas,[19] and is not to be considered as a reasonable inducer of AM immortalization. In our study, hTERT$^+$-AM cells were positive for pan-cytokeratin, a marker for epithelial cells, and weakly positive for vimentin, a marker for stromal cells. Besides, our data for hTERT expression and telomerase activity were consistent with those reported by Kumamoto, who suggested that telomerase activity might be associated with the proliferative potential of odontogenic epithelium.[20]

Since the discovery of hTERT, many studies have revealed that several proto-oncogenes and tumour suppressor genes, such as c-myc, Bcl-2, p21, Rb and p53, are involved in telomerase regulations.[21–23] It has been concluded that expression of p53, p21, p16 and hTERT is correlated with the biological behaviour of odontogenic tumours.[20,24–26] Furthermore, the hypermethylation of CpG islands of p16 in malignant transformation of AM has been reported.[27] In our experiments, we detected variations in the expressions of p53, p21 and p16 in hTERT$^+$-AM cells. The altered expressions of p53 and p21 were not detected at the mRNA or protein levels, while a differential expression of p16 during hTERT reactivation was observed. The roles of these cell cycle-related factors in immortalization of the parental cells have been the subject of controversy for many years. Some groups have demonstrated that hTERT alone can be sufficient for immortalization without any alteration of the p53/p21 or pRb/p16 pathways when the culture conditions were optimal, such as using a feeder layer.[28–30] However, others have argued that inactivation of p16 or p21 is not essential for immortalization, even though the cells were cultured on feeder layers or stimulated with growth-enhancing factors.[31–34] In this study, we used a culture system including collagen I-coated flasks and α-MEM, which contained insulin, transferrin and epidermal growth factor but not the feeder cells. In our opinion, collagen I may have a role similar to that of feeder layers, but the functions of mediation between them are slightly different. To date, there has not been a generally accepted culture system for AM cells. Here, we suggest that inactivation of p16, not p53 or p21 should be a critical event during hTERT-mediated immortalization of AM.

Taken together, we prolonged the lifespan of AM cells by transfection of hTERT. We suggest hTERT-immortalized AM cells should undergo an essential variation of p16 gene inactivation, which may have an important role in tumorigenesis and invasion of AM. It should be noted that details of the underlying mechanism regulating the detailed functions of hTERT in the development and invasion of AM remain to be determined.

## Conflict of Interest Statement

None declared.

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