BMI-1 activation is crucial in hTERT-induced epithelial-mesenchymal transition of oral epithelial cells

Bin Qiao^a, Zhifeng Chen^b, Fengchun Hu^b, Qian Tao^{b*}, Alfred K. Lam^{c*}

^a Department of Oral and Maxillofacial Surgery, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China

^b Department of Oral and maxillofacial Surgery, Guanghua School and Hospital of Stomatology and Institute of Stomatological Research, Sun Yat-sen University, Guangzhou 510055, China

^c Cancer Molecular Pathology, School of Medicine and Griffith Health Institute, Griffith University, Gold Coast, Queensland 4222, Australia

* Corresponding authors: Qian Tao & Alfred K. Lam

E-mail addresses:

a.lam@griffith.edu.au (A. Lam); taoqian@mail.sysu.edu.cn (Q. Tao);

Address for sending details:

Professor Alfred Lam, School of Medicine, Griffith University Gold Coast Campus. Gold Coast, Q4222, Australia

Abstract

BMI-1 (B lymphoma Mo-MLV insertion region 1 homolog) has been reported to be over-expressed in cell immortalization and the epithelial-mesenchymal transition (EMT) of cancer cells. The aim of this study is to study the roles of BMI-1 in the human telomerase reverse transcriptase (hTERT)-induced immortalization and EMT. In this study, hTERT+-OME cells and hTERT+-HaCaT cells were acquired by viral transduction of hTERT to primary cultured oral keratinocytes and HaCaT cells (skin epidermal cells). siRNA transduction was used for the inhibition of BMI-1 expression. RT-PCR and Western blots were performed to detect the expressions of twist, vimentin, BMI-1, hTERT and p16INK4a in these cell lines. EMT was assessed by immunohistochemistry (expressions of cytokeratin & vimentin), Western blots (expressions of Twist, vimentin & E-cadherin) and RT-PCR (expression of Twist). The results indicated that hTERT+-OME cells and hTERT+-HaCaT cells underwent EMT spontaneously with high expression of Twist. p16INK4a was silenced in both hTERT-transduced cells but could be detected in HaCaT cells. Moreover, BMI-1 was highly expressed in hTERT+-OME and hTERT+-HaCaT cells but was negative in HaCaT cells. When the expression of BMI-1 was blocked by siRNA transduction, the proliferations of hTERT+-OME and hTERT+-HaCaT cells were inhibited and the mono-spheroid colony formation of these hTERT-transduced cells was decreased. In addition, the expression of p16INK4a was regained while the expressions of EMT markers (twist and vimentin) were down-regulated in these two BMI-1 blocking cell lines. To conclude, this study suggests BMI-1 expression plays a role of hTERT-induced immortalization and EMT.

Keywords: BMI-1; *hTERT*; immortalization; EMT

INTRODUCTION

Cell death is often due to oncogenic stress as a result of an accumulation of cell divisions (Egbuniwe *et al*, 2011). Senescence and crisis are two check-points of cells overcoming cell death (Rheinwald *et al*, 2002). Senescence is a $p16^{\text{INK4a}}$ /Rb-dependent pathway that is activated in cell proliferation whereas crisis is caused by shortening of telomeres to a critical minimal length (McMurray and McCance, 2004). As cell division progresses, accumulation of $p16^{\text{INK4a}}$ and shortening of telomeres appear at the same time. The enrichment of $p16^{\text{INK4a}}$ may depend on telomere shortening for activation (Lundberg *et al*, 2000). Thus, the maintenance of telomere length of cells is the key step of cell immortalization.

Epithelial-mesenchymal transition (EMT) is recognised by changes in cell-shape, in which epithelial cells *in vivo* become detached from each other, penetrate the basement membrane and acquire a more flexible and migratory phenotype similar to mesenchymal cells (Nakamura and Tokura, 2011). EMT is a process that is associated with normal tissue development and organogenesis, as well as with tissue remodelling and wound healing. Inappropriate reactivation of EMT contributes to cancer cell invasion and metastasis.

BMI-1 (B lymphoma Mo-MLV insertion region 1 homolog) is an oncogene that regulates cell cycle inhibitor genes (Liu *et al*, 2012a). In addition, it represses the $p16^{\text{INK4a}}$ expression and induces EMT in human nasopharyngeal epithelial cells (Song *et al*, 2009). More importantly, BMI-1 regulates the self-renewing cell divisions of somatic stem cells (Park *et al*, 2004). Therefore, overexpression of Bmi-1 may lead to senescence bypass and immortalization.

Human telomerase has two major components: human telomerase reverse transcriptase catalytic subunit (*hTERT*), and human telomerase RNA. *hTERT* gene undergoes silencing as cells grow, which results in the block of telomere synthesis (Shay and Wright,

2005). Reactivation of telomerase activity occurred and is a prognostic marker in many human cancers (Lam *et al*, 2008b; Lam *et al*, 2008c; Lam *et al*, 2008d; Saleh *et al*, 2008; Tang *et al*, 2007; Saleh *et al*, 2007; Lam *et al*, 2001; Chow *et al*, 2001; Lam *et al*, 2000; Lo and Lam, 1999). Accumulating evidence has indicated that the maintenance of telomere length of cells in culture is transduction of *hTERT* gene into somatic cells (Jiang *et al*, 1999). However, there is argument that *hTERT*-transduced cells cannot be immortalized as the transduced cells may only divide for a finite number of cell doublings (Muntoni *et al*, 2003).

In our previous study, we established two epithelial cell lines (*hTERT*⁺-AM, *hTERT*⁺-OME) by *hTERT* transduction into cultured ameloblastoma cells and cultured oral keratinocytes, respectively (Tao *et al*, 2009; Qiao *et al*, 2012). Experiments in these cell lines showed that the loss of *p16*^{INK4a} expression and the maintenance of telomere integrity were pivotal mechanisms for cell immortalization. Moreover, it was found that *hTERT* transduction promoted EMT and stem cell-like properties in *hTERT*⁺-OME cells (Qiao *et al*, 2012). However, it is incompletely understood how *hTERT* contributes to cell immortalization. Given the clues of the functions of BMI-1 in carcinogenesis, we would like to investigate its roles in the *hTERT*-induced immortalization.

MATERIALS AND METHODS

Cell lines

Three cells lines, HaCaT, *hTERT*⁺-OME and *hTERT*⁺-HaCaT, were recruited. HaCaT is a spontaneously immortalized cell line generated by long-term incubation of a primary culture of human skin epidermal cells under selected culture conditions (Boukamp *et al*, 1997). *hTERT*⁺-OME is an immortalized cell line created by *hTERT* gene transduction into primary cultured oral mucosal epithelial (OME) cells (Qiao *et al*, 2012). In this study, we also established the *hTERT*⁺-HaCaT cell line. Ethic approval was obtained from the Ethics Committee of Zhengzhou University (reference number is 45005204-10-0) and from Ethics Committee of Griffith University (reference number is HERC/11/QGC/162).

The establishment of *hTERT*⁺-HaCaT cells

The use of retroviral vector (pLXSN-*hTERT*) and the transduction method were as previously described (Tao *et al*, 2009; Qiao *et al*, 2012). The empty vector pLXSN was used as the negative control. The virus particles were produced by PA317 packaging cells (ATCC, Manassas, VA, USA). Subsequently, the supernatant of the cells was harvested, passing through a 0.45 µm filter and was used to infect the HaCaT cells. The positive clones were acquired by G418 (Sigma, St.Louis, MO, USA) selection for 2 weeks. The survived cells under the G418 selection were named *hTERT*⁺-HaCaT cells.

Cell culture

All the cells (HaCaT, *hTERT*⁺-OME, *hTERT*⁺-HaCaT) were cultured in DMEM/F12 (Invitrogen, Grand Land, NY, USA) medium supplemented with 10% foetal bovine serum (Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma). In addition, 200µg/ml G418 was added in cultured *hTERT*⁺-OME cells and *hTERT*⁺-HaCaT cells.

Multi-cellular spheroid has closer characteristics to *in vivo* compared to monolayer cultured cells. Thus, the three cell lines were also studied in 3-D spheroid culture. In brief, cells were plated in ultralow attachment plates (Corning, Tewksbury, MA, USA) at a density of 20,000 viable cells per ml. Cells were grown in serum-free epithelial growth medium supplemented with B27 (Invitrogen) containing 20 ng/ml of epidermal growth factor and basic fibroblast growth factor (Invitrogen).

Immunocytochemistry

In *hTERT*⁺-HaCaT cells, cells that reached 70-80% confluence in 48-cell plates were fixed with 10% formaldehyde for 15 minutes and washed twice with phosphate buffered saline. Endogenous peroxidase activity was blocked by hydrogen peroxide for 30 minutes. 5% bovine serum albumin (Boster, Wuhan, Hubei, China) was added for 30 minutes to minimise antibody non-specificity. The cells were incubated overnight at 4 °C with primary antibodies, pan-cytokeratin (1:100) and vimentin (1:100) (Boster). They were then washed and treated with the secondary antibodies (horseradish peroxidase-labelled goat-anti mouse; Boster) for one hour at room temperature. The immunoreactive cells were coloured with diaminobenzidine for 5 minutes and cells negative for proteins were counterstained with haematoxylin (Sigma).

siRNA transduction assay

The expression of BMI-1 in *hTERT*-transduced cells was inhibited according to the manufacturer's instructions (Santa Cruz Biotechnology, Santa Cruz, CA, USA). In brief, cells were seeded in a six well-tissue culture plates. They were incubated in a humidified 5% carbon dioxide atmosphere until the cells are 60-80% confluent. After that, the growth medium was replaced by siRNA transduction medium containing 0.5µg siRNA (without

serum). The cells were then incubated for 24 hours at 37°C. After that, one ml of normal growth medium containing two times the serum concentration without removing the transduction mixture was added and incubated for 24 hours. The mRNA and protein of these cells were extracted for RT-PCR assay and Western blot analysis (see below). Also, the BMI-1-inhibited cells were used for methylthiazoletetrazolium (MTT) assay and monospheroid assay (see below).

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

Total RNA was extracted from the cultured cells (HaCaT, *hTERT*⁺–OME, *hTERT*⁺–HaCaT) and the cells underwent siRNA transduction by using of TRIZOL Reagent (Invitrogen). The cDNA was synthesized from 1 µg of RNA using a Promega RT-PCR kit (Promega, San Luis Obispo, CA, USA). The PCR products were subjected to electrophoresis in 1.5% agarose gels (Sigma) then stained with ethidium bromide (Sigma). The primers used were published previously (Liu *et al*, 2008; Rosivatz *et al*, 2002; Skrzypczak *et al*, 2007).

Western blot analysis

The total protein was extracted from the cultured cells using lysis buffer (Bio-Rad, Hercules, CA, USA). 40 µg proteins were subjected to SDS-PAGE with 10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were then incubated with primary antibodies overnight at 4°C, washed twice and incubated with horseradish peroxidase-conjugated secondary antibody (Bio-Rad) for two hours at room temperature. Subsequently, the protein bands were detected by enhanced chemiluminescence and visualised using a VersaDoc-MP Imaging System (Bio-Rad). The antibodies used were *hTERT* (Boster), vimentin (Boster), *p53* (Santa Cruz Biotechnology), *p16* (Santa Cruz Biotechnology), *p21* (Santa Cruz Biotechnology), Twist (Santa Cruz

Biotechnology), E-cadherin (Invitrogen), BMI-1 (Cell signalling Technology, Danvers, MA, USA) and GAPDH (Santa Cruz Biotechnology). The concentrations used were 1:500 (*hTERT*), 1:500 (vimentin), 1:500 (*p53*), 1:500 (*p16*), 1:500(*p21*), 1:500 (Twist), 1:1000 (E-cadherin), 1:500(BMI-1) and 1:1000 (GAPDH) respectively.

MTT assay

The cells (primary cultured oral keratinocytes, *hTERT*+–OME, HaCaT, *hTERT*+–HaCaT and the cells underwent siRNA transduction) were seeded in 96-well plates (Invitrogen) at a density of 7,000 cells per well. After cultured for 1 day, 2 days, 3 days, 4 days, 5 days, 6 days and 7 days, 3-(4, 5)-dimethylthiazol-2-yl-5-diphenyltetrazolium bromide (MTT, Sigma) at 5 mg/ml was added into each well and continuously incubated for 4 hours. The absorbance of these wells obtained every day was measured at 490 nm after added 150µl dimethyl sulfoxide (DMSO, Sigma).

Statistical analysis

The data from the MTT assay was analysed by paired Student's t-test for comparison between the non-transduced, *hTERT*-transduced and BMI-1 blocking cells. Statistical analysis was performed using the Statistical Package for Social Sciences for Windows (version 21, IBM SPSS Inc., New York, USA). A *p*-value of <0.05 was set as statistically significant.

RESULTS

Disclosure of EMT phenotypes and alterations of cell cycle inhibitor genes in *hTERT*⁺-HaCaT cells, HaCaT cells and *hTERT*⁺-HaCaT cells.

HaCaT cells were polygonal and arranged like cobblestones (Figure 1Aa). On the other hand, *hTERT*⁺-HaCaT cells were spindle-shaped when were cultured in low density (Figure 1Ab) but converted back to the epithelial cell phenotype at high confluence (Figure 1Ac). Immunocytochemistry showed that cytokeratin (Figure 1Ba) and vimentin (Figure 1Bb) were expressed in the *hTERT*⁺-HaCaT cells. At both mRNA and protein levels (Figure 1C), *hTERT* was noted in HaCaT cells and *hTERT*⁺-HaCaT cells. The expression of *hTERT* in *hTERT*⁺-HaCaT cells was higher in HaCaT cells. Also, *p16*^{INK4a} expression was downregulated and Twist expression was upregulated in *hTERT*⁺-HaCaT cells when compared to HaCaT cells. There was no obvious alteration of *p21* and *p53* in *hTERT*⁺-HaCaT cells.

The expression of BMI-1 in *hTERT*-transduced cells and the alterations of EMT traits as well as stem cell properties when BMI-1 was knocked down

BMI-1 was highly expressed in *hTERT*-transduced cells (*hTERT*⁺-OME or *hTERT*⁺-HaCaT). On the other hand, BMI-1 was negative in HaCaT cells (Figure 2A). When the expression of BMI-1 was inhibited in *hTERT*-transduced cells, the expression of *hTERT* was not changed (high expression). However, *p16*^{INK4a} expression was noted in BMI-1-inhibiting cells. In addition, the expressions of EMT markers (Twist and vimentin) were decreased in BMI-1-inhibiting cells, while E-cadherin (another EMT marker) was not changed (Figure 2B).

In the MTT assay, the knockdown of BMI-1 affected the growth of *hTERT*-transduced cells. *hTERT*⁺–OME cells almost stopped proliferation when BMI-1 was inhibited (Figure 3A).

To confirm the disappearance of stem cell traits in BMI-1 blocked cells, the *hTERT*-transduced cells (*hTERT*⁺–OME and *hTERT*⁺–HaCaT) and their paired BMI-1-inhibiting cells were studied by mono-spheroid colony formation. The HaCaT cells were used as a negative control. The *hTERT*-transduced cells survived in suspension and gave rise to mono-spheroid colony. On the other hand, their paired BMI-1-inhibiting cells had no ability to form mono-spheroid or only aggregated together (Figure 3B).

DISCUSSION

Immortalized cell lines of oral keratinocytes are created by high-risk human papillomavirus (HPV) 16 DNA infection, attributing to its E6 and E7 reading frames (Sdek *et al*, 2006). In 2010, an immortalized cell model of human oral keratinocytes was established by subculturing the cells (keratinocyte growth medium containing 0.15 mM calcium without serum) repeatedly until they reached the post-mitotic stage and bypassed check-points (Jang *et al*, 2010). The cell line was named Spi-HOK1 and was the first spontaneously immortalized oral keratinocytes without the use of HPV gene delivery (Jang *et al*, 2010). Similarly, Boukamp and his colleagues used epithelial cells from skin to acquire immortality by growing those cells in a modified cell-culture condition (low Ca^{2+} and high temperature) (Boukamp *et al*, 1988). These immortalized keratinocytes, which was named HaCaT, could simultaneously bypass senescence via up-regulation of *hTERT* as well as crisis via *p53* mutation (Boukamp *et al*, 1988). *p53* mutation was found both in the two spontaneously immortalized cell lines (Spi-HOK1 and HaCaT). However, the down-regulation of *p16*^{INK4a} was only appeared in Spi-HOK1 cells (Jang *et al*, 2010; Boukamp *et al*, 1988). *p16*^{INK4a} and *hTERT* were noted to be frequently expressed in cancer and correlated with grade, pathological staging and prognosis (Lam *et al*, 2008a; Yuen *et al*, 2002). In this study, *p16*^{INK4a} expression in *hTERT*-induced cell immortalization was studied.

In our previous report, *hTERT*+OME cells were immortalized successfully by the silencing of *p16*^{INK4a} pathway, and these cells could undergo EMT spontaneously (Qiao *et al*, 2012). The same phenomena were seen in *hTERT*+HaCaT cells. HaCaT cells however, expressed *hTERT* and *p16*^{INK4a} simultaneously, challenging the concept that p16 expression was negative regulated by *hTERT*. Due to the fact that *hTERT* can only be detected on basal cells of epithelium *in vivo*, some authors explained that HaCaT cell line probably came from

a single precursor basal cell in the primary culture (Harle-Bachor *et al*, 1996). This allows HaCaT cells express *hTERT* spontaneously.

BMI-1 was an essential constituent of the polycomb repressive complex 1, a key epigenetic regulator (Schuringa and Vellenga, 2010). Similar to *hTERT*, BMI-1 can suppress the *p16^{INK4a}/p19Arf* locus (Itahana *et al*, 2003). It was used combining with other triggers, e.g. HPV 16 E6 to immortalize somatic cells (Kim *et al*, 2007). HPV 16 E6 oncoprotein binds and inactivates p53 and then inhibits apoptosis via Bax to bypass crisis (Scheffner *et al*, 1990). Thus, BMI-1 may take the role of bypassing senescence in immortalization, akin to *hTERT*.

In this study, both *hTERT*-transduced cells (*hTERT*+/-OME, *hTERT*+/-HaCaT) highly expressed BMI-1 at mRNA and protein levels while it was not expressed in HaCaT cells. It is likely that BMI-1 strongly expressed in *hTERT*-transduced cells may be related to the exogenous gene transduction of *hTERT*. Also, the simultaneously expression of *hTERT* and *p16^{INK4a}* in HaCaT cells may be attributed to the absence of BMI-1. Maurelli and his colleagues found both *hTERT* and BMI-1 were up-regulated in *p16^{INK4a}* inactivated primary cultured skin keratinocytes (Maurelli *et al*, 2006). However, Lee and colleagues suggested that the elevated BMI-1 expression did not alter *p16^{INK4a}* expression (Lee *et al*, 2008). In the present study, we knocked down the expression of BMI-1 by siRNA assay in *hTERT*-transduction cells. The results revealed that the expression of *p16^{INK4a}* was regained, while the expression of *hTERT* was still high. Hence, *p16^{INK4a}* expression or silencing is not likely to be caused by *hTERT* alone. It is more likely that the effect is due to the combined activity of *hTERT* and BMI-1.

hTERT could stimulate EMT and induce stemness of cancer cells (Liu *et al*, 2012b). The mono-spheroid colony formation is a trait of self-renewal of stem cells (Short and Wagey, 2013). In our previous report, it was shown that the *hTERT*+/-OME cells could survive in 3-

D culture system and form mono-spheroid colony. In this study, mono-spheroids were noted in *hTERT*⁺-HaCaT cells. However, the HaCaT cells cannot survive in suspension culture. It seems that immortalization itself is not prerequisite for mono-spheroid colony formation. When the expression of BMI-1 was knocked down in the *hTERT*⁺-OME cells and *hTERT*⁺-HaCaT cells, akin to HaCaT cells (BMI-1 was silencing, *p16*^{INK4a} was active), the ability of mono-spheroid colony formation of these two *hTERT*-transduced cells was disappeared.

Conclusion

Our study confirms the silencing of *p16*^{INK4a} and the reactivation of BMI-1 are the results of *hTERT*-induced immortalization. The interplay between BMI-1 and *p16*^{INK4a} endows *hTERT*-transduced cells with stem cell traits. Thus, targeting BMI-1 inhibition has a potential role in treatment of oral cancer.

ACKNOWLEDGEMENTS

The authors declare no conflict of interest of this paper. This study was supported by the National Natural Science Foundation of China (Grant No. 81072227; Grant No. 81200796), funding from Youth Foundation of The First Affiliated Hospital of Zhengzhou University and funding from Griffith University.

REFERENCES

1. Boukamp P, Petrussevska RT, Breitkreutz D, *et al*, 1988. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell. Biol.* 106, 761-771.
2. Boukamp P, Popp S, Altmeyer S *et al*, 1997. Sustained nontumorigenic phenotype correlates with a largely stable chromosome content during long-term culture of the human keratinocyte line HaCaT. *Genes Chromosomes Cancer*. 19, 201-214.
3. Chow V, Law S, Lam KY, *et al*, 2001. Telomerase activity in small cell esophageal carcinoma. *Dis. Esophagus*. 14, 139-142.
4. Egbuniwe O, Idowu BD, Funes JM, *et al*, 2011. P16/p53 expression and telomerase activity in immortalized human dental pulp cells. *Cell Cycle*. 10, 3912-3919.
5. Härle-Bachor C, Boukamp P, 1996. Telomerase activity in the regenerative basal layer of the epidermis in human skin and in immortal and carcinoma-derived skin keratinocytes. *Proc. Natl. Acad. Sci. U. S. A.* 93, 6476-6481.
6. Itahana K, Zou Y, Itahana Y *et al*, 2003. Control of the replicative life span of human fibroblasts by p16 and the polycomb protein Bmi-1. *Mol. Cell. Biol.* 23, 389-401.
7. Jang da H, Oh JE, Kang HK *et al*, 2010. Spontaneous tumorigenicity of primary human oral keratinocytes with human papillomavirus negativity and impaired apoptosis. *Int. J. Oncol.* 36, 1491-1501.
8. Jiang XR, Jimenez G, Chang E *et al*, 1999. Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype. *Nat. Genet.* 21, 111-114.
9. Kim RH, Kang MK, Shin KH *et al*, 2007. Bmi-1 cooperates with human papillomavirus type 16 E6 to immortalize normal human oral keratinocytes. *Exp. Cell. Res.* 313, 462-472.

10. Lam AK, Ong K, Giv MJ, *et al*, 2008a. p16 expression in colorectal adenocarcinoma: marker of aggressiveness and morphological types. *Pathology*. 40, 580-585.
11. Lam AK, Ong K, Ho YH, 2008b. Aurora kinase expression in colorectal adenocarcinoma: correlations with clinicopathological features, p16 expression, and telomerase activity. *Hum. Pathol.* 39, 599-604.
12. Lam AK, Ong K, Ho YH, 2008c. hTERT expression in colorectal adenocarcinoma: correlations with p21, p53 expressions and clinicopathological features. *Int. J. Colorectal Dis.* 23, 587-594.
13. Lam AK, Saleh S, Smith RA, *et al*, 2008d. Quantitative analysis of survivin in colorectal adenocarcinoma: increased expression and correlation with telomerase activity. *Hum. Pathol.* 39, 1229-1233.
14. Lam KY, Law S, Tung PH, *et al*, 2001. Esophageal basaloid squamous cell carcinoma: an unique clinicopathological entity with telomerase activity as a prognostic indicator. *J. Pathol.* 195, 435-442.
15. Lam KY, Lo CY, Fan ST, *et al*, 2000. Telomerase activity in pancreatic endocrine tumours: a potential marker for malignancy. *J. Clin. Pathol. Mol. Pathol.* 53, 133-136.
16. Lee K, Adhikary G, Balasubramanian S *et al*, 2008. Expression of Bmi-1 in epidermis enhances cell survival by altering cell cycle regulatory protein expression and inhibiting apoptosis. *J. Invest. Dermatol.* 128, 9-17.
17. Liu Y, El-Naggar S, Darling DS, *et al*, 2008. Zeb1 links epithelial-mesenchymal transition and cellular senescence. *Development.* 135, 579-588.
18. Liu ZG, Liu L, Xu LH *et al.*, 2012a. Bmi-1 induces radioresistance in MCF-7 mammary carcinoma cells. *Oncol. Rep.* 27, 1116-1122.

19. Liu Z, Li Q, Li K *et al.*, 2012b. Telomerase reverse transcriptase promotes epithelial-mesenchymal transition and stem cell-like traits in cancer cells. *Oncogene* doi: 10.1038/onc.2012.441.
20. Lo CY, Lam KY, 1999. Telomerase activity in thyroid malignancy. *Thyroid*. 112, 263-270.
21. Lundberg AS, Hahn WC, Gupta P, *et al*, 2000. Genes involved in senescence and immortalization. *Curr. Opin. Cell Biol.* 12, 705-709.
22. Maurelli R, Zambruno G, Guerra L *et al.*, 2006. Inactivation of p16INK4a (inhibitor of cyclin-dependent kinase 4A) immortalizes primary human keratinocytes by maintaining cells in the stem cell compartment. *FASEB. J.* 20, 1516-1518.
23. McMurray HR, McCance DJ, 2004. Degradation of p53, not telomerase activation, by E6 is required for bypass of crisis and immortalization by human papillomavirus type 16 E6/E7. *J. Virol.* 78, 5698-5706.
24. Muntoni A, Fleming J, Gordon KE *et al.*, 2003. Senescing oral dysplasias are not immortalized by ectopic expression of hTERT alone without other molecular changes, such as loss of INK4A and/or retinoic acid receptor-beta: but p53 mutations are not necessarily required. *Oncogene*. 22, 7804-7808.
25. Nakamura M, Tokura Y, 2011. Epithelial-mesenchymal transition in the skin. *J. Dermatol. Sci.* 61, 7-13.
26. Park IK, Morrison SJ, Clarke MF, 2004. Bmi1, stem cells, and senescence regulation. *J. Clin. Invest.* 113, 175–179.
27. Qiao B, Gopalan V, Chen Z, *et al*, 2012. Epithelial-mesenchymal transition and mesenchymal-epithelial transition are essential for the acquisition of stem cell properties in hTERT-immortalised oral epithelial cells. *Biol. Cell.* 104, 476-489.

28. Rheinwald JG, Hahn WC, Ramsey MR, *et al.*, 2002. A two-stage, p16 (INK4A)- and p53-dependent keratinocyte senescence mechanism that limits replicative potential independent of telomere status. *Mol. Cell. Biol.* 22, 5157-5172.
29. Rosivatz E, Becker I, Specht K *et al.*, 2002. Differential expression of the epithelial-mesenchymal transition regulators snail, SIP1, and twist in gastric cancer. *Am. J. Pathol.* 161, 1881-1891.
30. Saleh S, Lam AK, Ho YH, 2008. Real-time PCR quantification of human telomerase reverse transcriptase (hTERT) in colorectal cancer. *Pathology.* 40, 25-30.
31. Saleh S, Lam AK, Gertraud Buettner P, *et al.*, 2007. Telomerase activity of basal cell carcinoma in patients living in North Queensland, Australia. *Hum. Pathol.* 38, 1023-1029.
32. Scheffner M, Werness BA, Huibregtse JM, *et al.*, 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell.* 63, 1129-1136.
33. Schuringa JJ, Vellenga E, 2010. Role of the polycomb group gene BMI1 in normal and leukemic hematopoietic stem and progenitor cells. *Curr. Opin. Hematol.* 17, 294-299.
34. Sdek P, Zhang ZY, Cao J, *et al.*, 2006. Alteration of cell-cycle regulatory proteins in human oral epithelial cells immortalized by HPV16 E6 and E7. *Int. J. Oral. Maxillofac. Surg.* 35, 653-657.
35. Shay JW, Wright WE, 2005. Senescence and immortalization: role of telomeres and telomerase. *Carcinogenesis.* 26, 867-874.
36. Short B, Wagey R, 2013. Isolation and culture of mesenchymal stem cells from mouse compact bone. *Methods Mol. Biol.* 946, 335-347.

37. Skrzypeczak J, Wirstlein P, Mikołajczyk M, *et al.*, 2007. TGF superfamily and MMP2, MMP9, TIMP1 genes expression in the endometrium of women with impaired reproduction. *Folia. Histochem. Cytobiol.* 45, S143-148.
38. Song LB, Li J, Liao WT *et al.*, 2009. The polycomb group protein Bmi-1 represses the tumor suppressor PTEN and induces epithelial-mesenchymal transition in human nasopharyngeal epithelial cells. *J. Clin. Invest.* 119, 3626-3636.
39. Tang WK, Chui CH, Fatima S *et al.*, 2007. Inhibitory effects of *Gleditsia sinensis* fruit extract on telomerase activity and oncogenic expression in human esophageal squamous cell carcinoma. *Int. J. Mol. Med.* 19, 953-960.
40. Tao Q, Lv B, Qiao B, *et al.*, 2009. Immortalization of ameloblastoma cells via reactivation of telomerase function: Phenotypic and molecular characteristics. *Oral Oncol.* 45, e239-244.
41. Yuen PW, Man M, Lam KY, *et al.*, 2002. Clinicopathological significance of p16 gene expression in the surgical treatment of head and neck squamous cell carcinomas. *J. Clin. Pathol.* 55, 58-60.

LEGENDS

Figure 1

The establishment of *hTERT*⁺-HaCaT cells and identification of their EMT traits

(A) Cell morphological alterations: (a) HaCaT cells show polygonal morphology and a cobblestone arrangement. (b) *hTERT*⁺-HaCaT grow scattered in low density. (c) *hTERT*⁺-HaCaT cells convert back to an epithelial cell phenotype at higher confluence.

(B) Identification of EMT traits by immunocytochemistry:

hTERT⁺-HaCaT cells express both cytokeratin (a) and vimentin (b).

(C) Markers of cell cycle and EMT are studied by RT-PCR and Western blots

(1) HaCaT cells; (2) *hTERT*⁺-HaCaT cells. GAPDH was used as controls. At both mRNA and protein levels, *hTERT* expression increases in *hTERT*⁺-HaCaT cells, while *p16* is almost completely downregulated. In contrast, expressions of both *p16* and *hTERT* proteins and mRNA are detected in HaCaT cells. Moreover, the expression of Twist (EMT-inducer) is found in *hTERT*⁺-HaCaT cells at both mRNA and protein levels.

Figure 2

Relationship between BMI-1 expression, cell cycle and EMT markers

(A) BMI-1 expression in different cell lines

The BMI-1 expression is upregulated in *hTERT*-transduced cells. Also, BMI-1 is positive expressed in primary cultured oral mucosal epithelial (OME) cells, but is negative in HaCaT cells.

(B) The alterations of differential markers of cell cycle and EMT when BMI-1 is inhibited.

At both mRNA and protein levels, the expression of *hTERT* is unchanged, while the expression of *p16* is regained in BMI-1 blocking cells. The EMT markers (Twist, vimentin) are decreased in BMI-1 blocking cells. The expression of E-cadherin (another EMT marker) keeps silent even if the BMI-1 is blocked.

Figure 3

The blocking of BMI-1 expression and cellular properties

(A) The alterations of cell kinetics curves on MTT assay.

hTERT⁺-OME cells stop proliferation when they undergo siRNA transduction at day-3 (left panel). Moreover, the BMI-1 blocked *hTERT*⁺-HaCaT cells grow much slower than uninhibited cells. The BMI-1 blocked *hTERT*⁺-HaCaT cells share the similar proliferation rate with HaCaT cells (right panel). * The significantly differences are set at $p < 0.05$.

(B) The mono-spheroid colony formation.

The mono-spheroid formation of *hTERT*-transduced cells is decreased in BMI-1 blocked cells: the HaCaT cells (control cells) cannot form mono-spheroid colony even they are cultured in suspension for 5 days. Similarly, when BMI-1 is knocked down in *hTERT*-transduced cells, the ability of spheroid formation of these cells is disappeared ($\times 100$).