

Enhanced Proliferative but not the Pro-Thrombotic Potential of Vascular Smooth Muscle Cells with Telomerase is Compromised Concurrent with p53 Induction

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Received Date: January 14, 2014 Accepted Date: March 01, 2014 Published Date: March 19, 2014

Citation: Vipul Chitalia, et al. (2014) Enhanced Proliferative but not the Pro-Thrombotic Potential of Vascular Smooth Muscle Cells with Telomerase is Compromised Concurrent with P53 Induction. J Cardio Vasc Med 2: 1-8

Abstract

Objective: Primary human cells undergo a finite number of cell divisions before entering a non-dividing state, which is contributed by an incremental attrition of telomeres. Replenishment of telomerase enzyme to restore telomere ends has been leveraged in biomedical research to prolong passages of primary human cells. Such cells still experience a non-proliferative state with variable differentiation features after a finite number of passages through incompletely understood mechanism. Here we show that compared to parental vascular smooth muscle cells (vSMCs), primary human aortic vSMCs stably expressing human telomerase reverse transcriptase (hTERT) maintain the biochemical and functional phenotypes of parental cells up to 30 passages. At this point, robust induction of p53 coincided with the proliferative failure despite intact telomerase enzyme activity. Intriguingly, the vSMCs maintained a stable profile of tissue factor, a potent procoagulant, spanning over all the passages. These data demonstrate a dissociation of proliferative and thrombotic functions of vSMCs and also raise a possibility of p53 as a potential inhibitor to induce a non-diving state even in the *milieu* of normal telomerase activity.

Keywords: Vascular smooth muscle cell line, p53, Telomerase, Immortalization

Introduction

Primary human cells experience a limited life span and attain an irreversible G1 cell cycle arrest [1]. At that stage, the cells cease to respond to mitogenic stimuli and change their morphological and functional profiles, both of which further limit their use for experimental purposes [1]. These functional changes in cells over time are induced by various physiological and pathological stresses of which the attrition of telomere is one of the major contributors [2, 3].

The molecular causes of telomere shortening are thought to originate from “end replication problem”, the inability of DNA replication machinery to complete DNA synthesis at the lagging strand [2]. Eventually, this shortening leads to telomere uncapping, which is the disruption of the

proper structure of the protective cap at the end of the telomere [2]. This capping is crucial as it is recognized as DNA breaks, in turn activating DNA damage pathways resulting in cell cycle arrest [4,5]. Forced uncapping of telomeres through inhibition of the telomere binding protein induces loss of the telomeric overhang and leads to permanent failure to proliferate, alters morphology and functional characteristics of cells, all of which characterize senescence [3, 6–9]. The introduction of double stranded DNA break results in p53 induction, through yet poorly understood mechanism [10, 11]. Ectopic expression of telomerase enzyme to ensure persistent regeneration of telomere ends at every passage circumvents the problem of telomere attrition [12]. This is expected to result in unlimited cell passages, thus this technique has been leveraged in biomedical research [13]. Despite the expression of telomerase enzyme, the cells still undergo proliferative failure after a finite number of passages through yet poorly characterized mediators.

Primary human vascular smooth muscle cells (vS-

MCs) are a widely used cell-type in vascular biological research. However, their limited passages (10-12 passages), batch-to-batch variation due to different donors introduce inter-experimental variability and high cost, limiting their use in biomedical research. We set out to obtain vSMCs cell lines that retain biochemical and functional characteristics of its parental cells and also allow more passages using stable expression of telomerase enzyme. Telomerase successfully retained the telomere length till 30-cell passages without compromising vSMCs thrombotic potential. However, despite intact telomerase enzyme, these cells failed to propagate beyond 30 passages coinciding with p53 induction, a potent cell cycle inhibitor. Intriguingly, this proliferative failure was not accompanied by the changes in morphology or thrombotic phenotype.

Methods

Cell culture

Vascular smooth muscle cells (vSMCs) (ATCC, USA) were grown in DMEM low glucose with 5% calf serum, 5% penicillin and streptomycin. The HEK-293T was grown as described previously [14, 15]. Human umbilical vein endothelial cells (HUVECs) (Promocell, Germany) pooled from 3 donors were grown in endothelial growth medium-2 (EGM-2) (Promocell, Germany). EGM-2 was prepared by supplementing endothelial basal medium (EBM-2) with fetal bovine serum (2%), hydrocortisone (1 μ g/ml), fibroblast growth factor-1 (10 ng/ml), epidermal growth factor (5 ng/ml), insulin-like growth factor (20 ng/ml), ascorbic acid (1 μ g/ml) and heparin (90 μ g/ml).

hTERT construct and generation of viral particles

Retroviral construct-plasmid from Addgene (Cat#1771) pBABE puro-hTERT(telomerase reverse transcriptase) was transfected in HEK293T packaging cells along with packaging, envelope and Reverse transcriptase vectors using Lipofectamine 2000 per manufacturer instructions. Medium containing active viral particle collected after 48 h was centrifuged, snapped frozen and stored at -80 °C. For viral transduction, the vSMCs seeded at 50-60% confluence were treated with viral medium along with Polybrene 1 μ g/ml (Sigma#107689). After determining the killing curve, Puromycin (Sigma#P9620) selection was initiated after 24 hours of transduction.

Immunoblotting

Cells were lysed in 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 30 mM EDTA, 0.5% Triton X-100 with complete protease inhibitor (Roche# 11873580001). Immunoblotting was performed as described previously[14] using antibodies; Vascular smooth muscle cell actin mouse (Sigma# A2547), myosine heavy chain rabbit (Santacruz# sc20641) and p53 mouse (Cell signaling# 9282) and smooth muscle cell kinase (Abcam # ab55475).

Immunofluorescence

Cells were grown in Chamber slides (BD falcon# 354656) and fixed with 4% PFA and processed as described previously[14]. Alexa 647 goat anti-mouse was used as secondary antibody.

vSMCs proliferation assay

5 \times 10³ vSMCs seeded in 48-well plates were synchronized overnight by serum starvation with 0% calf serum (CS). Medium was then switched after 16–18 hours to the 5% CS. One μ Ci of ³[H] thymidine (Perkin-Elmer, Boston, MA) was added to each well overnight and the cells were harvested after 16–24 h of treatment. Cells were washed with ice-cold PBS thrice followed by the precipitation of protein in 10% trichloroacetic acid in PBS for 30 min at 4 °C, washed with 90% ethanol and solubilized in 1 mL of 0.25 M NaOH with 0.1% SDS for 1 h. Samples were added to scintillation cocktail and radioactivity measured by liquid scintillation counting (Beckman Coulter).

TF activity assay

vSMCs were lysed using 50mM Tris buffered saline (pH 8.0) with 1% Triton X-100 and centrifuged at 14000g for 20 min. TF levels were measured using human tissue factor chromogenic activity kit* (Assaypro #CT1002b) using manufacturer's instructions. This kit measures the procoagulant activity/peptidyl activity in cell lysates and human plasma. The amount of factor Xa generated is measured by its ability to cleave highly specific chromogenic substrate for factor Xa, which is added to the reaction. TF levels are normalized to number of vSMCs and ug of protein.

Quantitative detection of telomerase activity by PCR

vSMCs were lysed with 1x Lysis buffer, centrifuged at 14000RPM for 30 minutes. The supernatant is assayed for Telomerase activity using real time PCR (Allied Biotech Inc # MT3010) per manufacturer's instructions. Telomerase activity is normalized to number of cells and ug of protein. Telomerase from the cells lysate adds telomeric repeats onto a substrate oligonucleotide and the resultant extended products are subsequently amplified by the polymerase chain reaction.

Statistics

Summary statistics are presented as mean and standard error of mean. Paired-t test was used to compare two groups and p <0.05 was considered significant.

Results

hTERT/vSMCs retain parental phenotype up to passage 30

Primary human aortic vascular smooth muscle cells (vSMCs) stop growing after 12 passages, while those stably expressing telomerase enzyme (hTERT/vSMCs) could be propagated up to 30 passages. We examined the markers of vascular smooth muscle cells in hTERT/vSMCs and compared to parental vSMCs at passage 3. HEK293T and human umbilical vein endothelial cells (HUVECs) served as controls. The results showed that hTERT/vSMCs even till passage 30 continue to express various smooth muscle cell markers such as alpha smooth muscle actin (Figure. 1A), heavy chain myosin (Figure. 1B) and smooth muscle light chain kinase (Figure. 1C) comparable to that of parental vSMCs. As expected, HEK293T and HUVECs showed no smooth muscle cell markers. These data strongly suggest that hTERT/vSMCs retain smooth muscle phenotype up to 30 passages.

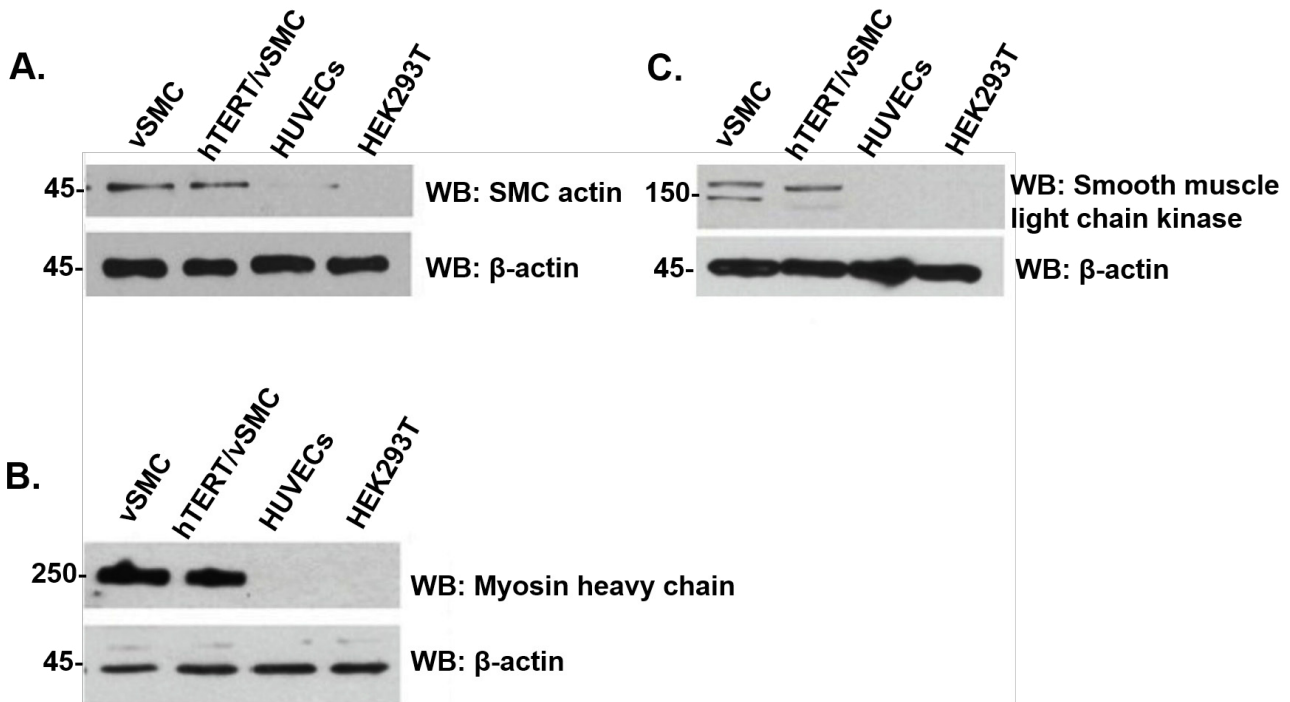


Figure 1: hTERT/vSMCs retain the contractile markers

A) vSMCs retain parental phenotype after 30 passages. Parental vSMCs (passage 3), stably transduced hTERT/vSMCs (passage 30), HUVECs and HEK 293T cells were harvested and the cell lysates were immunoblotted using SMC actin antibody and reprobed using β -actin antibody, where the later served as a loading control. Representative blot of three experiments is shown.

B) The SMC marker, myosin heavy chain expression remained unchanged up to 30 passages. Parental vSMCs (passage 3), hTERT/vSMCs (passage 30), HUVECs and HEK 293T cells were harvested and probed using heavy chain myosin and β -actin antibodies. Representative blot of three experiments is shown.

C) Smooth muscle cell myosin light chain kinase expression remained unchanged up to 30 passages. Parental vSMCs (passage 3), hTERT/vSMCs (passage 30), HUVECs and HEK 293T cells were harvested and probed as above. Representative blot of two experiments is shown.

hTERT /vSMCs tissue factor (TF) expression and activity remained unchanged up to passage 30

vSMCs are highly thrombogenic consistent with their high TF expression and activity compared to endothelial and THP-1 cells [14]. We compared TF expression in parental vSMCs, hTERT/vSMCs, HUVECs and HEK 293T using both immunoblotting and immunofluorescence. The expression levels of TF remained unchanged up to 30 passages in hTERT/vSMCs (Figure. 2A). TF expression was seen both in the cytosol and the membrane, suggesting proper localization of the protein in the hTERT/vSMCs (Figure. 2B). The expression of TF corroborated with TF activity (Figure. 2C). There was no significant difference in TF activity in parental compared to hTERT/vSMCs. All of the above data indicate that hTERT/vSMCs retain a stable TF profile and thrombogenicity for extended passages.

Cell proliferation reduced after passage 30

We examined the cell proliferation at different passages using ^3H thymidine inclusion, which is a direct measure of DNA replication (Figure. 3). The results revealed that there was no significant difference between parental vSMCs (passage 3) and hTERT/vSMCs 3, 7, 8, 11 and 15 passages. However, a trend towards reduced cell proliferation emerged with hTERT/vSMC at passage 21 ($p=0.058$) and became significant at pas-

sage 30 ($p=0.006$) (Figure.3). These results indicate that proliferative failure of hTERT transduced vSMCs after 30 passages.

hTERT /vSMCs retain telomerase expression up to passage 30

Reduced activity of telomerase and attrition of telomere length with further cell passages contribute to proliferative failure [2]. Therefore, we first confirmed the activity of telomerase in late passage hTERT/vSMCs using quantitative PCR (Figure. 4A). Telomerase activity showed no significant reduction with passage 30 of hTERT/vSMCs compared to parental or early passage of hTERT/vSMC. These data rule out the possibility of compromised telomerase activity as the cause of proliferative failure. On the other hand, it supports the notion that the cells lost the ability to proliferate despite normal telomere length and raises the possibility of other mediator.

Increase in p53 expression with extended passages of hTERT/vSMCs

We further probed for potential mediators of proliferative failure after passage 30. p53 is a well-established robust inducer of cell cycle arrest [16–20]. Therefore we posited that

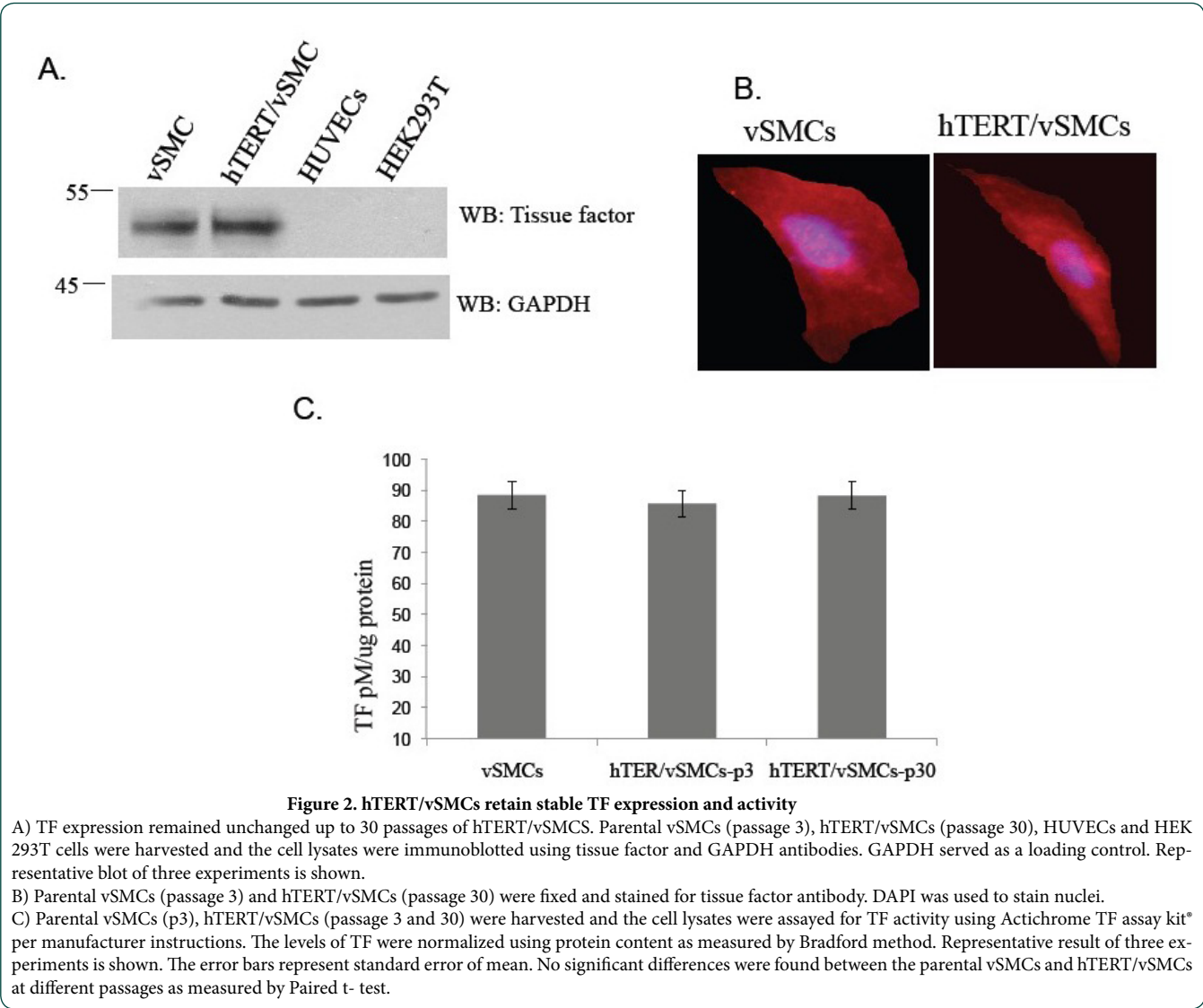


Figure 2. hTERT/vSMCs retain stable TF expression and activity

A) TF expression remained unchanged up to 30 passages of hTERT/vSMCs. Parental vSMCs (passage 3), hTERT/vSMCs (passage 30), HUVECs and HEK 293T cells were harvested and the cell lysates were immunoblotted using tissue factor and GAPDH antibodies. GAPDH served as a loading control. Representative blot of three experiments is shown.

B) Parental vSMCs (passage 3) and hTERT/vSMCs (passage 30) were fixed and stained for tissue factor antibody. DAPI was used to stain nuclei.

C) Parental vSMCs (p3), hTERT/vSMCs (passage 3 and 30) were harvested and the cell lysates were assayed for TF activity using Actichrome TF assay kit* per manufacturer instructions. The levels of TF were normalized using protein content as measured by Bradford method. Representative result of three experiments is shown. The error bars represent standard error of mean. No significant differences were found between the parental vSMCs and hTERT/vSMCs at different passages as measured by Paired t- test.

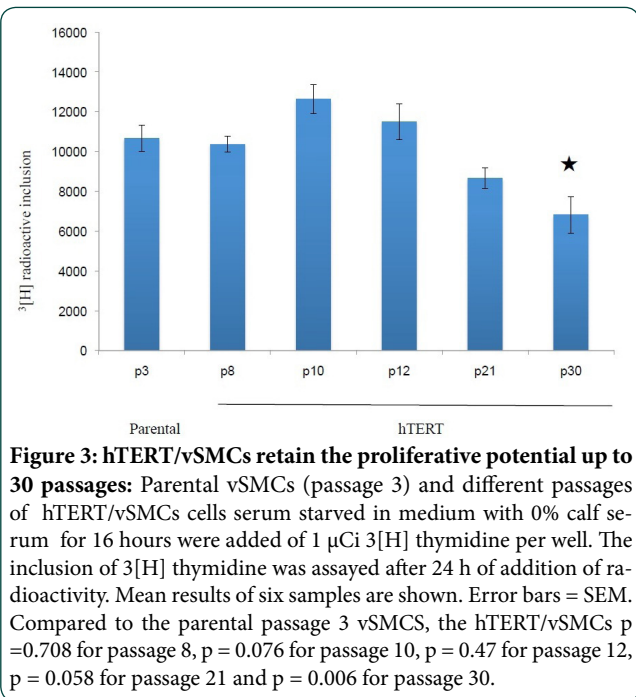


Figure 3: hTERT/vSMCs retain the proliferative potential up to 30 passages: Parental vSMCs (passage 3) and different passages of hTERT/vSMCs cells serum starved in medium with 0% calf serum for 16 hours were added of 1 μCi ³[H] thymidine per well. The inclusion of ³[H] thymidine was assayed after 24 h of addition of radioactivity. Mean results of six samples are shown. Error bars = SEM. Compared to the parental passage 3 vSMCS, the hTERT/vSMCs p = 0.708 for passage 8, p = 0.076 for passage 10, p = 0.47 for passage 12, p = 0.058 for passage 21 and p = 0.006 for passage 30.

p53 expression will be higher in later passages of hTERT/vSMCs compared to the parental vSMCs (Figure. 4B). Early passage hTERT/vSMCs and HUVECs were also included as controls. HEK293T cells served as a positive control. Indeed, p53 expression was virtually undetectable in parental and various passages of vSMCs and HUVECs. However, p53 increased with hTERT/vSMCs at passage 30 coinciding with the proliferative failure, raising a possibility of p53 as a candidate mediator of reduced proliferation of vSMCs after passage 30. Interestingly, TF expression remained unchanged till passage 30 (Figure. 4B).

Discussion

Proliferative potential of primary human aortic vSMCs abrogates after 12 passages. Our hTERT/vSMC cell lines not only were capable of extended proliferation up to 30 passages, but also retained the biochemical and the proliferative and thrombotic features similar to un-transduced parental primary human aortic vSMCs. Interestingly, in these cells, the proliferation still was inhibited with the intact telomerase activity concurrent with the induction of p53, a potent and well-established mediator of cell cycle arrest. In essence, passage 30 marked the dissociation of the proliferative and thrombotic potentials of vSMCs.

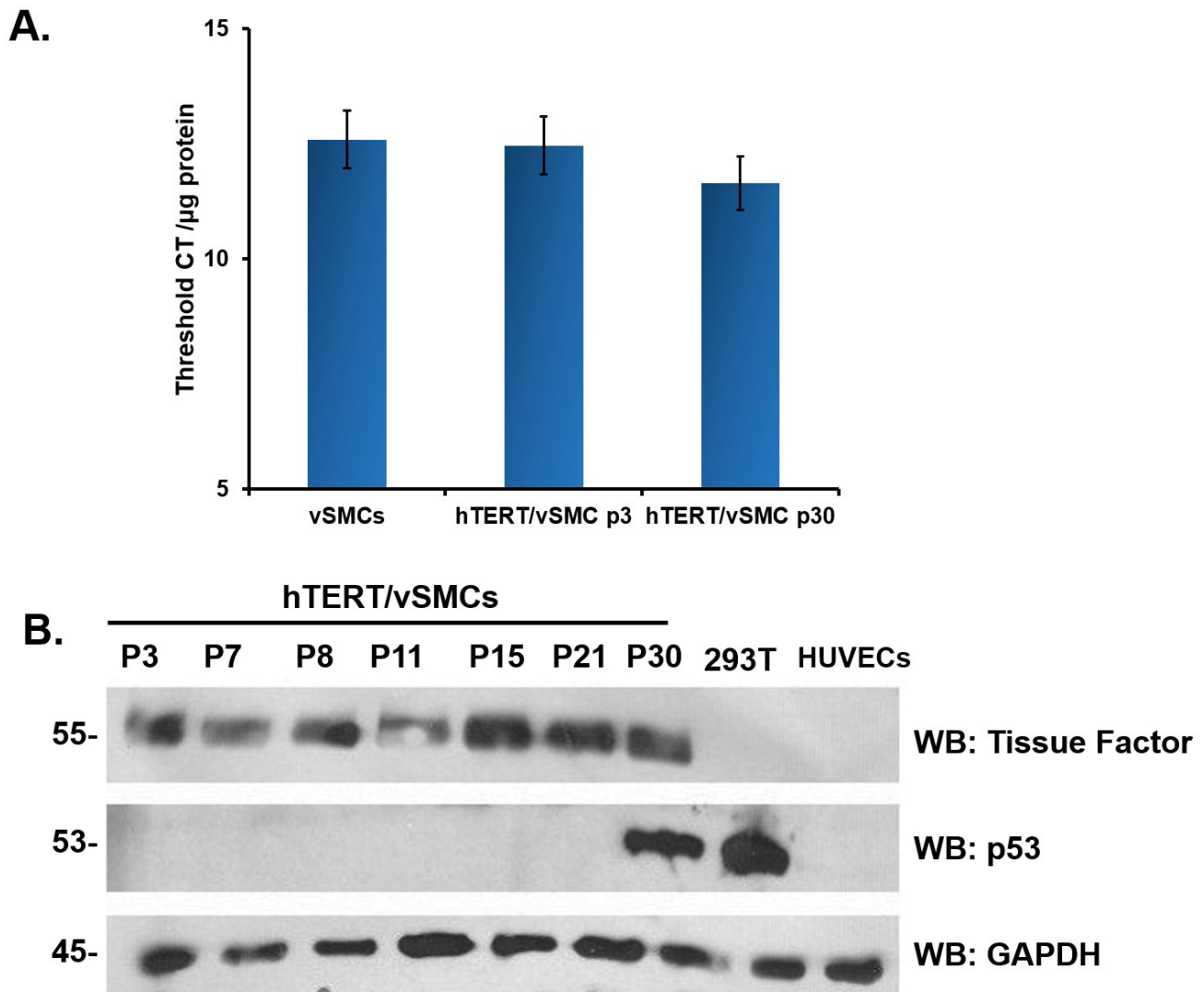


Figure 4. p53 induction in hTERT/vSMCs in the milieu of intact telomerase activity

A) vSMCs retain Telomerase activity up to passage 30. Parental vSMCs-passage 3 and stably transduced vSMCs/hTERT- passage 3 and 30 were lysed and assayed for telomerase activity using quantitative telomerase activity kit per manufacturer's instructions. Mean of two experiments is shown. Error bars = SEM. No significant difference was found among the samples.

B) Induction of p53 in vSMCs-hTERT and unchanged TF expression with various passages of hTERT/vSMCs. Parental vSMCs (passage 3) and stably transduced hTERT/vSMCs from various passages were harvested and the cell lysates were immunoblotted using p53 and TF antibodies and β -actin as loading control. Representative blot of three experiments is shown.

There are multiple approaches to immortalize cell lines such as by stably expressing simian virus (SV40) T antigen, hTERT, HPV E6/E7, EBV, MycT58A, and p53 cell immortalization systems with each method having specific limitations [21–28]. For example, Legrand et al used SV40 early region to immortalize arterial smooth muscle cells. Again those cells changed to multinucleated morphology, had chromosomes in the triploid range, and developed genomic aberrations and smooth muscle actin was significantly reduced [29]. The E6 and E7 open reading frames of human papillomavirus type 16 were also used to immortalize cells [21, 30]. Though the cells continued for 1 year for up to 180 passages, the trade off was loss of morphological features and markers of smooth muscle cells, suggesting that the cells underwent transformation in cell-type other than vSMCs. On the other hand, hTERT, extended the proliferative-life span of cells without compromising the original phenotype [31], and re-

duced the frequency of dicentric and abnormal chromosomes [32]. Hence, we selected hTERT over the other approaches.

Leveraging the findings that hypoxia increases the activity of the telomerase, Minamino T, et al. [13] increased proliferative potential of the vSMCs by hTERT overexpression. They examined vSMC population doubling (spanning over 1.5 months) and demonstrated the absence of dedifferentiation vSMCs, while achieving prolonged vSMC survival. Though our work exploits a similar strategy of hTERT, there remain several differences. First, the present body of work examines vSMC population doubling spanning over 3-4 months and the fate of vSMCs after that, the point where Minamino T, et al's study stopped. Second, the data indicate the dissociation of proliferation and thrombotic potentials of vSMCs. The vSMCs continued to robustly maintain their tissue factor expression and activity (Figure. 2

and Figure. 4c) even with the loss of proliferative phenotype.

The mechanism of telomerase extending cell's life span is well established [4,33]. Normal cells express low levels of telomerase enzyme and the inhibition of telomerase leads to decrease cell proliferation [5]. Telomerase is a reverse transcriptase that replaces the eroded telomeres. Its increased activity in germ line cells impart them potential to proliferate compared to the somatic cells during development. The enzyme telomerase is a ribonuclear protein composed of at least two subunits; an integral RNA that serves as a template for the synthesis of telomeric repeats and a protein (hTERT) that has reverse transcriptase activity. The RNA component is ubiquitous in human cells, but the presence of mRNA encoding hTERT is restricted to cells with telomerase activity [34]. Ectopic expression of telomere catalytic subunit prevents telomere uncapping and immortalizes the cell line. In immortal cells, loss of telomeric DNA is balanced by telomere elongation catalyzed by hTERT.

Maintaining the differentiation as vSMCs

Our confirmation of the hTERT/vSMCs cell lines maintaining phenotype, functionality and differentiation of primary human vSMCs arise from number of orthogonal tests. The cells retained 'signature' markers such as alpha smooth muscle cell and myosin heavy chain and smooth muscle myosine light chain kinase, which are the components of the contraction and relaxation machinery (Figure. 1). In addition, hTERT/vSMCs expressed comparable expression and proper localization of the thrombogenic marker - tissue factor, a critical feature of vSMCs (Figure. 2). The hTERT/vSMCs retained the proliferative potential in response to external stimuli like mitogenic factors and also the TF activity, an indicator of its thrombogenicity (Figure. 2 and 3). Interestingly, while the proliferative potential was compromised after passage 30, but the morphological and thrombogenic features remained unaffected suggesting that vSMCs retained their differentiation even while entering non-replicative phase. This is contrast with several other cell types such as adipose-derived stromal cells, which loses their differentiation while entering proliferative failure [35]. It is possible that hTERT expression helped the maintenance of vSMC differentiation, a phenomenon also observed in other cell-types [36].

Role of p53 in as an inhibitor of cell proliferation

The p53, a master tumor suppressor gene, is a sequence specific transcription factor, which senses various stress signal. It is a well-established and potent inhibitor of G1, G2 and S phases cell cycle by activating number of cyclin inhibitors and suppressing cyclin dependent kinases. Known targets of p53 include CIP1/WAF1, GADD45, WIP1, MDM2, EGFR, PCNA, CyclinD1, CyclinG, TGF α and 14-3-3s, an array of genes that influence cell cycle [17,19]. p53 expression substantially increased after passage 30 coinciding with the proliferative failure raises a tantalizing possibility of p53 as a candidate mediator of this effect (Figure. 4B). The interrelationship of p53 and telomerase is complex and likely to be different in primary human cells compared to cancer cells. For example, in cancer cell lines, the expression of p53 directly repressed telomerase mRNA and telomerase activity before inducing cell cycle arrest [37]. While, in human vSMCs cell line expression of p53 had no effect on telomerase activity

(Figure. 4A), possibly due to the overexpression of hTERT in these cells. On the other hand, the loss of telomerase activity and the exposed telomere ends are known to induce p53 [38]. However, in hTERT/vSMCs, p53 induction happened even in the presence of intact telomerase activity (Figure. 4A) suggesting other mechanism of p53 induction by passage 30.

There can be several possible explanations of increased p53 in hTERT/vSMCs beyond 30 passages. Proliferating cells are known to accumulate different types of stresses such as DNA damage, heat shock and hypoxia, which can all induce p53 due to post-translational modifications [39–41]. Increase in reactive oxygen levels with cell proliferation induces DNA damage and p53 resulting in cell-cycle arrest [42,43]. Enhanced lysosomal activity with increased cell passages also serves as a potent signal modulating p53 activity [44]. All of the above cellular stresses result in cell cycle arrest through p53 induction [45,46].

The present study has limitations. As mentioned above, this study does not perform a complete characterization of vSMCs at genomic level. Though p53 is a well-established mediator of cell cycle arrest and proliferative failure and the present data point towards this possibility, but further work is needed to demonstrate a conclusive role of p53 in the proliferative failure of vSMCs at passage p53.

The present study does not rule out the possibility of other mediators inhibiting cell proliferation. Overall, the above investigation demonstrates the generation of a functionally and morphologically intact human aortic vSMC cell line up to 30 passages. Our data further suggests p53 as a potential mediator of this proliferative failure in the *milieu* of intact telomerase enzyme. Overall, using telomerase is distinctly a better approach preserving the phenotype of primary human cells compared to other strategies.

Acknowledgments

We thank Mercedes Ballcells (MIT) and Jordi Martu (IQS) for providing human primary aortic vascular smooth muscle cells. We appreciate critical input of John Schwartz and Jean Francis (BUSM) for reviewing this manuscript. This work was supported by NIH/NIDDK K08DK080946 and Department of Medicine Young Investigator Career Investment Award to V.C.C.

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