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Dual expression of hTERT and VEGF prolongs life span and enhances angiogenic ability of aged BMSCs

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ABSTRACT

Previous studies have confirmed the therapeutic effects of bone marrow stromal cells (BMSCs) transplantation on cerebral ischemia. However, the proliferative, differentiative, and homing capacity of BMSC from the elderly are significantly reduced, especially after several passages expansion in vitro. In this study, by introducing lentivirus-mediated hTERT and VEGF genes to modify human BMSCs from aged donors, we observed extended lifespan, promoted angiogenic capacity while less enhanced tumorigenicity of the genetically engineering BMSCs. These results therefore suggest that the modification of aged BMSCs by dual expression of hTERT and VEGF may be used for autologous cell replacement for ischemic cerebrovascular disease in elderly patients.

1. Introduction

Though animal experiments [1–3] and preliminary clinical studies [4,5] have confirmed the therapeutic effects of BMSCs transplantation on cerebral ischemia, the low frequency of MSCs in bone marrow necessitate their in vitro expansion prior to transplantation. However, hBMSCs gradually senesce during expansion in vitro, which reflects in reduced proliferative capacity, differentiation potency and homing ability to focal zone [6–11]. Moreover, it is well understood that the quantity and quality of hBMSCs in elderly are further reduced [12,13], which suggests that the residual proliferative and repair potential is insufficient to maintain long-term expansion and tissue regeneration upon reinfusion, making it difficult to be directly applied in autologous transplantation therapy without modifications [14].

Though the mechanisms of hBMSCs underlying cell senescence are still unclear, several studies have documented that lack of telomerase activity in hBMSCs is a very important reason [11,15–17]. Human telomerase is mainly consisted by catalytic subunit (TERT: telomerase reverse transcriptase), an RNA template for reverse transcription (TERC: telomerase RNA component) and the protein to promote telomerase stability [18]. TERT catalyzes TTAGGG repeats to the terminal end of a chromosome to maintain telomere length, which is crucial to maintain the cellular reproductive activity. Since TERT expression in hBMSCs is negligible, it is considered the main rate-limiting factor for telomerase activity.

Vascular endothelial growth factor (VEGF) is the key factor of angiogenesis during embryo development and vessel regeneration in adults. It has been proved to play an important role in the neuroprotection, neurogenesis and angiogenesis after focal cerebral ischemia [19–21]. With genetic modification of hBMSCs by introducing VEGF, it has been reported that the life span of modified cells could be prolonged [19], while the combinative advantages of introducing VEGF and other factors including hTERT in hBMSCs has not yet fully investigated.

Given the fact that the elderly are a high-risk population for stroke, it is necessary to explore ways to improve the quality of the BMSCs from elderly in order to facilitate autologous transplantation. To overcome the weakness of BMSCs from aged donors, we performed dual transfection of lentivirus-mediated hTERT and VEGF 165 genes into BMSCs from aged donors. Our results suggested that enhanced expression of hTERT and VEGF successfully extended the lifespan of aged hBMSCs, promoted their angiogenic capacity, but did not enhance their tumorigenicity. The modification of aged BMSCs by lentivirus-mediated hTERT and VEGF dual

Abbreviations: BCA, bicinchoninic acid; hBMSC, human bone marrow stromal cell; MSC, marrow stromal cell; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; hTERT, human telomerase reverse transcriptase; IS, internal standard; LP, lentiviral particle; MOI, multiplicity of infection; PDL, population doubling level; TERC, telomerase RNA component; TRAP, telomeric repeat amplification protocol; VEGF, vascular endothelial growth factor.

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gene transfection may promote the therapeutic ability of these cells in treatment of ischemic cerebrovascular disease.

2. Materials and methods

2.1. Ethics statement

The human study was approved by institutional review board of Southern Medical University affiliated Zhujiang Hospital and all the participants signed consent form. All animal procedures were performed under compliance with a protocol approved by the Animal Care and Use Committee (ACUC) at the Southern Medical University in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

2.2. Lentiviral vectors expressing human TERT and VEGF165 gene

The human TERT (hTERT) cDNA and VEGF165 cDNA were obtained by PCR amplification from pReceiver-M02-hTERT and pReceiver-M02-VEGF respectively, which were generously provided by Dr. Zhenzhou Chen from Southern Medical University. The cDNA sequence of hTERT or VEGF was subcloned into pDONR221 by using BP recombination system, and then was cloned from pDONR221 into ViraPower lentiviral expression system™ (pLenti6.3/V5-DEST, Invitrogen) by using LR clonase. The two recombinant plasmids and packaging mixture were transfected into 293FT using Lipofectamine 2000 reagent, and the lentiviral particles were collected. ELISA was employed to detect the lentiviral titers. Quantitative PCR, Western blot and fluorescence immunocytochemistry were carried out to detect the expression of hTRET and VEGF 165 in human BMSCs after lentiviral transfection. The average virus physical titer of hTERT was 1.07×10^9 LP/mL, while VEGF165 was 1.2×10^9 LP/mL.

2.3. Human BMSC preparation, transfection with lentivirus and culture expansion

The human BMSCs were harvested from three healthy male donors, aged 64, 67 and 71 respectively according to the previous description [22,23]. Since P1 cells were not enough for the follow-up experiments, the passage 2 (P2) cells were used for subsequent transfection. When P2 cells reached 50–60% confluence, transfection of hBMSCs was carried out with hTERT lentivirus (MOI: 3, PU/cell) or VEGF165 (MOI: 3, PU/cell) or both two constructs (MOI: 3, PU/cell) in the presence of 8 μg/mL polybrene (sc-134220, Santa Cruz, CA 95060, USA). Then the cells were divided into four groups: hBMSC (untreated), hBMSC-V, hBMSC-T and hBMSC-V&T. Successfully transfected cells were selected with blasticidin at a final concentration of 5 μg/mL (Blasticidin, Invitrogen) for 7 days. Afterwards, the culture medium was changed to normal full medium for further maintenance.

2.4. Angiogenesis assay in vitro and in vivo

Analysis of capillary formation in vitro was performed as described previously [24]. Totally 12 visual fields of 3 wells were selected and calculated in four groups. Percentage of cells form capillary structures was calculated by capillary structure cells divided by all the cells in a visual field.

Analysis of capillary formation in vivo was performed as described previously [24]. Briefly, 15 8-week-old male athymic nude mice weighing 25–30 g were used in specific pathogen-free conditions. The DiI labeled PD10 cells (5×10^5) of four groups suspended in 0.5 mL Matrigel were injected subcutaneously in the back of recipient mice. Cell-free Matrigel plugs with serum-free culture medium of the same size were used as control here. Three weeks later, the plugs were removed, fixed with formalin for 24 h in room temperature, and subsequently cut into 10-mm thick sections on a freezing microtome. The sections were stained with immunofluorescence stained for vWF. Nine visual fields of each group were selected and photographed under an immunofluorescence microscope. Vessel circumference length was calculated by Image-Pro Plus. Vessel density was calculated by numbers of vessels divided by the area of every visual field.

2.5. Soft agar anchor-independent assay and tumorigenicity assay

Soft agar anchor-independent assay and tumorigenicity assay were carried out as described previously [25,26]. HeLa cells were used as a positive control. The colony-forming efficiency was calculated by dividing the number of colonies with the number of plated cells. Each assay was performed in triplicate. 3×10^6 PD10 cells of hBMSC, hBMSC-V, hBMSC-T, hBMSC-V&T or HeLa cells were subcutaneously injected at the flank of abdomen for each athymic nude mouse and observation of tumor growth at the injection site lasted for 3 months.

2.6. Karyotypic evaluation

Cells in four groups at PD10 in metaphase were arrested with 0.05 mg/mL colcemid (Gibco BRL) for 1 h and then harvested according to the standard methods [27]. Slides for chromosomes painting were dehydrated sequentially with 70%, 90%, and 100% ethanol (10 min each) at room temperature and were then stored at −20 °C prior to use. Cytogenetic analysis was performed using Giemsa-banding. Interpretation of karyotypes was based on the International System for Human Cytogenetic Nomenclature (ISCN 2005 standard).

2.7. Data presentation and statistical analysis

All values were represented as average values with the standard error of mean. All significant differences were evaluated using one-way ANOVA (Dunnett's t test was used for comparison between groups) with the following nomenclature is used: $^*p < 0.05$.

3. Results

3.1. The expression of VEGF and hTERT changed the lifespan and morphology of hBMSCs

The RT-PCR and Western blot result revealed that VEGF and hTERT constructs were successfully transfected and transcribed (see Supplementary material). Comparing with cells in hBMSC group, cells in hBMSC-T and hBMSC-V&T group presented significantly extended life spans, while cells in hBMSC-V group did not (Fig. 1A). Cells in hBMSC and hBMSC-V group ceased to grow at PDL 15 and 16, respectively. Cells in hBMSC-V&T group ceased to grow at PDL 32 and even cells in hBMSC-T group extended to grow to PDL 38. The hBMSC-T and hBMSC-V&T grew faster than two other groups at early expansion.

Besides, the cell morphology was also changed when the expression of VEGF/hTERT was introduced. From passage 1–6, hBMSC showed the same morphological changes during expansion. After passage 6, cells in hBMSC and hBMSC-V groups started to exhibit morphological changes including thin and flat shape, polygranulation and polynucleation, while cells in hBMSC-T and hBMSC-V&T groups kept the spindle-shaped form at least till to passage 10 (Fig. 1B).

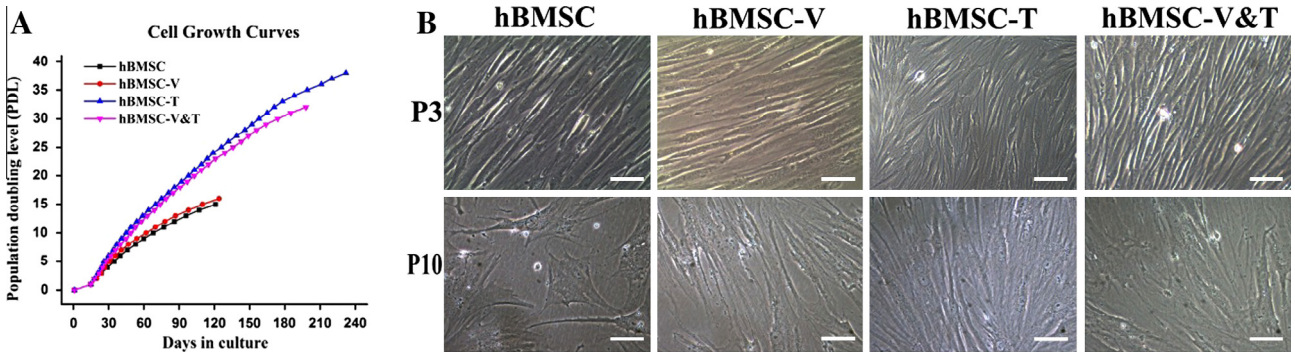

Fig. 1. Exogenous expression of *VEGF* and *hTERT* changed the lifespan and morphology of hBMSCs. (A): cell growth curves of four cell groups as indicated; (B): phase-contrast photomicrographs of hBMSCs at the confluent stage; first row, passage 3 (transfected passage 1), second row, passage 10; bar = 100 μm.

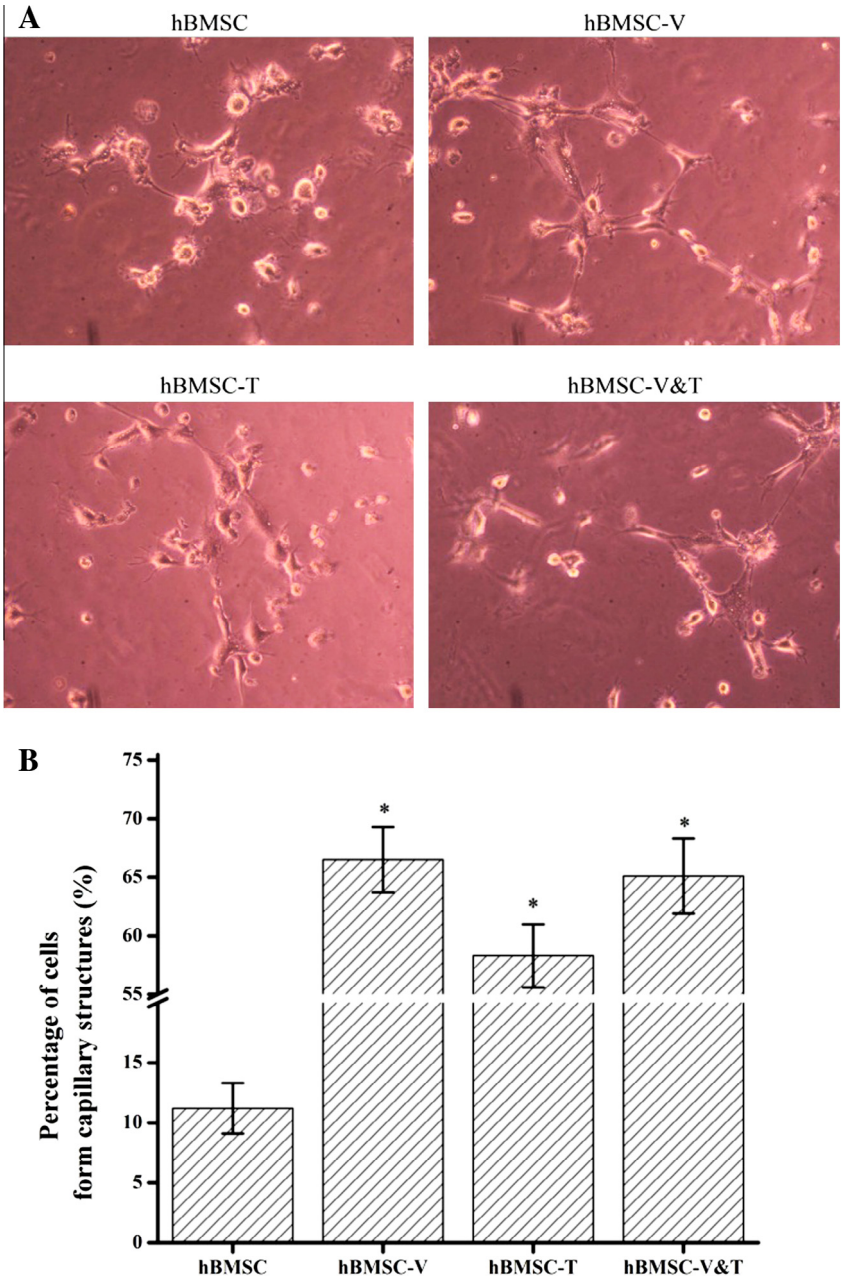

Fig. 2. The expression of *VEGF* and *hTRET* promoted angiogenesis in vitro. Matrigel matrix was added to a 24-well plate at 0.5 mL/well and incubated for 3 h at 37 °C. Four groups hBMSCs (10^4/well) were added in 0.5 mL serum-free medium containing 10 ng/mL *VEGF* and incubated for 6 h at 37 °C. (A): Typical light microscopic images of cell capillary formation on matrigel (×100 magnifications); (B): Statistical quantification result of cell percentages of formed capillary structure. Compared with hBMSC group, $^*P < 0.05$.

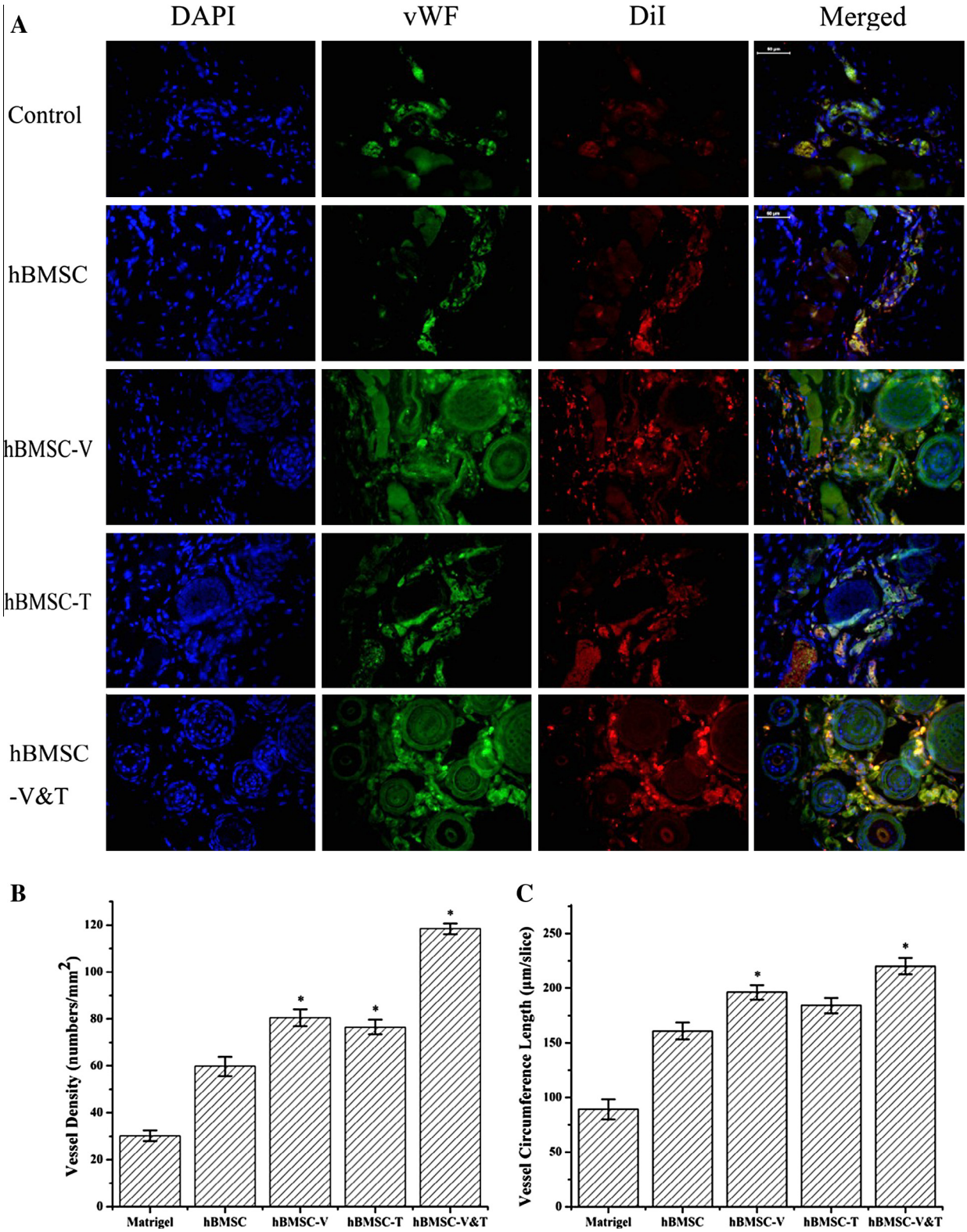

Fig. 3. Immunodeficient mice were injected subcutaneously with hBMSCs mixed with Matrigel. Plugs were removed after 21 days. (A): Immunofluorescence staining of human vWF revealed that the DiI-labeled hBMSCs (red) had migrated and interacted to form varying calibers of capillaries, which expressed vWF (green) (×200 magnification). Cell nuclei were counterstained with DAPI (blue). (B): Vessel density increased in *VEGF* and/or *hTERT* transfected groups. (C): Vessel circumference length increased in *VEGF* and *VEGF*&*hTERT* transfected groups. Compared with hBMSC group, $^{*}P < 0.05$. (For interpretation of the reference to color in this figure legend, the reader is referred to the web version of this article.)

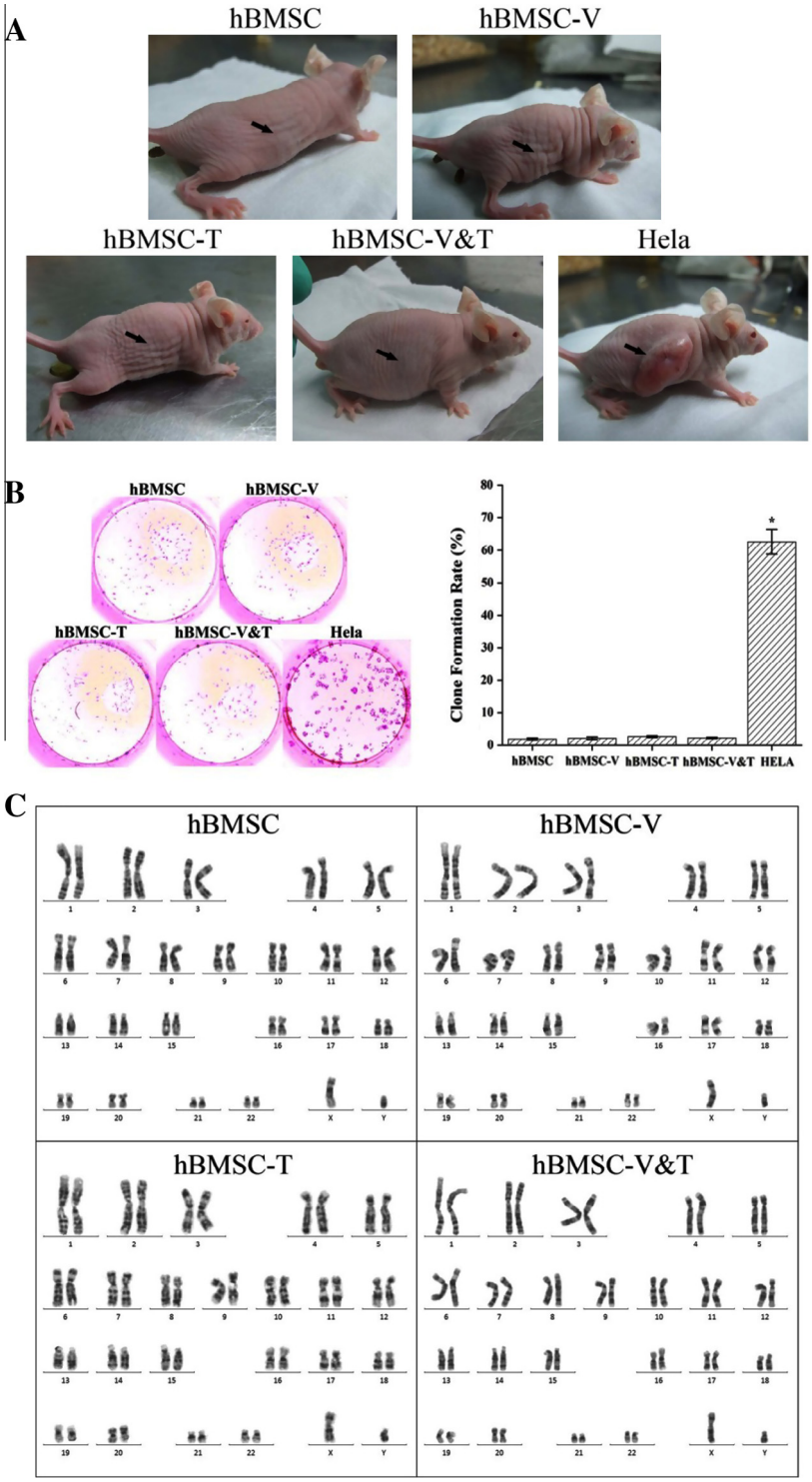

Fig. 4. The expression of *VEGF* and *hTRET* in hBMSCs has few effects on tumorigenicity. (A): Cells were grafted into subcutaneous tissue of NOD-SCID mice. (B): Soft agar anchor-independent assay. hBMSCs from four groups were separately seeded 50, 100 or 200 cells/well into soft agar and incubated for 3 weeks. HeLa cells were used as a positive control. Each assay was performed in triplicate. Photos show cell were seeded 100 cells/well. Compared with hBMSC group, $^{*}P < 0.05$. (C): Karyotypic (46, XY) analysis of parental and transfected cells with extended life spans. Normal diploid was seen in representative four groups.

3.2. The expression of VEGF and hTRET promoted angiogenesis in vitro and in vivo

More tube-like structures were observed in VEGF or hTERT transfected groups (Fig. 2A); suggesting overexpression of VEGF or hTERT could significantly increase angiogenic ability of hBMSCs in vitro (Fig. 2B). In order to investigate the interaction between cells in plugs and the recipient murine circulation, perfusion was not performed, which made the nonspecific red fluorescence presented in the vessels from plugs. Since this nonspecific red fluorescence existed, the ratio of DiI-labeled grafted cells integrated into the vessel was not calculated, only the definitely formed vessel density and vessel circumference length were calculated. Immunofluorescence revealed that the DiI-labeled grafted cells accumulated and interacted to form varying calibers of vessel-like structures expressing vWF. In contrast, few blood vessel

formations were observed in cell-free plugs (control) and hBMSC group (Fig. 3A). VEGF or hTERT expression significantly increased vessel formation, which displayed in rise of both vessel density and vessel circumference length (Fig. 3B, C).

3.3. The expression of VEGF and hTRET in hBMSCs had few effects on tumorigenicity

None of the hBMSCs version grafted into subcutaneous tissue of athymic nude mice formed tumors during the observation period (more than 90 days), while the control mice with HeLa cells graft formed a very big neoplasm at the injection site 30 days after injection (Fig. 4A) and died 60 days after injection. HE staining of nude mice skin also showed no tumors formed in all hBMSCs transplantation group (see Supplementary material). Further soft agar anchor-independent assay revealed that hBMSCs groups have very low clone formation rate, compared with HeLa cell group (Fig. 4B). Karyotypic (46, XY) analysis revealed no chromosome abnormality in representative four groups (Fig. 4C). Taking together, these results demonstrate that either VEGF or hTERT expression in hBMSCs cells has few effects on tumorigenicity.

4. Discussion

Cell therapy for cerebrovascular disease is a practically clinical application. In this practice, BMSCs are being increasingly appreciated because their application in autologous transplantation can avoid self-immune reactions and ethics concerns. Aged people are always the high-risk population with cerebrovascular disease. Clinically, the best cell therapy for aged patients with the disease is to apply their own hBMSCs. However, the cells derived from the aged donors generally do not fit the autologous transplantation due to their aged characteristics [12–14,28], including shorter life span, loss of angiogenic ability and few telomerase activity. Aging of hBMSCs may due to many mechanisms including telomerase activity Mounting studies showed that hBMSC from young donors were effective in therapeutic application, while these from elder ones were not. However, considering that the elderly are potential autologous hBMSCs transplantation population, studies on hBMSCs from the elderly are important.

FACS analysis and immunofluorescence assay in this study revealed that genetically-engineering hBMSCs with enhanced expression of VEGF and hTERT maintained their surface antigens as parental hBMSCs, still positive for mesenchymal stem cell markers including CD29, CD 44, and CD90, and negative for hematopoietic markers including CD34 and CD45 (see Supplementary material) [29,30]. Meanwhile we noticed that all groups of cell were negative for CD34, which is an important common marker used to characterize and isolate human stem cells [29]. During BMSC differentiation into vascular endothelial cells CD34 should keep negative [24,30–32]. Though three germinal layer induced differentiation experiments were not performed, these results suggested lentivirus-mediated transfection did not change characteristics of mesenchymal stem cell of hBMSCs.

VEGF is a neurotrophic, neuroprotective and neuroproliferative factor, which has been testified critically for cell-induced functional recovery in the post stroke brain [19,21,33–36]. The transplanted cells in plugs were with new vessels in athymic nude mice and functionally linked to the murine circulation, as attested by the presence of recipient red blood cells (Fig. 3A). These findings confirmed that these cells have certain functional angiogenesis ability in vivo. The observations therefore not only demonstrated the modified cells are with normal physiological features but also may serve as safe and effective tools to treat vascular complications.

There is always an important concern for the genetically modified hBMSCs: whether the cell karyotype is changed and can any tumorigenicity risk be induced? Especially when using lentivirus as transfection vector, since lentivirus may integrate their genome into the host chromosome [37]. In this study, we observed that VEGF and hTERT overexpressed hBMSCs did not increase tumorigenicity and kept normal karyotype. hTERT is not an oncogene [38], different from using other oncogene to modify cells to increase life span [39,40], hTERT will not increase tumorigenicity [27,41–44]. Lentivirus-mediated hTERT gene transfection on hBMSC did not show malignant transformation in vitro and in vivo after extended culture periods. There were no changes observed in the expression of tumor suppressor genes and karyotype [45].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.053.

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