Immortalization of human dermal microvascular endothelial cells

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Research Article

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Immortalization of human dermal microvascular endothelial cells

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Abstract

Objectives: Microvascular endothelial cells (MECs) have been proved by increasing studies to play important roles in the process of endocrine, immune response, and pathogenic microorganism infection. However, most types of MECs have a limited number of divisions. Therefore, the immortalization of primary MECs may provide a better cell model for research. And the present research is aimed to establish an immortal human dermal microvascular endothelial cells (HDMECs).

Methods: To immortalize HDMECs, the telomerase reverse transcriptase (hTERT) gene was transferred into the primary HDMECs by lentiviral infection. The passages of HDMECs transfected with hTERT or without hTERT were analyzed. At the same time, the relative telomerase activity and telomere length in HDMECs transfected with hTERT were detected by RT-PCR assay. And the β-galactosidase (β-GAL) activity in HDMECs transfected with hTERT was detected by ELISA kits. Finally, karyotype and tube formation analysis were used to evaluate the effects of transfection with hTERT on the characteristics of HDMECs.

Results: The results showed that the number of passages of HDMECs transfected with hTERT was significantly increased. The telomerase activity of HDMECs transfected with hTERT gene was enhanced, and β-GAL activity was significantly reduced. Moreover, the transfection of hTERT gene has almost no effect on the karyotype and tube formation of HDMECs.

Conclusion: These data indicate that transfection of hTERT gene could successfully enhance the cleavage ability of HDMECs, and the characteristics of hTERT-HDMECs remain almost unchanged.

Key words: HDMECs, hTERT, β-GAL, karyotype and tube formation

Introduction

MECs are distributed between capillaries and tissues, forming a barrier between blood vessels and

tissues, which have various physiological functions such as regulating vasomotor, blood coagulation, vascular permeability [1-3]. MECs are also involved in a series of physiological processes such as regeneration, wound healing, inflammation, immune regulation and angiogenesis [4]. HDMECs has become an important cell type in studies, and its division ability and activity directly affect the accuracy of the experiment [5, 6]. However, after multiple passages, the primary HDMECs will begin to age or even stop proliferating. In order to ensure the number and stable state of HDMECs used for research, we choose to immortalize HDMECs cultured in vitro.

Telomerase is a DNA polymerase that extends the 3' ends of chromosomes by synthesizing multiple telomeric repeats [7]. It is a unique ribonucleoprotein (RNP) containing a specialized telomerase reverse transcriptase (TERT) and telomerase RNA (TER) [8]. Telomerase is active in most human tumors but not expressed in most non-immortalized somatic cells, which can inhibit telomere erosion and prevent cell cycle senescence and apoptosis due to telomere length shorting [9]. At present, many cell lines have been successfully established with transfected telomerase, which can maintain the basic physical and chemical properties of primary cells [10, 11]. In the present study, hTERT was used to immortalize HDMECs with lentivirus.

Materials and Methods

Cell culture

HDMECs were purchased from Sciencell (#2000, California, USA) and cultured in ECM (Sciencell, California, USA) supplemented with 5% FBS (Sciencell, California, USA) and 1% penicillin G/streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2. Hela cell line were cultured in DMEM (Gibco, Thermo Fisher Scientific, Beijing, China) supplemented with 5% FBS and 1% penicillin G/streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2.

Plasmids and Lentivirus

The pLV-hTERT-P2A-Puro plasmid was constructed by pLV-hTERT-IRES-hygro (Addgene, Massachusetts, USA) and CAS9-gRNA-g1 (Huahengjian, Beijing, China). Then the plasmid was transformed into Trans5α competent cells. Afterwards, plasmid DNA was isolated using TIANpure Mini Plasmid Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions. Lentivirus was produced using HEK 293F cells (Invitrogen, Thermo Fisher Scientific, Beijing, China) transfected with packaging constructs pLV-hTERT-P2A-Puro by LV-MAX™ Lentiviral Production System (Invitrogen, Thermo Fisher Scientific, Beijing, China) according to the instructions.

Minimum killing concentration of puromycin on HDMECs

To optimize the concentration of puromycin (Solarbio, Beijing, China), the 5% ECM medium was supplemented with increasing doses of puromycin (0.1, 0.5, 0.6, 1 and 10 μg/mL). HDMECs were seeded in a 12-well dish and covered by ECM medium containing puromycin. Then cells were incubated in an incubator containing 5% CO_2 for 48, 72 and 96 h.

hTERT transfection

HDMECs ($1x10^5$) were cultured in a well of 6-well plate, 100 μL of lentivirus was added to the well and incubated for 1 h. Subsequently, ECM medium containing 5% FBS was added and cultured for 24 h. Replace with ECM medium containing 5 μg/mL Polybrene (Vector Builder, Chicago, USA) for another 48 h.

Cell growth assay

Cells were seeded at $5x10^4$ cells per well in a 12-well plate for 24, 48, 72 and 96 h. Then the cells were harvested and counted. All experiments were repeated 3 times.

β-GAL activity by ELISA

The β-GAL activity of HDMECs transfected with or without hTERT at passage 10 were analyzed by using the ELISA Kit (ZCI Bio, Shanghai, China) according to the manufacturer's instructions.

Telomerase activity and telomere length

Total RNA was extracted according to the instruction of Total RNA Extraction Kit (GenePool, Beijing Jipu Biotechnology Co., Ltd, China). The integrity of the RNA sample was assessed by electrophoresis of 5 μL RNA on 1% agarose gel. The total RNA was reverse transcribed into cDNA using the mRNA cDNA Synthesis Kit (GenePool, Beijing Jipu Biotechnology Co., Ltd, China) according to the manufacturer's instructions. Real-time quantitative PCR reactions were carried out with Telomerase activity detection kit (GenePool, Beijing Jipu Biotechnology Co., Ltd, China). Genomic DNA was extracted by Isopropanol precipitation. The integrity of the DNA sample was assessed by electrophoresis of 3 μL DNA on 1.0 % agarose gel. Real-time quantitative PCR reactions were carried out with Telomere length detection kit (GenePool, Beijing Jipu Biotechnology Co., Ltd, China). Hela cell line was selected as a positive control.

Karyotyping

HDMECs were treated with 0.2 μg/mL Demecolcine (Sigma, Merck KGaA, Germany) for 72 h, harvested with TrypLE Express (Gibco, Thermo Fisher Scientific, Beijing, China) and incubated in

hypoosmotic 0.075 mol/L KCl solution for 25 min. Following the incubation, the cells were fixed in methanol : acetic acid (3:1) and metaphase spreads were produced by dripping the cell suspension on glass slides positioned at a slight angle over a steaming water bath. The glass slides were stained in Giemsa solution (Sigma, Merck KGaA, Germany) for 8 min. The chromosome counting and metaphase spreads were observed by microscope.

Tube Formation Assay

Growth factor-reduced Matrigel (Corning, NY, USA) was placed 70 μL in each well of a 96-well dish and incubated at 37 ◦C for 30 min. Cells were seeded at 1.0×10^4 per well in 5% ECM, and incubated at 37 ◦C for 9 h. Tube formation was observed using an inverted microscope (Olympus, Tokyo, Japan).

Statistics

All date were expressed as means ± standard deviations (SD). The statistical significance of differences was determined using the Student's t-test or one-way analysis of variance (ANOVA) as appropriate. All the statistical tests were performed using GraphPad Prism soft ware 8.2 (GraphPad software, USA). The p-value < 0.05 was considered statistically significant.

Results

Cultivation of HDMECs

As shown in Fig. 1A, the HDMECs of passage 2 were observed to be oval and fusiform. Different degrees of stretching can be observed in the cells of passage 6 (Fig. 1B). After the 11th passage, the cells almost stopped proliferating (Fig. 1C).

Screen HDMEC positive for hTERT

As shown in Fig. 2, after treatment with 0.1 μg/mL puromycin for 48, 72 and 96 h, about 50% of HDMECs survived. After treatment with 0.5 μg/mL puromycin for 48, 72 and 96 h, about 10% of HDMECs survived. Little HDMECs still survived at 48 and 72 h after treatment with 0.6 μg/mL puromycin. However, all HDMECs were dead at 96 h treated with 0.6 μg/mL puromycin. HDMECs were dead at 48 h treated with 1 μg/mL puromycin. Therefore, it is determined that 0.6 μg/mL puromycin is the lowest concentration that can be used to screen HDMECs carrying the puromycin resistance gene. In order to screen the HDMECs successfully transfected with hTERT, we treated the HDMECs inoculated with lentivirus with 0.6 μg/mL puromycin. Live HDMECs can still be observed after 96 h.

HDMECs proliferation

To the 10th generation, HDMECs are obviously deformed, senile and dead (Fig. 4A). All of the hTERT-HDMECs exhibited an elongated, “cobblestone like” shape, while maintaining their proliferation activity even at high numbers of passages (Fig. 4B-C). As shown in Fig. 4D, the number and growth rate of the 10th generation HDMECs decreased compared with the 10th generation hTERT-HDMECs, whereas that of the 10th generation hTERT-HDMECs was consistent compared with the 5th generation HDMECs.

The activity of β-GAL

As shown in Fig. 5, the β-GAL activity of HDMECs have increased senescence compared to hTERT-HDMECs at passage 10 ($P<0.01$).

Analysis of telomerase activity and telomere length

The telomerase activity in hTERT-HDMECs and Hela cells were much higher than levels seen in the HDMECs (Fig. 6A, $P<0.01$). However, the enhancement of telomerase activity did not extend the length of telomeres. The telomere length in hTERT-HDMECs and Hela cells were shorter than that in HDMECs (Fig. 6B, $P<0.01$).

Karyotype analysis

To test for potential cooperating genetic aberrations, karyotype of the HDMECs and hTERT-HDMECs were analyzed. As shown in Fig. 7, in comparison with 6th passage HDMECs karyotype, karyotype analyzed of the 12th passage hTERT-HDMECs showed no aberrations. In all of the examined chromosomal spreads ($2n = 46$), hTERT-HDMECs have normal chromosome number and chromosome length in all examined metaphase spreads.

Tube formation analysis

The ability of tube formation on Matrigel was analyzed by tube formation assay. Under the appropriate stimulation of Matrigel, HDMECs and hTERT-HDMECs migrated and proliferated, then align and finally formed tubes. The results show that hTERT-HDMECs can form the same tubes as HDMECs, and they are no change in the cell characteristics [12].

Discussion

At present, most studies on endothelial cells use HDMECs or human umbilical vein endothelial cells (HUVECs) [13]. Since HDMECs is prone to aging and has a limited number of divisions, it needs to be purchased again or separated from the tissue [14]. In addition, if the source of ECs is different, the physiological characteristics of ECs will be different, which is determined by the heterogeneity of

endothelial cells [15]. In addition, spontaneous immortalization is rare in most cells. Therefore, the immortalization of primary MECs is a way to solve the limitations of MECs.

Immortalizing cells by introducing foreign genes into cells through viruses is a commonly used method of immortalization, such as simian virus 40 (SV40) or hTERT [16]. However, compared with primary cells, SV40 transfection may induce cell chromosomal abnormalities, cell cycle control changes and increased carcinogenic risk [17]. Some studies have confirmed that hTERT transfection does not cause malignant transformation of cells, and does not change cell characteristics and karyotype stability [18, 19]. Previous studies have confirmed that cells can be immortalized by transfection with hTERT, which is usually used to increase the possibility of cell immortalization and ultimately obtain an immortalized cell line [20]. Therefore, in this experiment, we introduced hTERT into HDMECs through lentivirus infection to produce immortalized HDMECs.

The number of passages and senescence are important indicators for evaluating cell viability. The proliferation ability of HDMECs transfected with hTERT was enhanced. Compared with the original HDMECs, hTERT-HDMECs clearly shows higher proliferation potential without any signs of aging. Cell senescence refers to the stable stagnation of cell proliferation. β-GAL activity is enhanced in senescent cells, which means that increased β-GAL activity implies cell senescence [21]. Analysis of β-GAL activity showed that HDMECs, but not hTERT-HDMECs, showed signs of aging. It can be seen that HDMECs transfected with hTERT slowed down senescence and maintained the ability of cell division and proliferation.

Telomerase is an enzyme responsible for maintaining the length of telomeres. Since telomerase is inactivated or not expressed in most somatic cells, the replication potential of the cell is limited [13]. When telomeres are shortened, the cell cycle is inhibited. In this study, we observed an increase in telomere activity in HDMMCs transfected with hTERT compared with HDMECs. However, transfection of hTERT did not lead to telomere lengthening of HDMMCs. Studies have shown that the space of the longer telomeres in the immortalized cell nucleus overlaps, even if the same intensity value is maintained, the volume of telomeres seems to be variable, which indicates that telomeres of the same length can be more or less compact [22]. In our study, the telomere length in hTERT-HDMECs was shorter than that in HDMECs. This may be because hTERT-HDMECs can maintain division and proliferation through the spatial overlap of longer telomeres. hTERT may only extend the shortest telomere length, rather than all telomere lengths.

Karyotype analysis and tube formation analysis showed that there is no difference in the number and relative length of chromosomes between hTERT-HDMECs and HDMECs, and they both have a pro-angiogenic phenotype that activates proliferation and migration. It shows that the characteristics of HDMECs transfected with hTERT have not changed. This is consistent with studies that the karyotype and tube formation of immortalized cells do not change and do not cause tumors, indicating that they can be safely used in future studies [23, 24]. In summary, transfection with hTERT can significantly increase the number of passages of HDMECs without changing the characteristics of endothelial cells.

Acknowledgments

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Conflicts of Interest

The authors declare no conflicts of interest.

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Fig. captions and legends

Fig. 1 Cultivation of HDMECs. (A) Figure of the 2th generation HDMECs (20×). (B) Figure of the 6th generation HDMECs(20×). (C) Figure of the 11th generation HDMECs (20×).

Fig. 2 Survival results of Puromycin cells at different concentrations (10×).

Fig. 3 Establishing an immortal HDMECs (10×). (A) The 2th generation of HDMECs before immortalization. (B) 24 h after transfection with hTERT. (C) 24 h after joining Polybrene. (D) 48 h after joining Polybrene. (E) 24 h after adding puromycin. (F) 48 h after adding puromycin.

Fig. 4 Value-added situation of hTERT-HDMECs (10×). (A) The morphology of the 10th passage of HDMECs. (B) The morphology of the 10th passage of hTERT-HDMECs. (C) The morphology of the 20th passage of hTERT-HDMECs. (D) Cell growth curve.

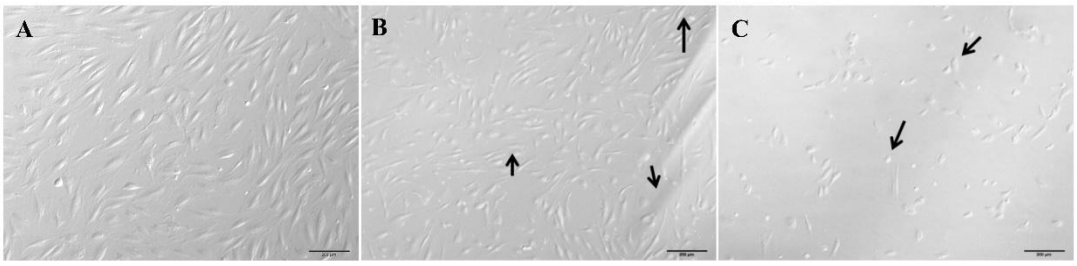
Fig. 5 The β-GAL viability of HDMECs.

Fig. 6 Telomerase activity and telomere length of HDMECs. (A) Relative telomerase activity. (B) Relative telomere length.

Fig. 7 Karyotype analysis results of HDMECs (P6) and hTERT-HDMECs (P12).

Fig. 8 Tube formation analysis results. (A) HDMECs (P10). (B) hTERT-HDMECs (P10).

289 **Fig. 1**



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291 **Fig. 2**

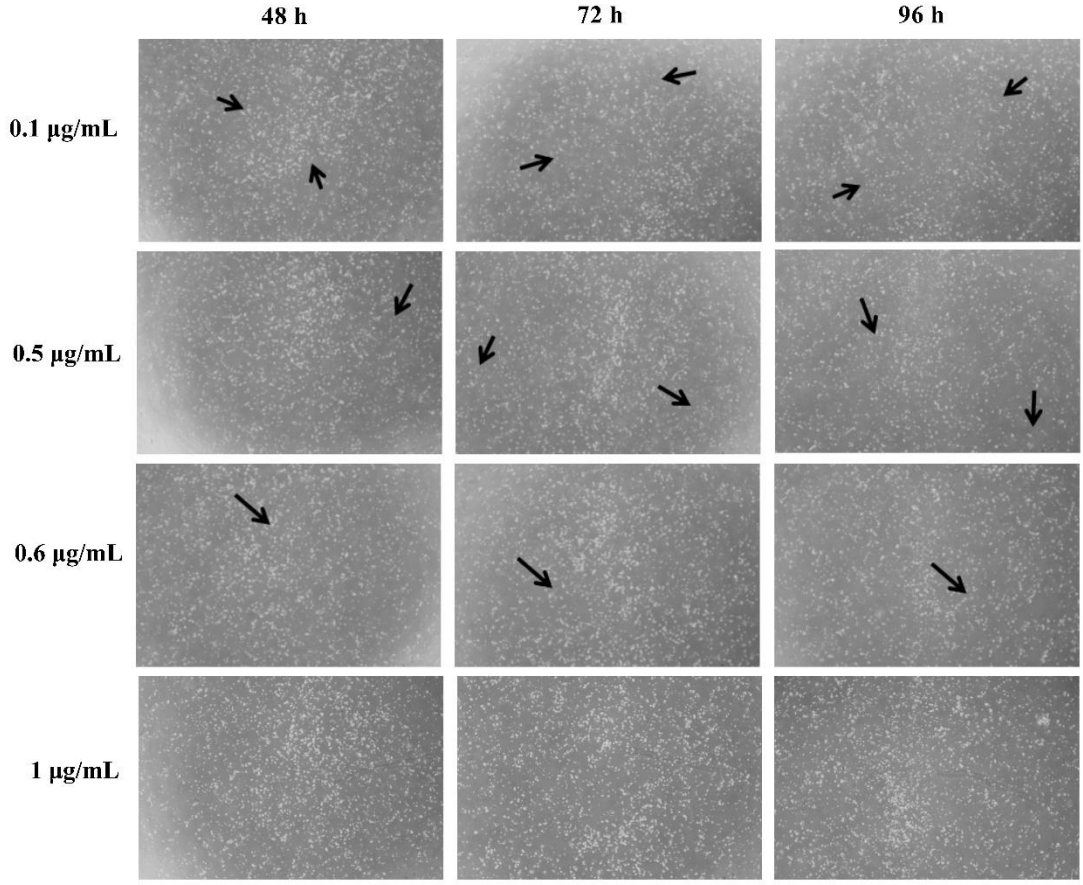

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Fig. 3

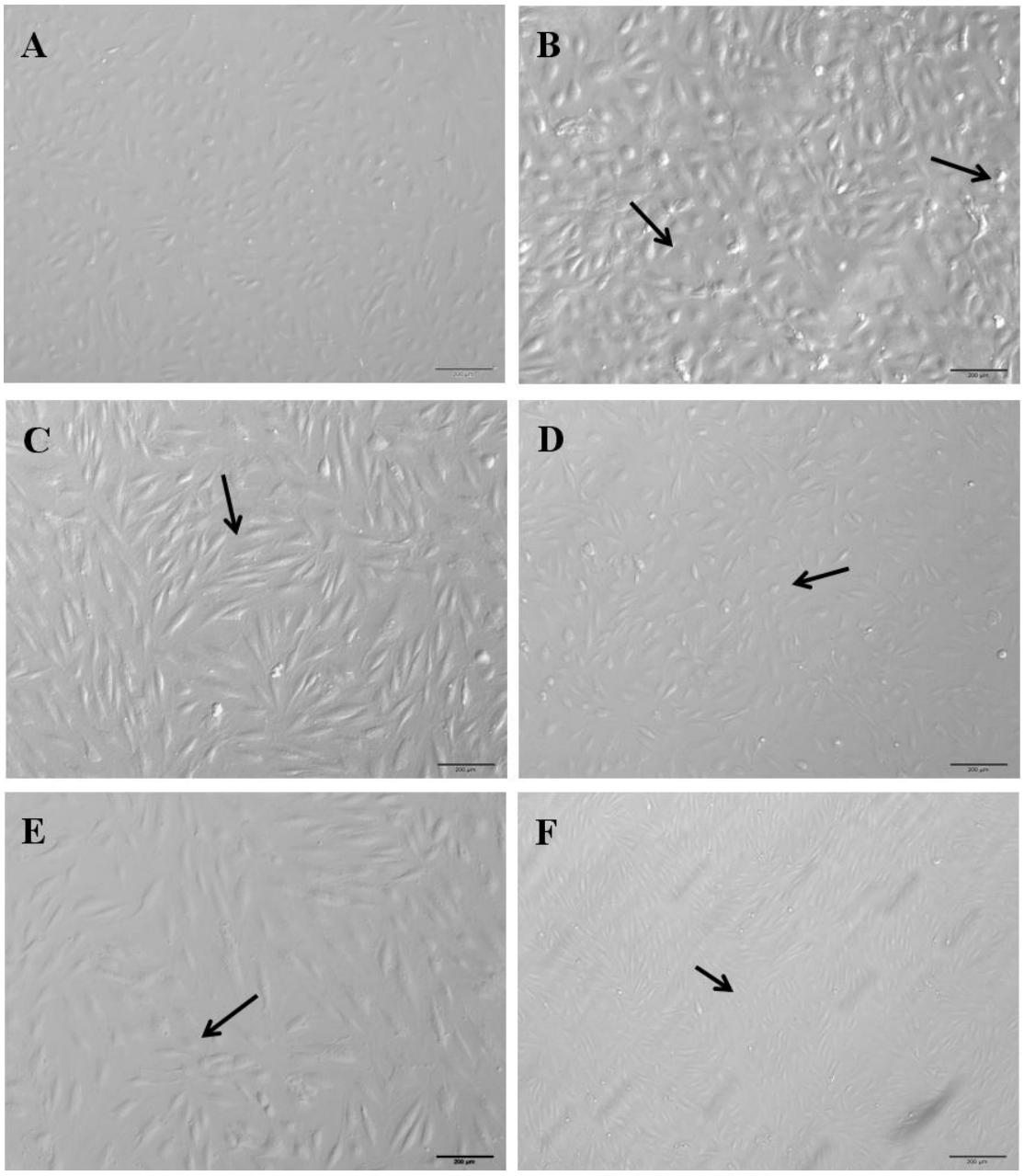
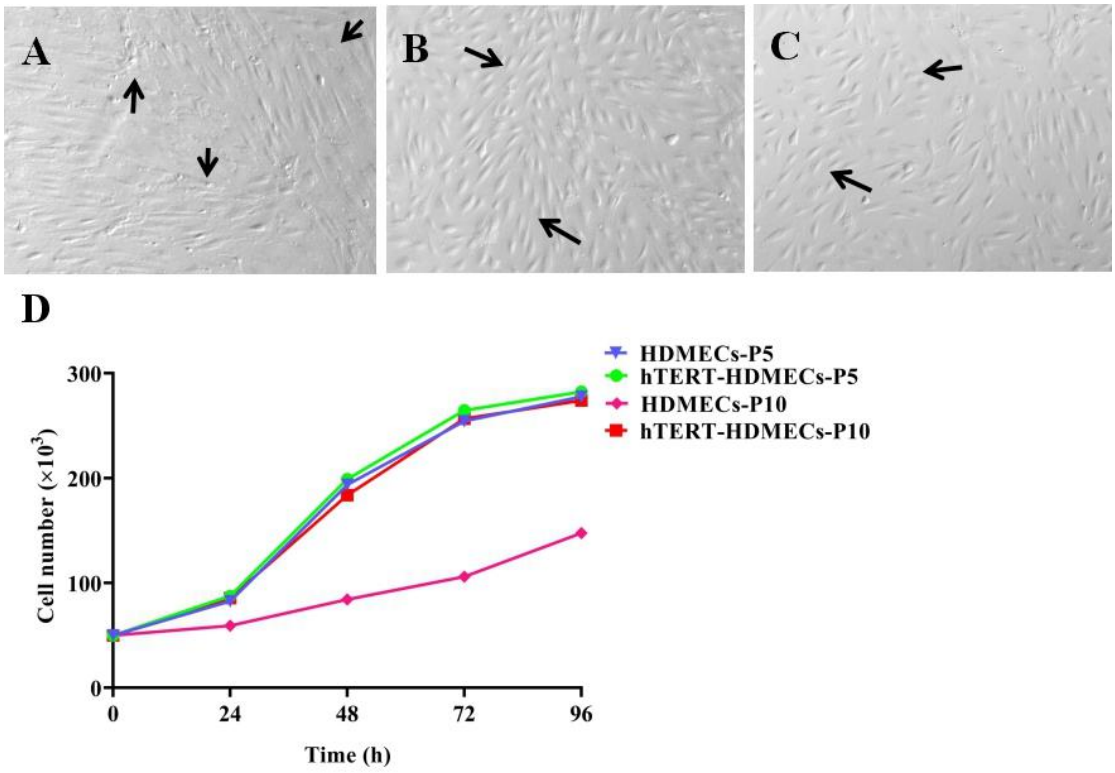
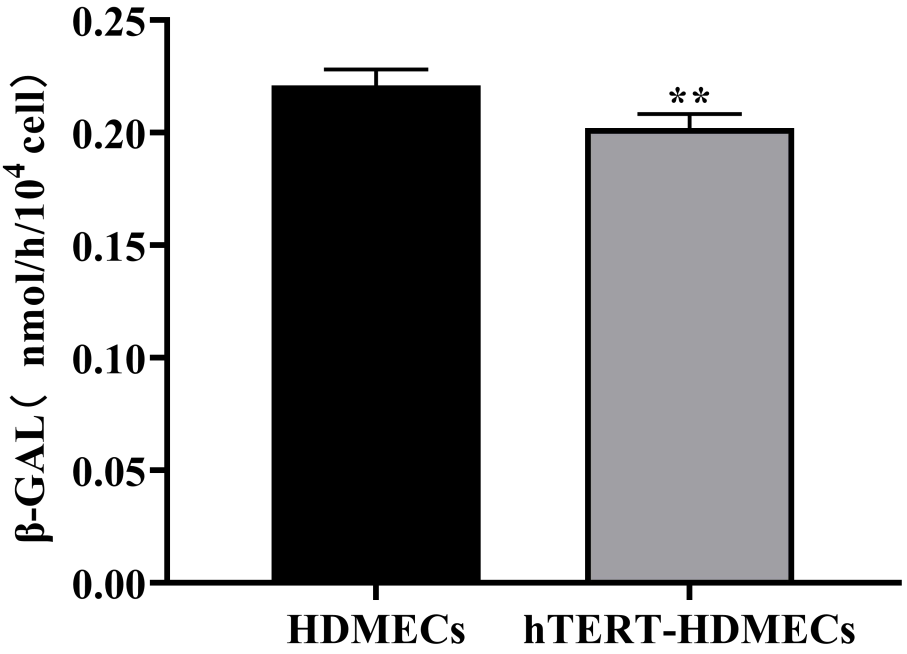


Fig. 4


A
B
C
D
HDMECs-P5
hTERT-HDMECs-P5
HDMECs-P10
hTERT-HDMECs-P10
Cell number (×10³)
300
200
100
0
0
24
48
72
96
Time (h)

328 **Fig. 5**



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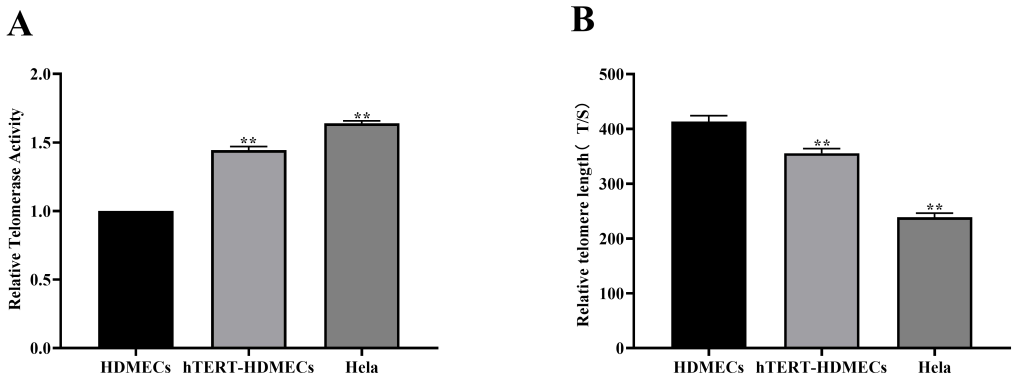
330 **Fig. 6**



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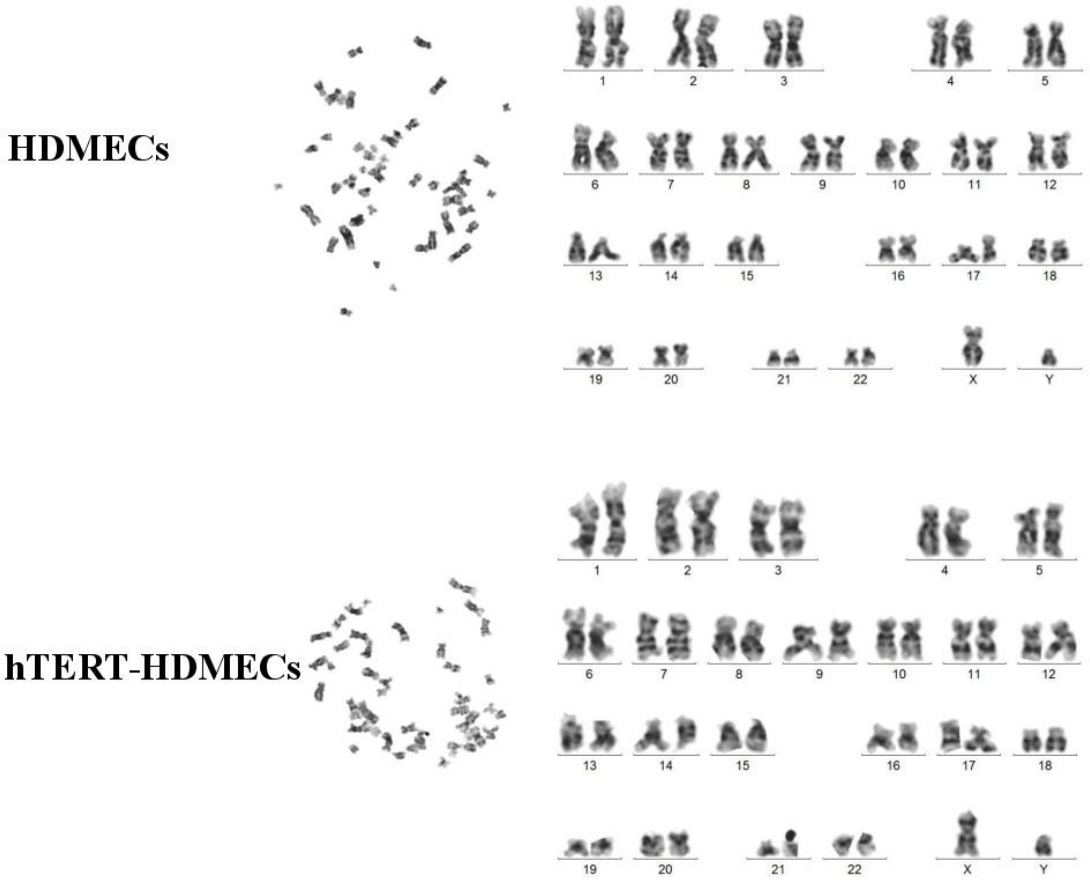
340 **Fig. 7**



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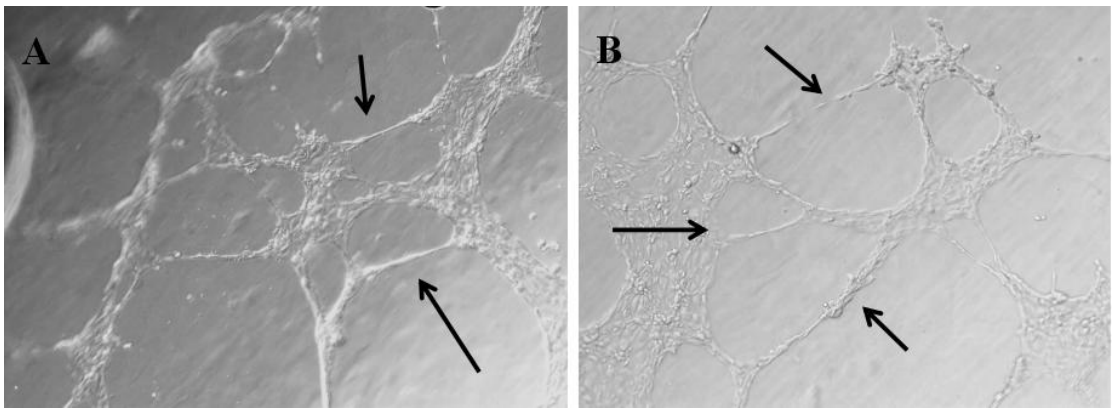
342 **Fig. 8**



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Figures

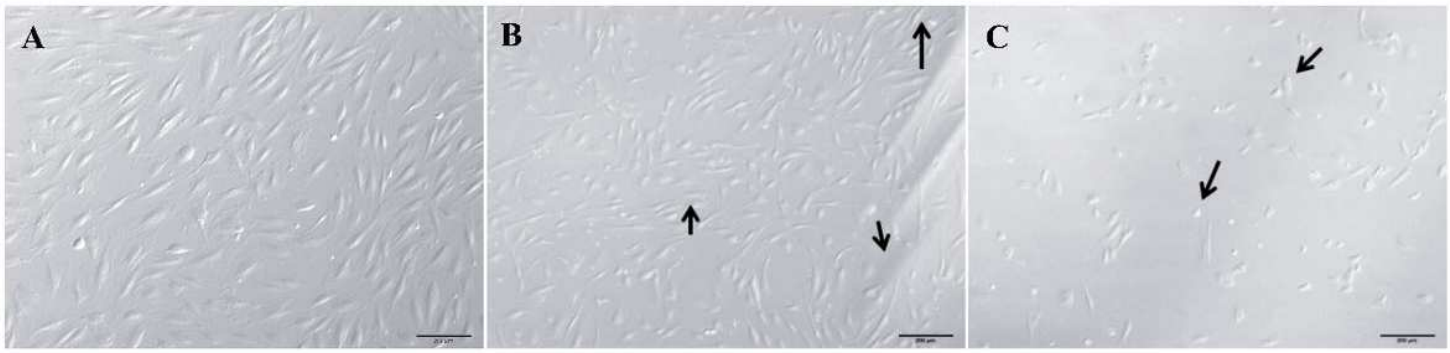

Figure 1

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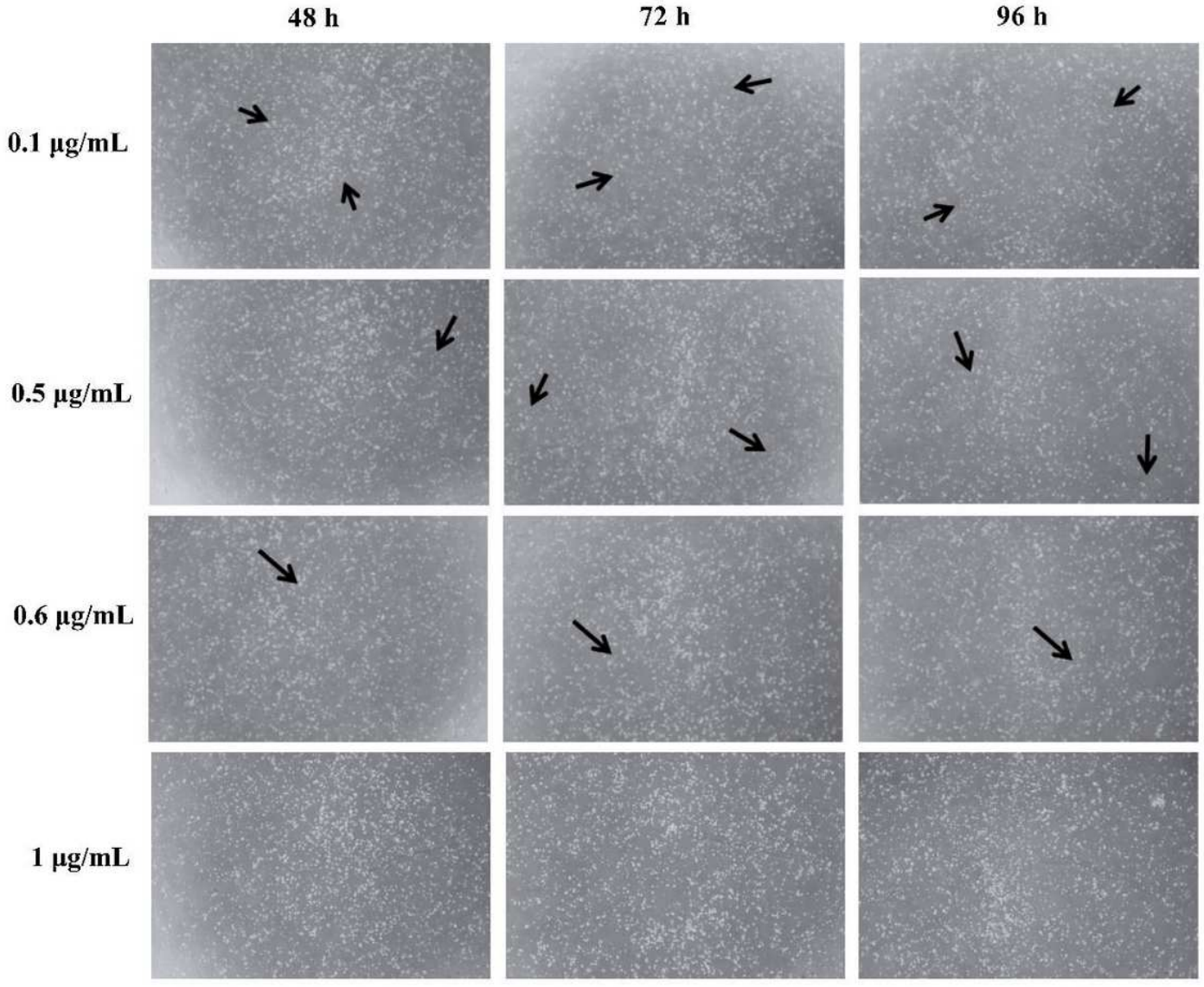

Figure 2

Survival results of Puromycin cells at different concentrations (10×).

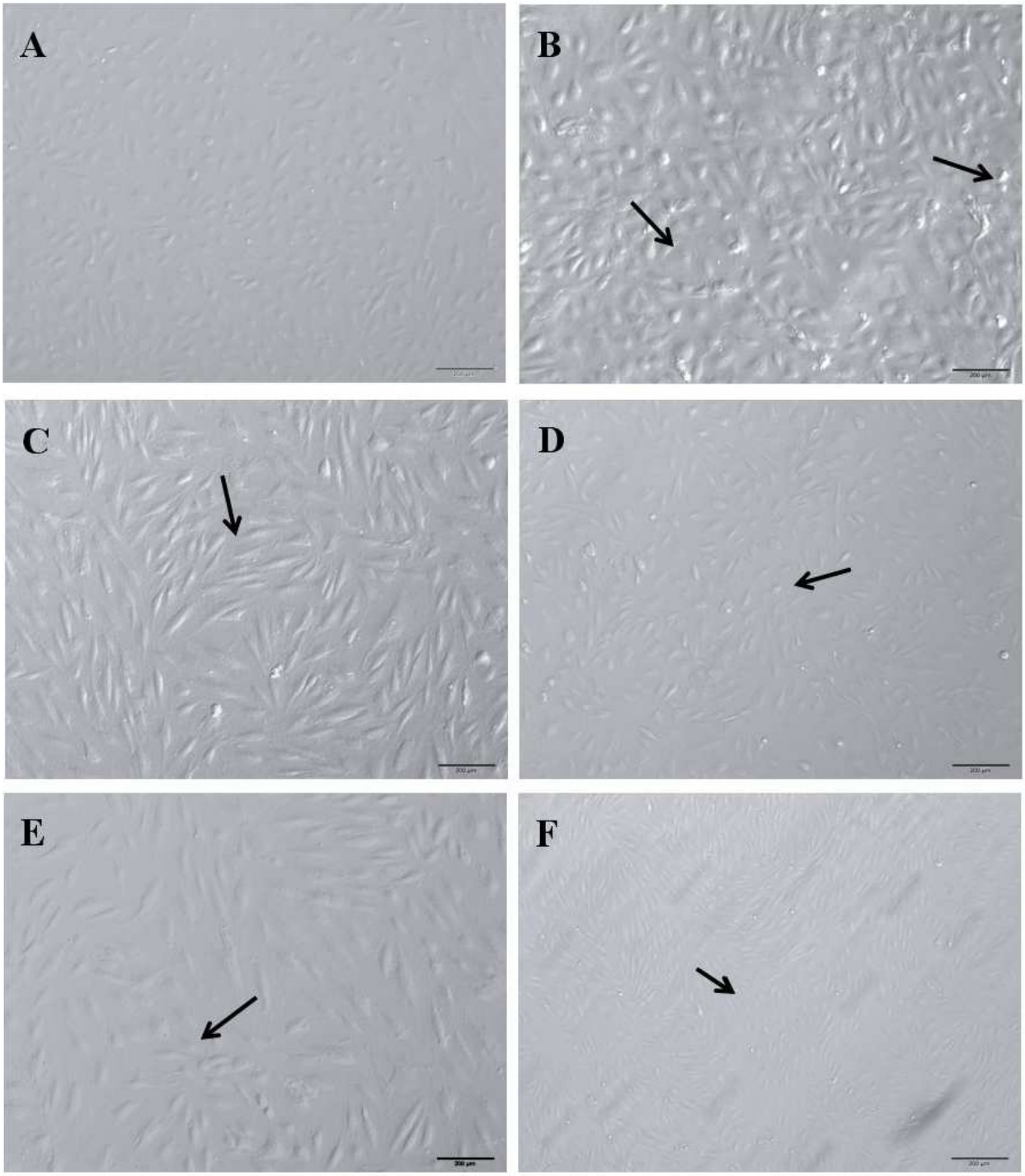

Figure 3

Establishing an immortal HDMECs (10×). (A) The 2th generation of HDMECs before immortalization. (B) 24 h after transfection with hTERT. (C) 24 h after joining Polybrene. (D) 48 h after joining Polybrene. (E)

24 h after adding puromycin. (F) 48 h after adding puromycin.

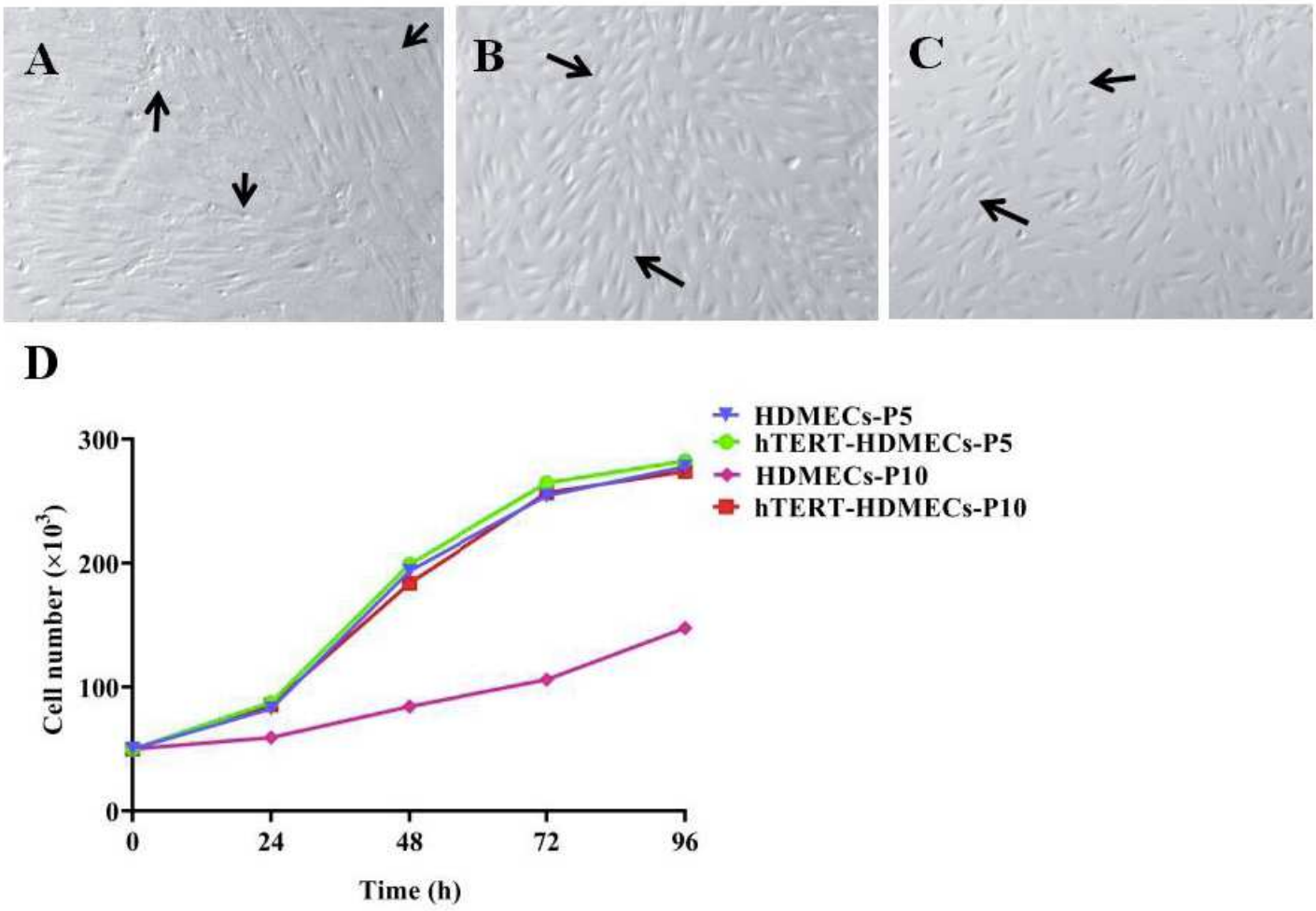

Figure 4

Value-added situation of hTERT-HDMECs (10×). (A) The morphology of the 10th passage of HDMECs. (B) The morphology of the 10th passage of hTERT-HDMECs. (C) The morphology of the 20th passage of hTERT-HDMECs. (D) Cell growth curve.

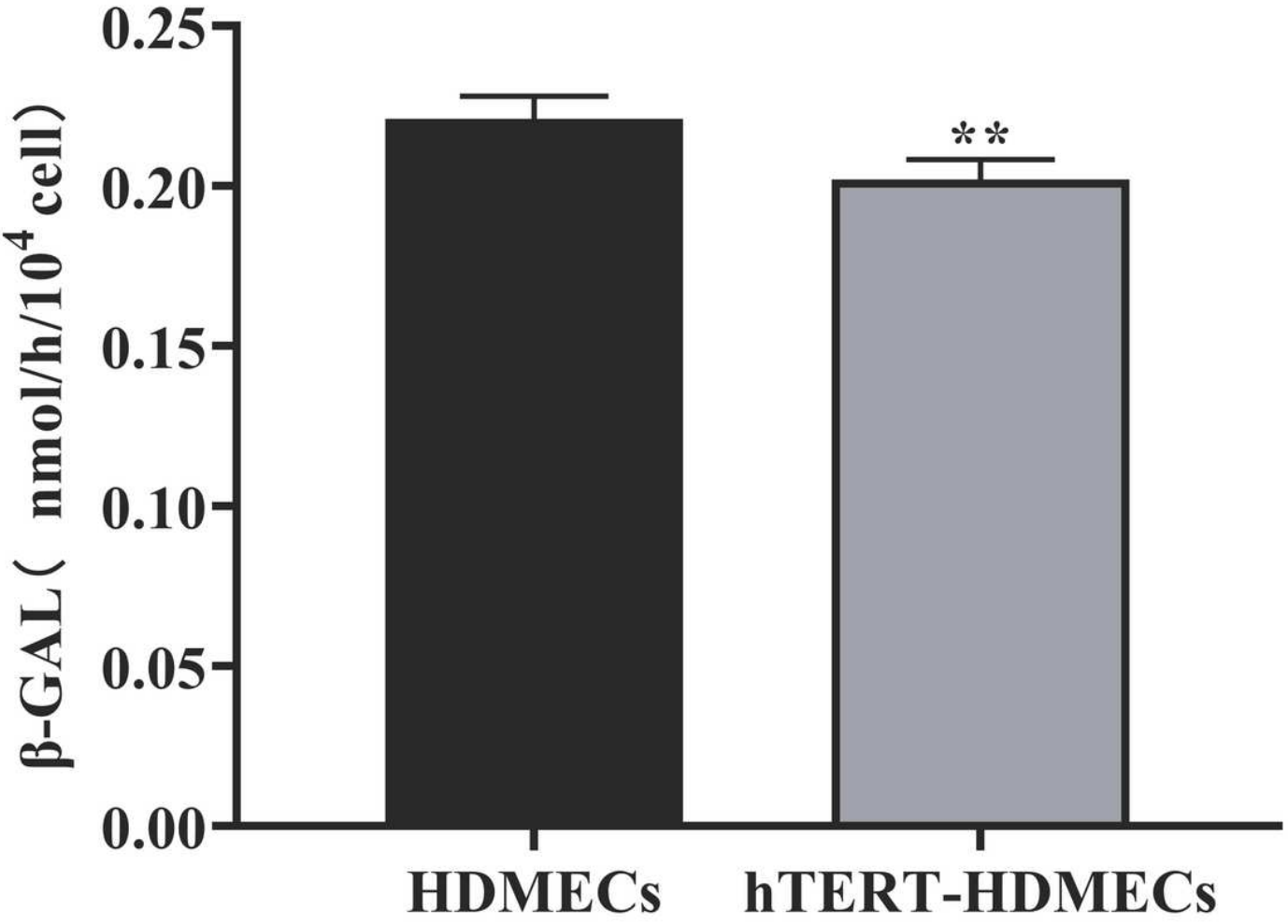

Figure 5

The β-GAL viability of HDMECs.

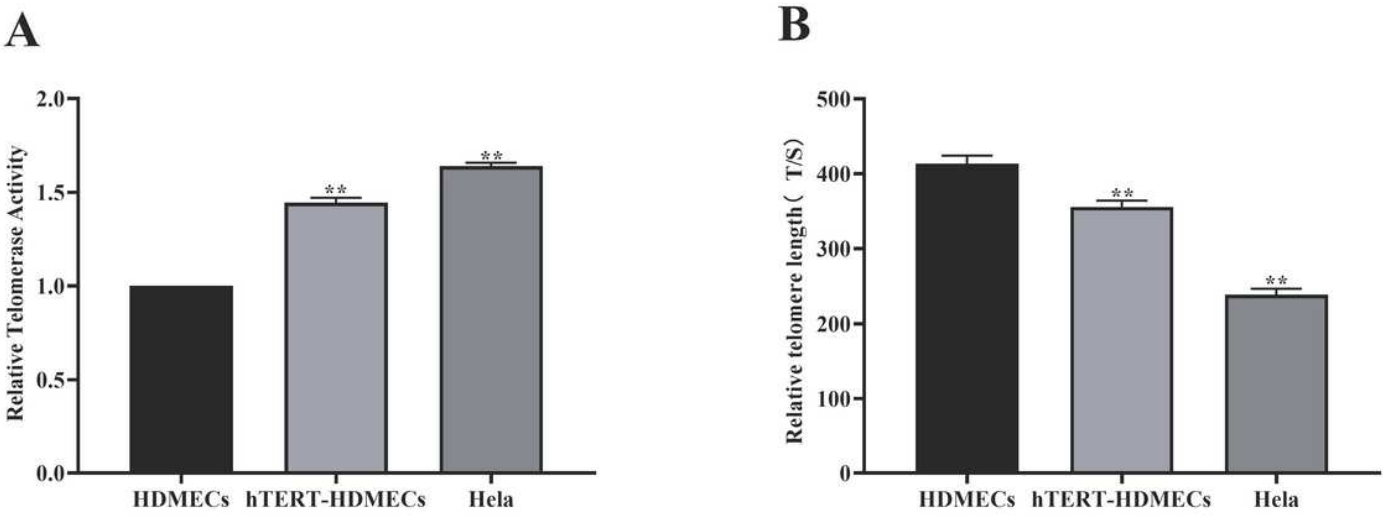

Figure 6

Telomerase activity and telomere length of HDMECs. (A) Relative telomerase activity. (B) Relative telomere length.

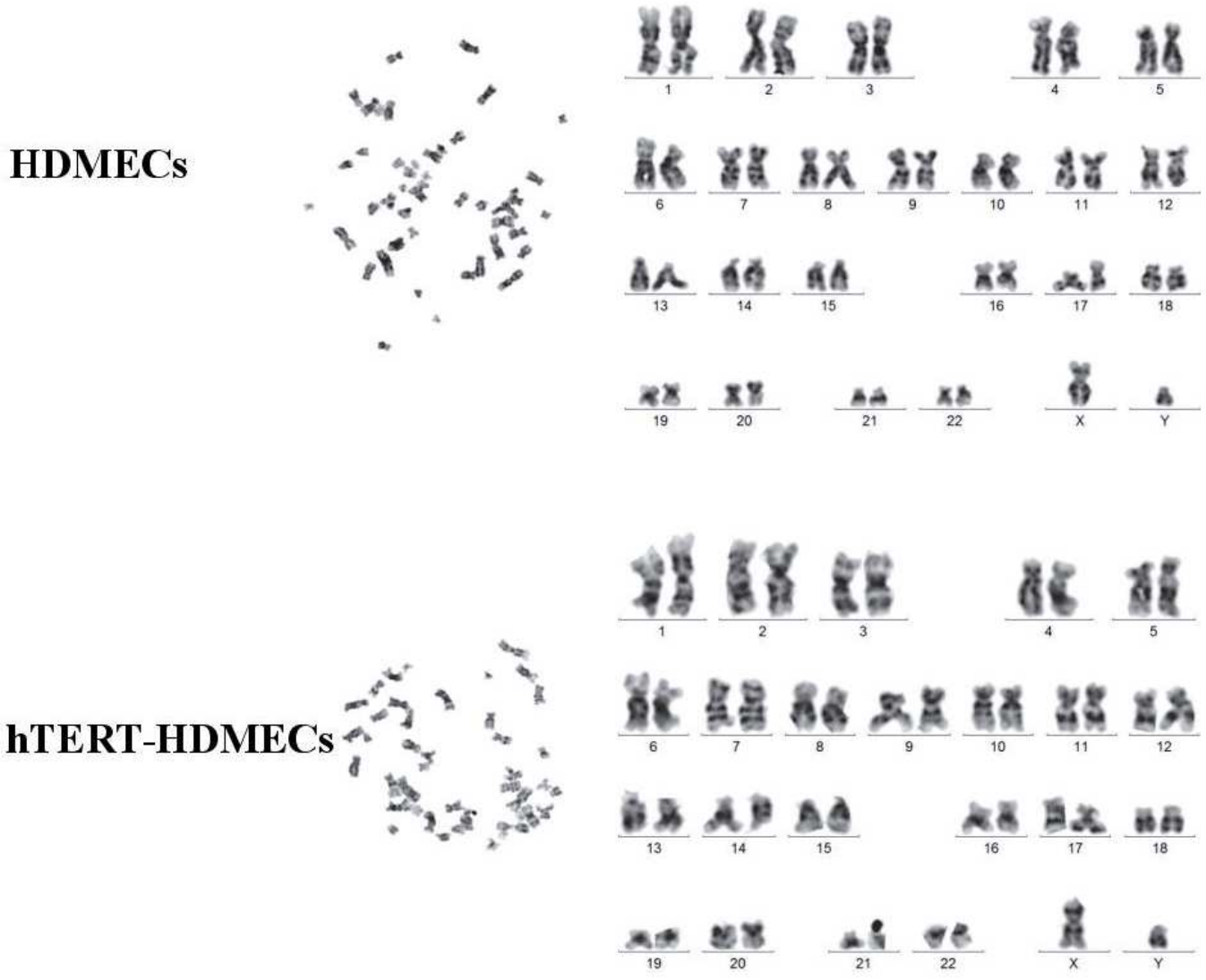

Figure 7

Karyotype analysis results of HDMECs (P6) and hTERT-HDMECs (P12).

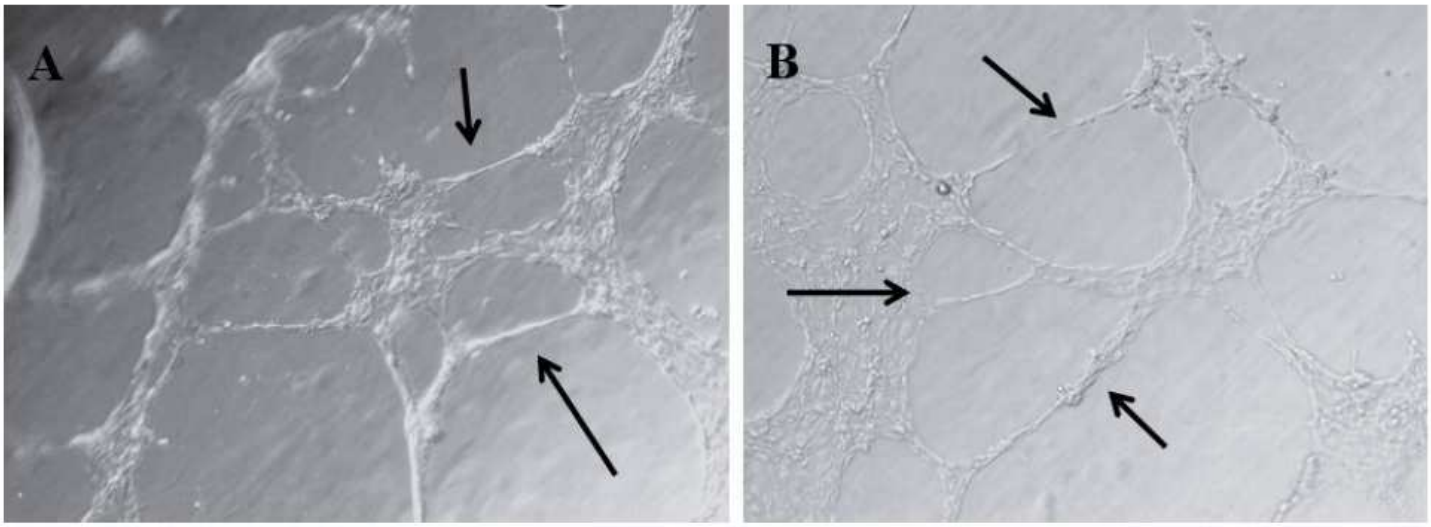

Figure 8

Tube formation analysis results. (A) HDMECs (P10). (B) hTERT-HDMECs (P10).