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Original Article

Exogenous hTERT gene transfected endothelial progenitor cells from bone marrow promoted angiogenesis in ischemic myocardium of rats

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Abstract: Objective: To explore the biological behavior and the revascularizative ability of endothelial progenitor cells (EPCs) transfected with human telomerase reverse transcriptase (hTERT) gene. Methods: EPCs were isolated from mononuclear cells in bone marrow by using the method of density gradient centrifugation, then cultured with differential velocity adherent method, EPCs were transfected by recombinant plasmid carrying GFP report gene EGFP-hTERT. The EPCs secretion and proliferation ability were detected before and after transfection. The expression of EPCs mRNA were detected by RT-PCR before and after transfection. The new capillaries of infarct area were observed. Results: After transgenesis, the proliferation of EPCs were increased, and the secretion of NO, LDH, iNOS by EPCs were significantly increased compared to the non-transgenesis group. After transplanted the transfected EPCs into the ischemic myocardial of rats, revascularization were increased obviously. Conclusion: EPCs maintained the original biological characteristics after transfecting exogenous hTER gene, the proliferation and survival rate were up-regulated significantly, and the revascularization ability of EPCs were significantly strengthen.

Keywords: Endothelial progenitor cells, human telomerase reverse transcriptase (hTERT), transfection, myocardial infarction

Introduction

With the highest morbidity and mortality, coronary heart disease (CHD) is threatening the health of people worldwide. Recent years, scholars proposed that, the stem cell can be used to treat acute myocardial infarction so as to make up the function resulting from the necrosis of cardiomyocytes. Endothelial progenitor cells (EPCs), one of adult stem cells, are precursor cells of endothelial cells. EPCs in peripheral blood were first identified in 1997 [1], it can be proliferated, migrated, differentiated in vitro, and possessed the ability of angiogenesis in vivo. But the number of EPCs in bone marrow, peripheral blood, umbilical cord blood are very few, only 15000 EPCs in 1 ml cord blood approximately [2], which cannot meet the requirement of transplantation. This shortage is the primary problem restricted the usage of EPCs. To obtain a large number of EPCs, proliferation method are widely used in vitro. There is another weakness in this method. Cells cultured in vito will probably have proliferation receding, apoptosis, even karyotype changing. Telomeres are structural nucleoprotein located in the end of eukaryotic linear chromosomes, which maintain the integrity of mitotic genome by the protecting the DNA located in terminal chromosome [3]. The length of telomere was shortening along with the cell division, when telomeres shorten to a critical length, cells will be senescent and apoptosis. Telomere's length is maintained by telomerase, which is a RNA nuclear protease complex with reverse transcriptase activity, and hTERT is the catalytic subunit on telomerase that activate the telomere. In this study, exogenous hTERT gene was transfected to bone marrow EPCs of rats, and the biological behavior of transfected EPCs was observed.

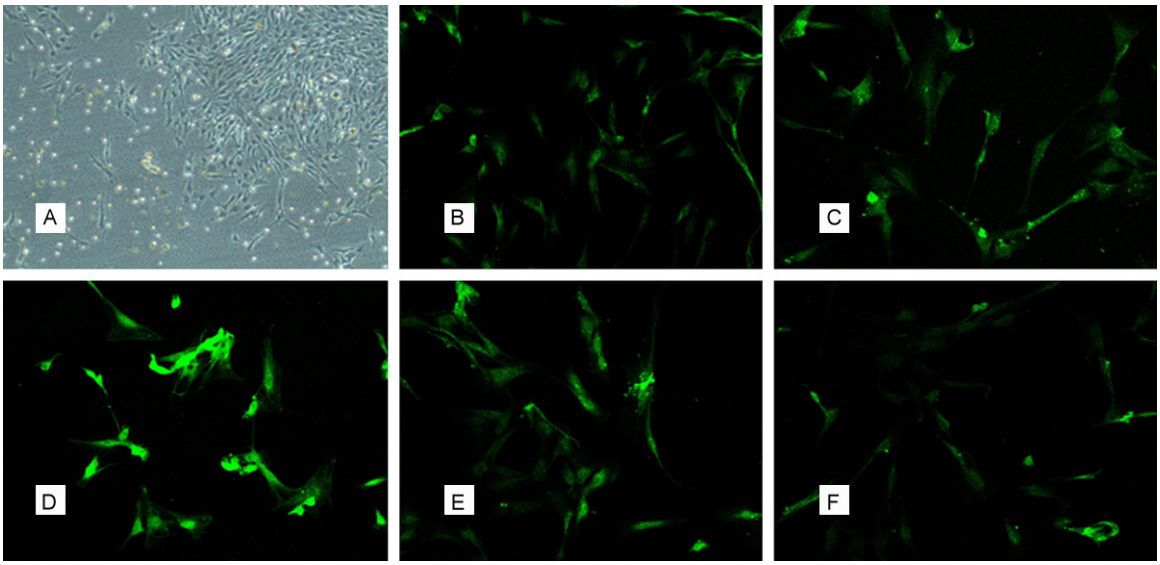

Figure 1. Molecules expressed on the surface of EPCs. A. EPCs on 7th day (×40); B. CD31 (×100); C. CD34 (×100); D. CD133 (×100); E. vWF (×100); F. FLK-1 (×100).

Materials

Animals

Sprague Dawley rats (SPF, 4-week-old, male and female, 80-100 g) were provided by the Experimental Animal Center of Guangdong Medical College.

Reagents

EGM-2 all medium were purchased from Hyclone Company in USA. Hematoxylin stain and eosin stain were purchased from Maixin Company in China. EGFP-hTERT eukaryotic expression vector were constructed by Shanghai Genechem co. Ltd, China. The primary rabbit anti-rat antibody including vWF, FLK-1 (Santa Cruz Biotechnology Co. Ltd, USA), CD133, CD31, CD34 (Beijing Boosen Biological Technology Co. Ltd). The secondary goat anti-rabbit antibody for the molecular mention above were conjugated with fluorochrome FITC (Beijing Boosen Biological Technology Co. Ltd, China).

Methods

Cultivation of EPCs

Femur, humerus, tibia bone marrow from rats were taken out and mixed with cold physiological saline. Bone marrow mixed fluid gently added into the FICOLL lymphocyte separation liquid (1:1), and the cells were extracted follow the methods used previously [4]. The extracted cells resuspended with EGM-2 and cultivated in incubator with the condition of 37°C, 5% CO_2 for fifteen days.

Identification of EPCs

EPCs growing on glass coverslips were fixed with 4% paraformaldehyde for 30min first, and then permeabilized with 0.3% Trion-X100, sealed with 10% goat serum, added the primary antibody of anti-CD31, 34, 133, VWF, FLK-1 and PBS solution, incubated overnight in the temperature of 4°C. Secondary antibody were marked with FITC, cells were incubated in dark at room temperature for 1 h. After sealing the piece, cells were observed under confocal laser scanning microscopy.

Transfection of cationic liposome and selection and amplification of the resistant clone

The third generation of EPCs was inoculated into 24 well-plates, liposome were transfected when 80% cells were fusion. Cells were divided into 2 groups: transfection group and empty vector group. G418 (neomycin) were used to screen and amplify the resistant clones.

EPCs cycle detection

According to the instruments of cell cycle detection kit, the cell cycle of EPCs was detected at the 5th generation and the 15th generation before and after transfection.

Table 1. NO, LDH and iNOS concentration in supernatant of EPCs cultured ($\bar{x}$±s, *n*=4)

Group	NO (μmol/L)	LDH (μmol/L)	iNOS (U/ml)
EPCs	14.673±0.507	3.577±0.085	3.95±0.132
EGFP-EPCs	15.087±0.684[&]	3.532±0.034[&]	3.407±0.132[&]
hTERT-EPCs	17.17±0.686[&]	3.98±0.141[&]	4.397±0.170[&]

[&]*P*<0.01 (compared with EPCs group).

EPCs proliferation detection

Cells were dissociated and implanted in 96 well-plates. Each group contained parallel wells, and were detected daily by using MTT method. Experimental procedures outlined below: First, 5 mg/ml MTT fluid were added to each well, 150 μL dimethylsulfoxide (DMSO) were added into each well at last 4 h of culture, low-speed oscillation in dark for 10 min. OD value of cells in each well was detected with microplate reader when wave length was 492 nm.

Concentration of NO, LDH and iNOS analysis

Cells were divided into 3 groups: Control group (group EPCs), Transfection group (hTERT-EPCs group), NO-loaded group (EGFP-EPCs group). Cells dissociated and implanted in 96 well-plates. Each group contained parallel wells, NO, LDH, iNOS detection were performed according to the instrument of kits.

Determination of the optimal screening concentration of G418 and EGFP-hTERT recombinant plasmid transfected EPCs

Gradient medium in different concentration of G418 (100, 200, 300, 400, 500, 600, 700, 800 μg/mL) were prepared. Cells in 24 well-plates were cultured in different concentration of G418 when there were 80% fuse in each well. The optimal screening concentration of G418 were that can kill all cells within 10-14 days; And the EGFP-hTERT recombinant plasmid transfection were performed according to the instruments of Lipofectamine 2000 Regent kit from Invitrogen company.

G418 selection and positive cells obtaining

Cells divided into 2 groups: normal group (EPCs), transfection group (hTERT-EPCs); cells transferred to the medium with optimal screening concentration of G418 when there were 80% fuse in each well. Changed the medium into maintaining concentration (half the optimal concentration) when cells in control group alldied,continuedcultureuntil the positive cells appear, and then transfer the positive cells into 6 well-plates for expansion by using filter paper method.

hTERT mRNA expression before and after transfection analyzed by reverse transcription PCR (RT-PCR)

hTERT mRNA of EPCs from the 5th, 15th gen eration before transfection and the 5th, 25th generation after transfection were analysis. Specific primers were designed by Primer5.0 software according to the hTERT sequence. The length of target fragment is 410 bp, the se quence of the primers listed below: F 5'-GTATGCCGTGGTCCAGAAGG-3', R 5'-CGTGGGTG-AGGTGAGGTGTC-3'. Total RNA was extracted by RNA extraction kit from TAKARA Company, and reverse transcription was also performed by relevant kit.

Observation of capillary regeneration of myocardial infarct area in rats

Rats were divided into 3 groups: Control, EPCs and transfection group (hTERT-EPCs). Myocardial infarction model were built in rats according to the method descript previously [5]. Three areas of infarction lesions were selected to transplant EPCs and hTERT-EPCs through intramyocardial injection, and PBS were injected into rats in control group. All rats were sacrificed and removed the heart after 4 weeks, capillary regeneration of myocardial infarct area was observed in paraffin sections of the myocardium stained with HE.

Statistical analysis

All data were stated as $\bar{x}$±s, and analyzed using SPSS 19.0 software with One-way ANOVA and Scheffe test, The relationship between the concentration of NO and the activity of iNOS were tested by pearson correlation analysis. *P*<0.05 was considered to be of statistical significance.

Results

The growth and molecules expressed on the surface of identified EPCs

The separated mononuclear cells before culture presented a circular profile. After cultured, cells attached and grew well on the 4th day, and

Table 2. Number of vessels in myocardial infarction area ($\bar{x}$±s, *n*=5)

Group	Control	EPCs	hTERT-EPCs
Number of vessels (number/HP)	8.04±0.385#	11.52±0.756#	13.68±0.701

#*P*<0.01 (compared with EPCs group).

Table 3. Quantitative data for cells circle detected by flow cytometry ($\bar{x}$±s, *n*=4)

group	G1 (%)	G2 (%)	S (%)	G2/G1	CV (%)
Control-t (the 5th generation)	89.24±9.81	3.11±0.67	7.65±2.33	1.77±0.22	8.23±1.96
Control-t (the 15th generation)	73.61±6.49	5.17±1.96	6.94±2.11	2.15±0.94	7.46±1.03
EPCs-hTERT-t (the 5th generation)	66.44±7.88★	7.31±3.36	17.13±5.66★,Δ	2.97±1.08★	9.64±2.11Δ
EPCs-hTERT-t (the 15th generation)	69.43±8.32	6.91±3.06	10.46±3.19★,Δ	1.97±0.72	7.91±1.77

★*P* < 0.05 (compared with the Control-t group of the 5th generation). Δ*P* < 0.05 (compared with the Control-t group of the 15th generation).

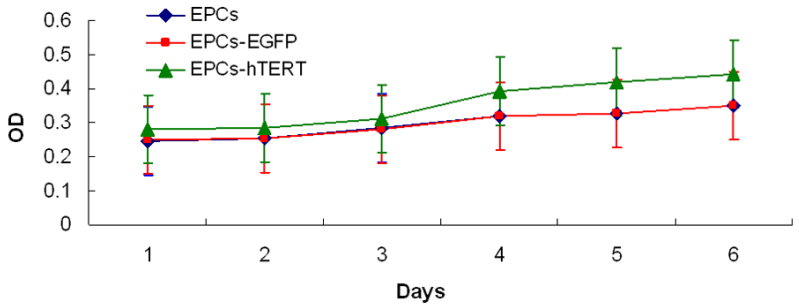

Figure 2. Cells growth in each group.

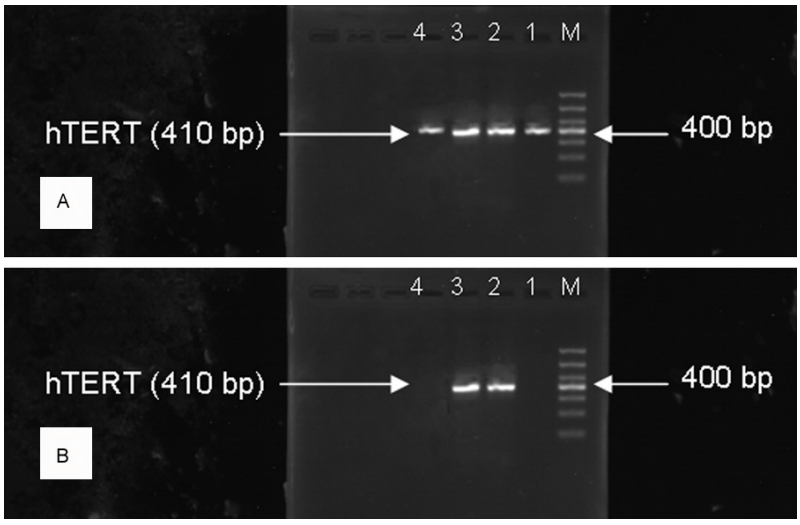

Figure 3. hTERT mRNA expression analyzed by RT-PCR. M for Marker; 1, 4 for EPCs group; 2, 3 for the hTERT-EPCs group; A. The 5th generation of EPCs and hTERT-EPCs; B. The 15th generation of EPCs and 25th generation of hTERT-EPCs.

grew confluence on 7th day. EPCs presented a cord-like profile and even capillary network like profile on 14th day, some of which presented a typical cobblestone look. Molecules expressed on the surface of EPCs were CD31, CD34, CD133, vWF, FLK-1 (**Figure 1A-F**).

Proliferation of transfected cells was much stronger

The results of cell cycle detection showed that, after transfection, cell proliferative ability is much stronger than before transfected, and the proliferative ability of EPCs decreased while hTERT-EPCs group had no obvious change with the prolongation of culture time before transfection (**Table 3**). Cells proliferation in hTERT-EPCs group was significantly higher than which in EPCs and EGFP-EPCs group (**Figure 2**).

Secretory function of transfected EPCs were much stronger

Secretion of NO, LDH and iNOS in transfected group were much more than other two groups (**Table 1**).

hTERT mRNA expressed continuously after transfection

The results of RT-PCR showed that, EPCs aged early in the 15th generation, but hTERT-EPCs still expressed hTERT mRNA strongly on the 25th generation, which indicate that survi-

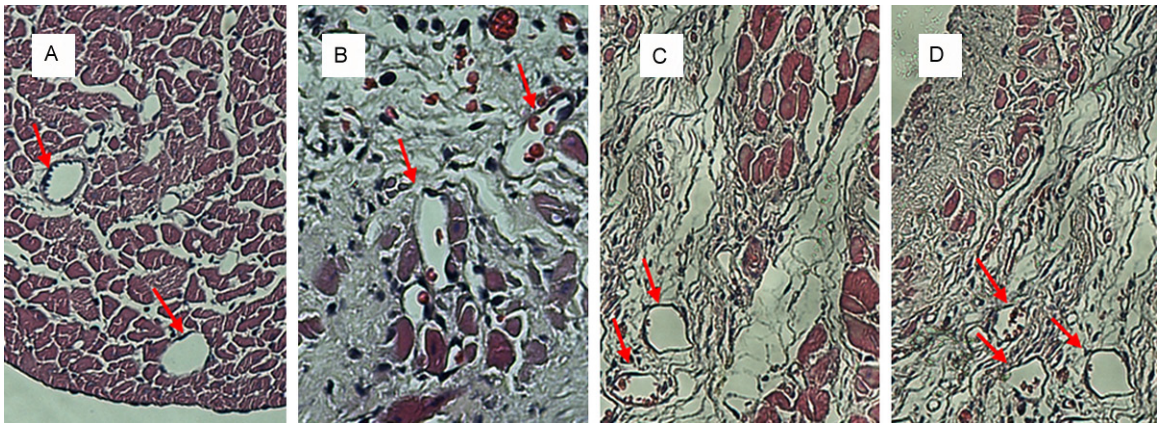

Figure 4. Pathology analysis of Myocardial (HE staining, ×400). A. Control group; B. PBS group; C. EPCs group; D. hTERT-EPCs group. Arrow shows the vascular.

val ability of EPCs were enhanced by transgenosis (**Figure 3**).

Capillary regenerated more in the infarct area of myocardium after transplanted the transfected EPCs

Angiogenesis in all 3 groups, but capillary regenerated in transfection group increased significantly than the other two groups (**Table 2**; **Figure 4**).

Discussion

Early research has shown that [6] EPCs plays an important role in repair process of the damaged vascular wall. It was reported that, the decrease of EPCs level may be an independent predictive factorof patients with coronary heart disease who had poorly prognosis [7]. The transplantation of EPCs can make up for the necrosis of the myocardial cells and endothelial cells. Plenty of researches had focused on EPCs, but the surface marker of which had not reached a consensus. Hur [8] believes that naive EPCs mainly expressed CD133 (also AC133), CD34 and VEGFR-2 (also KDR or FLK.1). and mature EPCs expressed VE-cadherin, Fh-1, e-NOS, CD31 and etc. Some scholars defined the cells that expressed CD34/CD133/VEGFR-2l (KDR or Flk-1) as EPCs [9]. However, CD34 CD133, Flk 1, c-Kit, Sca-1, DiL Ac-LDL, vWF, VEGFR2 are used for the major cell surface markers of EPCs in most research [10, 11]. So we have chocen the molecular of CD31, CD34, CD133, FLK-1 and VWF as the surface marker for EPCs identification.

Transfected hTERT somatic cells can be used as seed cell for tissue engineering. Masato Sato [12] transfected the hTERT gene to rabbit chondrocyte, and found that, the transfected cells showed higher levels of cell proliferation despite of the donor age, and expressed type II collagen. In addition, cells growth increased in vitro after transfection. Wang [13] found that exogenous hTERT gene could be transfected to human epidermal stem cells in vitro. After transfection, hTERT mRNA and protein expressed stronger than non-transfected cells. Our experimental results showed that, transfected EPCs secreted NO, LDH and iNOS well, which indicated EPCs not only possess the function of endothelial cells, but the proliferation and the secretion also were stronger than endothelial cells. In our study, the results of MTT analysis indicated a much more stronger proliferation ability of transgenic EPCs, and the results of RT-PCR showed an increase in quantity and duration of hTERT mRNA expression in transfected cells. Our results are consistent with that of Murasawa [14].

EPCs differentiate to vascular endothelial cells and independently, that is to say EPCs does not need to rely on the intrinsic vascular system and form a new vascular all by itself. Kalka [15] transplanted athymic mice with EPCs cultured in vitro to mice with lower limb ischemia and found that, capillaries in ischemic area increasedsignificantly,andtissueperfusionalsoincreased,theamputationratewassignificantlydecreased. Kawamoto [16] injected EPCs to myocardial ischemia mice and found the blood supply of ischemic myocardium increased significantly

and the left ventricular function improved. Those evidences suggested EPCs as a vascular endothelial precursor cells was potent to regenerate vessels, it played an important role in the angiogenesis in adult individuals. This discovery provided an important clue to explore the mechanism of angiogenesis, and a new strategy in the therapy of coronary heart disease. However, the application of EPCs was restricted because the little number was in peripheral blood. Exogenous hTERT gene transfection may provide a solution to this restrict. Various cells were proven to be prolonged life-span and increased the survival rate after hTERT gene transfection. In our experiment, we found the capillary regeneration increased in rats' infracted myocardial tissue after transgenic EPCs were transplanted.

In summary, EPCs have the treatment prospects for coronary heart disease, but there are still many issues to be resolved. Identified surface markers of EPCs are essential for improving the purity of EPCs in isolation. In addition, whether transgenic EPCs well mutate or not needs to be further studied.

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Disclosure of conflict of interest

None.

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References

[1] Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G and Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997; 275: 964-967.

[2] Murohara T, Ikeda H, Duan J, Shintani S, Sasaki K, Eguchi H, Onitsuka I, Matsui K and Imaizumi T. Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization. J Clin Invest 2000; 105: 1527-1536.

[3] Atturu G, Brouilette S, Samani NJ, London NJ, Sayers RD and Bown MJ. Short leukocyte telomere length is associated with abdominal aortic aneurysm (AAA). Eur J Vasc Endovasc Surg 2010; 39: 559-564.

[4] He J, Xiao Z, Chen X, Chen M, Fang L, Yang M, Lv Q, Li Y, Li G, Hu J and Xie X. The expression of functional Toll-like receptor 4 is associated with proliferation and maintenance of stem cell phenotype in endothelial progenitor cells (EPCs). J Cell Biochem 2010; 111: 179-186.

[5] Formiga FR, Pelacho B, Garbayo E, Abizanda G, Gavira JJ, Simon-Yarza T, Mazo M, Tamayo E, Jauquicoa C, Ortiz-de-Solorzano C, Prosper F and Blanco-Prieto MJ. Sustained release of VEGF through PLGA microparticles improves vasculogenesis and tissue remodeling in an acute myocardial ischemia-reperfusion model. J Control Release 2010; 147: 30-37.

[6] Werner N, Priller J, Laufs U, Endres M, Bohm M, Dirnagl U and Nickenig G. Bone marrow-derived progenitor cells modulate vascular reendothelialization and neointimal formation: effect of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibition. Arterioscler Thromb Vasc Biol 2002; 22: 1567-1572.

[7] Schmidt-Lucke C, Rossig L, Fichtlscherer S, Vasa M, Britten M, Kamper U, Dimmeler S and Zeiher AM. Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events: proof of concept for the clinical importance of endogenous vascular repair. Circulation 2005; 111: 2981-2987.

[8] Hur J, Yoon CH, Kim HS, Choi JH, Kang HJ, Hwang KK, Oh BH, Lee MM and Park YB. Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. Arterioscler Thromb Vasc Biol 2004; 24: 288-293.

[9] Gehling UM, Ergun S, Schumacher U, Wagener C, Pantel K, Otte M, Schuch G, Schafhausen P, Mende T, Kilic N, Kluge K, Schafer B, Hossfeld DK and Fiedler W. In vitro differentiation of endothelial cells from AC133-positive progenitor cells. Blood 2000; 95: 3106-3112.

[10] Miyata T, Iizasa H, Sai Y, Fujii J, Terasaki T and Nakashima E. Platelet-derived growth factor-BB (PDGF-BB) induces differentiation of bone marrow endothelial progenitor cell-derived cell line TR-BME2 into mural cells, and changes the phenotype. J Cell Physiol 2005; 204: 948-955.

[11] Friedrich EB, Walenta K, Scharlau J, Nickenig G and Werner N. CD34-/CD133+/VEGFR-2+ endothelial progenitor cell subpopulation with potent vasoregenerative capacities. Circ Res 2006; 98: e20-25.

[12] Sato M, Shin-ya K, Lee JI, Ishihara M, Nagai T, Kaneshiro N, Mitani G, Tahara H and Mochida J. Human telomerase reverse transcriptase and glucose-regulated protein 78 increase the

life span of articular chondrocytes and their repair potential. BMC Musculoskelet Disord 2012; 13: 51.

[13] Zhang Y, Nuglozeh E, Toure F, Schmidt AM and Vunjak-Novakovic G. Controllable expansion of primary cardiomyocytes by reversible immortalization. Hum Gene Ther 2009; 20: 1687-1696.

[14] Murasawa S, Llevadot J, Silver M, Isner JM, Losordo DW and Asahara T. Constitutive human telomerase reverse transcriptase expression enhances regenerative properties of endothelial progenitor cells. Circulation 2002; 106: 1133-1139.

[15] Kalka C, Masuda H, Takahashi T, Kalka-Moll WM, Silver M, Kearney M, Li T, Isner JM and Asahara T. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc Natl Acad Sci U S A 2000; 97: 3422-3427.

[16] Kawamoto A, Gwon HC, Iwaguro H, Yamaguchi JI, Uchida S, Masuda H, Silver M, Ma H, Kearney M, Isner JM and Asahara T. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. Circulation 2001; 103: 634-637.