Clinical Research

Administration of mesenchymal stem cells after rat sciatic nerve defect reconstruction with amnion tube expresses higher s100 protein

IGK Satrio Adiwardhana,[1] I Ketut Suyasa,[2] Putu Astawa,[3] IKG. Mulyadi Ridia,[4] Made Bramantya K[5]

[1,2,3,4,5]Department of Orthopaedic & Traumatology, Faculty of Medicine, Universitas Udayana, Sanglah General Hospital, Bali, Indonesia

ABSTRACT

Introduction: Peripheral nerve injury is still a difficult challenge. Amnion tube construction guides the regeneration of axons in peripheral nerve injury defects. Schwann cells have an important role in the healing of nerves, characterized by the presence of S100 proteins. This study aims to assess the expression of S100 protein after reconstruction of sciatic nerve defect using amnion tube and mesenchymal stem cells.

Methods: This study was an experimental study with a post-test only control group using 3-month Wistar white rats weighing 200-250 grams. Neurectomy procedure of sciatic nerve with a defect of 10 mm was performed and subjects were randomized into 2 groups: control group (n = 18) treated with amnion tube and treatment group (n = 18) treated with amnion tube combined with mesenchymal stem cells. Ten days post-treatment, S100 protein expression was examined immunohistochemically in the proximal nerve, the middle part of the amniotic tube, and the distal part. The data obtained was analyzed using SPSS.

Results: Statistical analysis by Chi square test found that the expression of S100 in the nerve cells was significantly higher (P <0.05) on the proximal, middle, and distal ends in the treatment group treated with a combination of amnion tube and mesenchymal stem cells when compared to the control group given only amnion tube.

Conclusion: Mesenchymal stem cells treatmentin post-reconstruction of sciatic defect of rats combined with amnion tube provides better regeneration ability, characterized by higher S100 protein expression, compared to amnion tube treatment without mesenchymal stem cells.

ABSTRAK

Pendahuluan: *Cedera saraf tepi saat ini masih jadi tantangan. Konstruksi dengan* amnion tube *dapat menuntun regenerasi akson pada defek cedera saraf tepi. Sel schwann memiliki peranan penting dalam penyembuhan saraf, ditandai dengan adanya protein S100. Penelitian ini bertujuan untuk menilai ekspresi protein S100 pasca-rekonstruksi defek saraf ischiadikus tikus dengan menggunakan* amnion tube *yang disertai sel punca mesenkim.*

Metode: *Penelitian ini adalah penelitian eksperimental dengan* post-test only control group *design dengan subjek tikus putih* wistar *berumur 3 bulan dengan berat200-250 gram. Subjek penelitian yang memenuhi kriteria inklusi akan dilakukan neurektomi saraf ischiadikus dengan defek 10 mm dan diacak menjadi 2 kelompok: kelompok kontrol (n = 18) yang diberi* amnion tube *dan kelompok perlakuan (n = 18) yang diberi kombinasi* amnion tube *dan sel punca mesenkim. Sepuluhhari pasca-perlakuan, ekspresi protein S100 diperiksa secara immunohistokimia pada saraf bagian proksimal, bagian tengah* amnion tube, *dan distal. Data yang diperoleh dianalisis dengan menggunakan SPSS.*

Hasil: *Analisis statistik menggunakan* Chi square test *menunjukkan hasil ekspresi S100 pada sel saraf yang lebih tinggi secara signifikan (P<0,05) pada ujung proksimal, tengah, dan distal pada kelompok perlakuan yang diberi kombinasi* amnion tube *dan sel punca mesenkim jika dibandingkan dengan kelompok kontrol yang hanya diberi* amnion tube.

Kesimpulan: *Perlakuan sel punca mesenkim pasca-rekonstruksi defek saraf ischiadikus tikus bersama amnion tube memberikan kemampuan regenerasi yang lebih baik, ditandai dengan ekspresi protein S100 yang lebih tinggi dibandingkan tanpa sel punca mesenkim.*

Keywords: sciatic nerve defect, Mesenchymal stem cell, Amnion Tube, Protein S100.

Corresponding author : IGK Satrio Adiwardhana, MD, ryo.wardhana7@gmail.com

INTRODUCTION

The peripheral nerves are an axon group that sends motor stimuli derived from the anterior horn of the spinal cord to the muscles and the sensory stimulation of the side receptor through the cell on the dorsal root ganglia to the cord. Peripheral nerve injury occurs due to interruption of communication between the central nerves and the peripheral nerves, including compression, laceration, and nerve retention.[1] Peripheral nerve injury occurs in 2.8% of all trauma patients, where the incidence in developing countries is estimated 13 - 23 per 100.000 people per year. In the United States, 360.000 people have experienced upper extremity nerve injury, resulting in 8 million days of restriction of activity and 5 million days of rest.[2]

The problem of peripheral nerve injury is a defect which can be repaired by nerve autograft asthe golden standard. There are some disadvantages of nerve autograft: sacrificing donor tissue that affects the nerve function in the donor network, morbidity arising in donor tissue, limited diameter and length, possible misdirection of axons, and infection.As an alternative, nerve conduit can be used for peripheral nerve defect treatment and serves as a scaffold for regeneration of axons from proximal to distal, without disturbance due to poor suturing (mismatching) and avoiding the outward growing or extra neural (misdirection) axons.

Amnion is an example of a nerve conduit derived from a layer of human fetal rich in collagen, fibronectin, and laminin. Amnion has several advantageous properties such as pluripotent, anti-inflammatory, bio-absorbable, low immunogenic reaction, does not induce cancer properties, and does not cause ethical problems. One important factor that determines the prognosis of peripheral nerve injury is Schwann cells. Schwann cells marker, S100 protein, is a structural protein produced by oligodendroglia cells and astroglia cells that have biological effects such as protein phosphorylation, enzyme activity, Ca^{2+} balance, and inflammatory response to peripheral nerves.S100 protein is also thought to function as a promoter of nerve cells elongation and nerve regeneration.[2,3]

Stem cells are used as one of the nerve cell injury therapies because of their low immunogenicity. Stem cells are able to differentiate into multiple mesodermal lineage cells, including chondrocytes, osteocytes, and adipocytes, also into neuronal phenotypes, including astrocytes, oligodendrocytes, microglia, and neurons. BMSC (Bone Marrow Stromal Cells) is one example of adult stem cell that can differentiate into Schwann cells.

This study investigated the management approach of peripheral nerve injury with mesenchymal stem cells to accelerate nerve regeneration by using amnion tube as the nerve conduit, and analyzed the expression of S100 protein by Schwann cells in the proximal, distal, and injured amniotic part of the tubes. The originality of this study is the use of BMSC in amnion tube to overcome peripheral nerve injury defect.

METHODS

This research was conducted by using a pure experimental research design on animals employing randomized post test only control group design, utilizing a control group. This research was conducted from 1 February 2018 to 30 March 2018 at the Biomaterial Center installation – Bank network of Dr. Soetomo Hospital for production of amnion tube, Laboratory of the Centre for Research and Development of Stem Cells, Airlangga university,

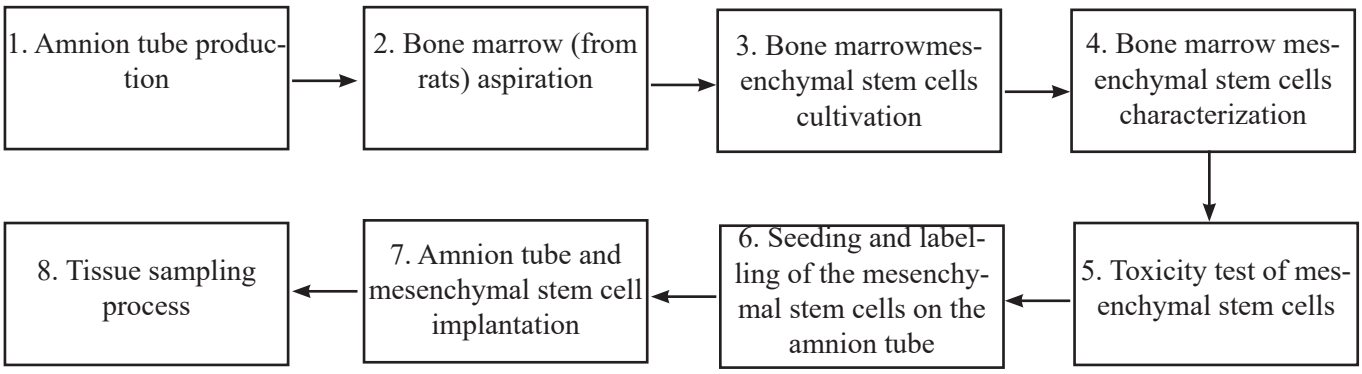

Figure 1. The steps of the research describe every aspect that was done in the process.

Surabaya, for the preparation of mesenchyme stem cells, Laboratory of Pharmacology, Faculty of Medicine, Udayana University and Laboratory of Veterinary Pathology, Faculty of Veterinary Medicine, Udayana University, for immunohistochemical examination of S100 protein.

The data obtained is analyzed by the following steps, including descriptive analysis, normality - homogeneity analysis and inferential analysis.The steps of this research can be seen in the diagram shown in Figure 1.

Each of the step in this research will be theoretically explained below.

1. Amnion tube production

The amnion used has passed biocompatibility and biodegradation tests. Proteolytic enzyme was added to a 100mm^2 rectangular amnion fragment, washed with distilled water, packed circularly in a 1.0mm diameter of stainless steel, and then immersed in a cross-linking agent. After being dried at 37°C, the fragment was removed from the iron rod, washed with water to remove the remaining cross-linking agent, dried again, and then stored in a vial and sterilized with gamma radiation of 25kGy.

2. Bone marrow (rats) aspiration

Experiment rats wereanesthetizedby intramuscular injection of 20mg/kg BW ketamine and intramuscular injection of 3mg/kg BW xylazine. After disinfection procedure, lateral incision was performed, the femoral bone was identified, and the hip joint in proximal part and the distal partof the knee joint were then separated. The femur was rinsed with 0,9%NaCl solution twice. The bone marrow was taken from the femur part with intramedullary flushing method.

3. Bone marrow mesenchymal stem cells cultivation

The bone marrow was resuspended by adding PBS in 1:1 ratio and agitated until homogeneous. The suspension was poured into a cyclic tube filled with 5 cc of ficol by slowly passing it through the tube wall.The mixture was then centrifuged for 30 minutes at a speed of 1600 rpm with a temperature of 26ºC. The mixture resulting in4 separated layers, and the aspiration of the 2nd layer gave a buffycoat thatlooked like a fog ring. The buffycoat solution was then poured slowly into a 15 ml tube cone and then washed with 10cc of PBS. The solution was then centrifuged for 5 minutes at 1600 rpm. Once pellet was formed, culture medium was then added to thepellet. Resuspensionwas performed until homogeneous, and the suspension was then plated on a 10cm diameter dish. Identity label in the form of name and processing date was then attached to the dish and then incubated in a CO_2 incubator.

4. Bone marrow mesenchymal stem cells characterization

Mononuclear cell was isolated from the harvested bone marrow using sentrifugal hystopaque-density method. All mononuclear cells obtained were cultured in Dulbecco's Eagle medium, with 10% fetal bovine serum (Sigma Chemical) at 37ºC plastic dish in 5% CO_2 moisture. Not-attached cells were removed, and the attached cells were cultivated. For identification, cells were incubated using FITC anti-human CD90 antibody (Biologened, USA), PE anti-human CD29 antibody (Biologened, USA), PerCp/Cy5.5 anti-human CD34 and FITCanti-human CD45. MSC characterization was done by using fluorescence-activated cell sorting (FACS Calibur).[4,5] Monolayer cells were split into single cells and then plated on a COOKE glass object and incubated at 37ºC for 2 hours. The cellswere further fixed with 4% formalin buffer for 15 min, and the glass object was washed with PBS and dried, followed by 10% FBS blocking for 15 min. The cells were then added with 10 µl secondary antibody dye as desired, for example the characterization of mesenchymal stem cells with CD 105, haemopoietic stem cell with CD 45, cancer cells with CD 133, and so on, the CD staining was performed in a dark room since it was sensitive to light. The cells were then incubated at 37ºC for 1 hour and readby using a Fluorescence microscope. CD 105 gave positive result of ICC staining.

5. Toxicity test of mesenchymal stem cells

The monolayer cells from step 4were split into single cells and then plated on M-96plate,3000-5000 cells/ well, and then incubated in CO_2 incubator at 37ºC for approximately 24 hours. The cells grown on the M-96 plates were added with a certain material (200 µl/well, at a desired concentration) to determine if they were toxic to cells, or induce cell proliferation, and incubated for 20 h in CO_2 incubator at 37°C. MTT (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) (5mg/ml) was dissolved in PBS and then added to each well, 25µl/ well, and incubated for 4 hours. The MTT assay result was the formation of Formazan crystals as a product of tetrazolium dye reduction by the dehydrogenase enzymes secreted by the cells's mitochondria. The

more the formazan crystals formed,the higher the cell viability; or cell proliferation may also indicate that the tested material was not toxic to the cells.

6. Seeding and labelling of the mesenchymal stem cells on the amnion tube

The monolayer mesenchymal stem cells from step (4) was split into single cells with cell tripsinization. The mesenchyme root labeling on the amnion tube was performed using the PKH26 protocol, 2 mL final staining containing of $2x10^{-6}$ M PKH26 and $1x10^{7}$ cells/mL solution. All steps were done in a room temperature at 20-25ºC. The suspension containing $2x10^{7}$cells was poured into a conical bottom polypropylene tube and washed once using the medium without serum. The tube was centrifugedat 400 x g for 5 minutes. The supernatant was poured with a pipette, leaving the remaining medium no more than 25/ml. The pellet was added with 1 ml Diluent C solution and resuspended by pipettingit slowly. After the dyeing, 2x Dye solution (4 x 10^{-6} M) was prepared in Diluent C solution by adding 4 µL ethanol dye solution PKH26 (No. Catalog: P9691) to 1 ml Retail C Solution into a polypropylene centrifuge tube and then mixedthoroughly. After that, 1 ml of 2x suspension solution was immediately added into 1 ml 2x dye solution and then the solution was mixed with a pipette. The final concentration of the mixturewas $1x10^{7}$ cells/mL and $2x10^{-6}$ M PKH26. The cell/dye solution was incubated for 1-5 minutes. Because color developmenttook place quickly, longer mixing duration would not have any more benefits. Color development was stopped by adding 2 ml serum or commonly used protein solution (e.g. 1% BSA) and the solution was incubated for 1 min to allow adhesion of the dye. The cells were centrifuged at 400 x g for 10 minutes at 20-25ºC, the supernatant was gently separatedwhile tried not to move the cells. The cell's pellet was re-suspendedin 10 ml prepared media, transferredinto a sterile poly-propylene tube, centrifuged at 400 xg for 5 min at 20-25ºC, and the pellet was washed twice with 10 ml medium to ensure the removal of the remaining dyes. After the final wash, the pellet was re-suspended with 10 ml of ready-to-check media from cell recovery, cell viability, and fluorescence intensity. The suspension was centrifuged and re-suspendedin order to get the final concentration of the desired living cells.

7. Amnion tube and mesenchymal stem cell implantation

Skin incision was performed from the right knee to the pelvis, the gluteal musclewas separated. The sciatic nerve was exposed and cut in the mid thighs. The tip of the proximal and the distal pieces were separatedfor 10mm long. In the control group (n1 = 18), the neural defect was attached to the amnion tube, stitched with 10-0 monolilamide nylon thread with 2 stitches at each end, proximal and distal, and in the treatment group (n2 = 18) the neural defect was attached to a combination of amnion tube and mesenchymal stem cells.The muscle and fascia cells are covered with a 4-0 resorbable single suture and the skin is glued using a stapler.

8. Tissue sampling process

The rat's sciatic nerve was re-explored through the previous surgery path, 10^{th} day post-implantation. Three slices of tissues perpendicular to the long axis of the nerve were prepared: 5mm from the proximal end (Sp), in the middle (Sm) and 5mm from the distal end (Sd). The sampling was conductedcompletely in a sterile condition and the materials and the tools used were disposable ones. Each ingredient was filled in a 1.5ml container and then put in a container containing formalin and took to the veterinary laboratory. The rest of the unutilized body of rats' were burned.

9. Statistical analysis was performed with SPSS for Windows version 22.0 and the hypothetical method used was Chi-Square test.

RESULTS

Table 1. The frequency distribution of research subjects of each group

Group	Frequency (n)	Percentage (%)
Treatment (Amnion Tube and MSC)	16	50.00
Control (Amnion Tube)	16	50.00
Total	32	100

From the Table 1, it can be seen that the total number of the study subjects was 32 with the treatment group treated with amnion tube combined with stem cells after reconstruction ofsciatic nerve defects consisted of 16 subjects or 50.00%.

Table 2. Comparation test results of post-test data of S100 variable for treatment and control group

Protein S100 expression		Group		p-value
		Treatment (n=16) n (%)	Control (n=16) n (%)	
Proximal				
	Weak	4 (25)	9 (56,3)	
	Moderate	5 (31,3)	6 (37,5)	
	Strong	7 (43,8)	1 (6,3)	
Middle				0,033
	Weak	6 (37,5)	13 (68,4)	
	Moderate	8 (50)	3 (18,8)	
	Strong	2 (12,5)	0 (0)	
Distal				0,011
	Weak	6 (37,5)	14 (87,5)	
	Moderate	7 (43,8)	2 (12,5)	
	Strong	3 (18,8)	0 (0)	

From The table 2, it can be seen that the S100 protein expression in the proximal nerve stump section in the treatment group showed strong expression (43.8%), whereas in the control group showed weak expression (56.3%).In the middle of the amnion tube, the expression of S100 protein in the treatment group showed moderate expression (50%) while in the control group showed weak expression(68.4%).At the distal nerve stump, S100 protein expression showed moderate expression in the treatment group (43.8%) while in the control group showed weak expression(87.5%).

The statistical test used in this research was Chi-Square. The results in Table 2 demonstrated that the difference of S100 protein expression in the proximal, middle, and distal portions of nerve stump between the treatment group with the addition of mesenchymal stem cells compared to the control group without mesenchymal stem cells was statistically significant with $p < 0.05$.

DISCUSSION

From the results of this study, we found that by adding mesenchymal stem cells to the amnion tube, the process of nerve cell regeneration can be accelerated as evidenced by the expression of S100 protein, which is a marker of Schwann cells found in myelin.These results are consistent with a study conducted by Wakao,*et al*., (2010) on auto-cell transplantation therapy with MSC in non-primate animal nerve injury that evidenced by histologic, electromyographic and functional improvements by utilizing collagen nerve conduits packed with autologous bone marrow derived from MSC differentiated into Schwann cells *in vitro*.[6] Sunderland,*et al*. (2004) reported that the expression of S100 protein in the peripheral proximal segment of the peripheral nerves that was destroyed did not change significantly compared to the control group.[7]On the contrary, the expression of S100 protein in the distal segment decreased in the initial 2 weeks, in parallel, the S100 level of the nerve in the distal stump on the transparent nerve decreased significantly by the end of the first week. Another study by Guo and Dong (2009) showed positive results in the rabbit group with a 10 mm facial nerve defect after the addition of stem cells to the allograft chitosan tube.[8]

Increased expression of S100 proteins signifies the proliferation of Schwann cells in post-recurrent rat's sciatic nerve.[4,9] In the nervous system, proteins Sl00a and S100b are found in the glial cells. Protein S100b is the only protein present in the peripheral nerves.[10,11]Perez,*et al*., observed the level of the S100 protein in optic nerve degeneration and concluded that the S100 protein subunits were associated with the Schwan Cells. Immunohistochemical and immunocytochemical studies confirmed that in the peripheral nervous system, S100 protein is found especially in the glial cells (Schwann). The expression of S100 is related to axon diameter and the degree of myelination process. S100 expression is also found in the development or in the nerve injury response associated with biological processes of Schwann Cells.[2,9-11]Several other previous studies have shown that proliferating Schwann cells promote sustained regeneration and functional recovery of the sciatic nerve.

The use of a nerve conduit as a scaffold of axon regeneration guide from proximal to distal may reduce some of the possible problems, such as disorders due to mismatching and outward growth of axons or extra-neural growth (misdirection). Another advantage of nerve conduit use is that no secondary injury is created. The use of nerve conduit will provide minimal stress on the proximal and distal nerve defects, if compared to the primary repair operation where the pull is unavoidable. The voltage in the connecting area will interfere with the blood flow

which will eventually inhibit the regeneration.[12] Based on some of the above description, post-mesenchymal stem cell treatment in post-reconstruction of rats with sciatic nerve defects combined with amnion tube provides better regeneration ability, characterized by higher S100 protein expression,compared to without mesenchymal stem cells.

The planting of mesenchymal stem cells into the scaffold has been shown to stimulate nerve regeneration by providing an unlimited source of Schwann cells.[4,13-14]S100 protein expression in the proximal, middle and distal of nerve stump after reconstruction of rats with sciatic nerve defect using amnion tube and mesenchymal stem cells is higher than without mesenchymal stem cells.

CONCLUSION

Mesenchymal-stem cell treatmentafter reconstruction of sciatic nerve defects in ratscombined with amnion tube providesbetter regenerationability, characterized by higher S100 protein expression compared towithout mesenchymal stem cells. However, further research is needed by using human-like research subjects, such as monkeys or human, in order forthe results can be applied as a treatment for peripheral nerve defects.

Limitation of the Study

Although this study has concluded a successful improvement of nerve regeneration using amnion tube in rats, this study has not been tested on human. Further study is required to analyze the effect of the amnion tube on thenerve regeneration in human.

REFERENCES

1. Kelsey J, Praemer A, Nelson L, Felberg A, Rice L. Upper extremity disorders. Frequency, impact, and cost. New York, NY: Churchill Livingstone Inc.; 1997.
2. Li Y, Zhang L, Zhang J, Liu Z, Duan Z, Li B. Morphological study of Schwann cells remyelination in contused spinal cord of mices. Chinese J Traumatol = Zhonghua chuang shang za zhi. 2013;16(4):225–9. 4.
3. Mata M, Alessi D, Fink DJ. S100 is preferentially distributed in myelin-forming Schwann cells. J Neurocytol. 1990 Jun;19(3):432–42.
4. Chen C-J, Ou Y-C, Liao S-L, Chen W-Y, Chen S-Y, Wu C-W, et al. Transplantation of bone marrow stromal cells for peripheral nerve repair. Exp Neurol. 2007 Mar;204(1):443–53.
5. Ishikawa N, Suzuki Y, Dezawa M, Kataoka K, Ohta M, Cho H, et al. Peripheral nerve regeneration by transplantation of BMSC-derived Schwann cells as chitosan gel sponge scaffolds. J Biomed Mater Res - Part A. 2009;89(4):1118–24.
6. S. Wakao, T. Hayashi, M. Kitada et al., "Long-term observation of auto-cell transplantation in non-human primate reveals safety and efficiency of bone marrow stromal cell-derived schwann cells in peripheral nerve regeneration"Experimental Neurology. 2010.vol. 223, no. 2, pp. 537–547.
7. Sunderland IRP, Brenner MJ, Singham J, Rickman SR, Hunter DA, Mackinnon SE. Effect of Tension on Nerve Regeneration in Mice Sciatic Nerve Transection Model. Ann Plast Surg. 2004 Oct;53(4):382–7.
8. Guo, BF and Dong, MM. "Application of neural stem cells in tissue-engineered artificial nerve." Otolaryngology–Head and Neck Surgery.2009.Vol 140, Issue 2, pp. 159 - 164 https://doi.org/10.1016/j.otohns.2008.10.039
9. Belkas JS, Shoichet MS, Midha R. Peripheral nerve regeneration through guidance tubes Neurol Res. 2004 Mar;26(2):151–60.
10. Donato R, R. Cannon B, Sorci G, Riuzzi F, Hsu K, J. Weber D, et al. Functions of S100 Proteins. Curr Mol Med [Internet]. 2013;13(1):24–57. Available from: http://openurl.ingenta.com/content/xref?genre=article&issn=1566-5240&volume=13&issue=1&spage=24
11. Isobe T, Ichimori K, Nakajima T, Okuyama T.The alpha subunit of S100 protein is present in tumor cells of human malignant melanoma, but not in schwannoma. Brain Res. 1984;294:38
12. Davis GE, Blaker SN, Engvall E, Varon S, Manthorpe M, Gage FH. Human Amnion Membrane Serves as a Substmiceum for Growing Axons in vitro and in vivo. Science (80-). 1987;236(4805):1106–9.
13. Dezawa M, Takahashi I, Esaki M, Takano M, Sawada H. Sciatic nerve regeneration in mices induced by transplantation of in vitro differentiated bone-marrow stromal cells. Eur J Neurosci. 2001 Dec;14(11):1771–6.
14. Prockop DJ. Marrow Stromal Cells as Stem Cells for NonhematopoieticTissues. Science.1997;276(5309):71–4.