Granulocyte colony-stimulating factor enhances the proliferation of endogenous neural stem cell after cerebral ischemia-reperfusion injury in rats

Jun Tan[1], Juan Li[2], Chengbiao Lu[2], Tingtong Yang[3], Yanyan Han[2], Jianke Xu[4], Yuming Xu[*]

[1] The First Affiliated Hospital of Zhengzhou University, Zhengzhou, PR China
[2]Department of Neurology, The Third Affiliated Hospital of Xinxiang Medical University, Xinxiang, Henan, China
[3]Department of Pathology, Xinxiang Medical University, Xinxiang, Henan, China
[4] Department of Neurology, Kaifeng First People's Hospital, Kaifeng, Henan, China
E-mail: 13903711125@126.com

Abstract: This study was to evaluate the effects of recombinant human granulocyte colony-stimulating factor (rhG-CSF) on the proliferation of endogenous neural stem cells (NSCs) after cerebral ischemia-reperfusion injury (I/R injury) in rats. The animal model of I/R injury was established by middle cerebral artery occlusion (MCAO) method. Rats were divided into 3 groups: Sham operation group, ischemia-reperfusion group (I/R group) and I/R + rhG-CSF group. NSCs proliferation activity in hippocampus was evaluated using BrdU (5-bromo-2'-deoxyuridine) cell proliferation assay. The expression of two key molecules, Wnt1 and β-catenin, which play an important role in the signal transduction pathway of mediating NSCs proliferation, was determined by RT-PCR and Western Blot. Our study showed that the number of BrdU positive cells in I/R group significantly increased after 3d, and reached the peak after 7d, compared with the sham group. When the rats were treated with rhG-CSF (30 μg/kg/d), the NSCs proliferation activity were enhanced significantly, compared with the other two groups. Furthermore，the mRNA and protein expression levels of Wnt1 and β-catenin also were significantly higher in I/R + rhG-CSF group than that in the other two groups. These findings demonstrated that ischemic brain damage can induce the NSCs proliferation in the hippocampal dentate gyrus of adult rats and suggested that rhG-CSF could be used as an potential agent to repair the cerebral injury induced by ischemia-reperfusion.

[Jun Tan, Juan Li, Chengbiao Lu, Tingtong Yang, Yanyan Han, Jianke Xu, Yuming Xu. **Granulocyte colony-stimulating factor enhances the proliferation of endogenous neural stem cell after cerebral ischemia-reperfusion injury in rats.** *Life Sci J* 2013:10(1):3155-3159]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 394

Keywords: Neural stem cells, Proliferation, I/R injury, rhG - CSF, BrdU

Introduction

Ischemic cerebrovascular diseases account for about 60-80 percent of the cerebral vascular diseases in human [1]. After ischemia injury, apoptosis and inflammatory response were observed at the peripheral area of cerebral ischemia [2]. How to reduce hemorrhagic brain injury and protect the injured brain with the potential of reversibility becomes the hotspot of current neuroscience research.

Neural stem cells (NSCs) mainly exist in the dentate gyrus of hippocampus, subventricular zone (SVZ) and subgranular zone (SGZ) in adult animal brains. NSCs have the potential of differentiating into neurons and glial cells [3, 4]. In the recent years, stem cell therapy has become a new strategy for treating various cerebrovascular diseases [5, 6]. NSCs treatment for isomeric cerebral injury has two main ways: NSCs transplantation and autologous in-situ activation[7]. The recent finding that endogenous NSCs reside in the adult brain opened plausible options for cell replacement strategies directed to CNS diseases [8, 9]. The controlling process of stem cell proliferation and differentiation at the molecular level has been explored [10, 11] . NSCs can be stimulated to proliferate by certain substances released during the injury [12]. However, the proliferative capability of NSCs is very limited, which restricted their role for repairing the brain damage.

G-CSF, a glycoprotein whose molecular weight is 20 kD, is generated by hematopoietic cell such as monocyte/macrophage and lymphocyte. At its high concentration, G-CSF could promote hematopoietic stem cells to proliferate, influence the mutual adhesion between stem cells and stromal microenvironment of bone marrow, and mobilize lots of hematopoietic stem cells into the peripheral blood circulation[13]. The recent studies have found that G-CSF could promote stem cell proliferation after myocardial infarction, being captured in the place of myocardial injury [14-16]. In this study, middle cerebral artery occlusion (MCAO) method was used to establish the I/R model in rats[17], NSCs proliferation activity in the ischemia area of hippocampus was evaluated using BrdU marker method. The expression of two key molecules in the signal transduction pathway of adjusting the NSCs proliferation, Wnt1 and β-catenin, was determined in the presence an absence of G-CSF. This could provide exprimental envidence for

the clinical treatment of ischemic cerebrovascular diseases.

Materials and Methods

Animals: Regional cerebral ischemia was induced by MCAO for 120 min, followed by reperfusion for 1d, 3d, 7d, 14d and 21d [18]. 120 healthy male Wistar rats (weighing 250-300g, provided by Laboratory Animal Center of Zhengzhou University, SCXK Henan 2010-0002) were randomly divided into 3 groups: Sham operation group; I/R group; I/R+ rhG-CSF group. In Sham group, carotid artery (CA) and external carotid artery (ECA) were isolated without occlusion. In I/R+ rhG-CSF group, the rats were given hypodermic injection of rhG – CSF 30μg/kg/d after occlusion but before reperfusion. In I/R group, animals were given equal amounts of physiological saline. Rats were killed on 1, 3, 7, 14, 21 days. Use of the animals was reviewed and approved by the guidance suggestion of caring laboratory animals, introduced by the Ministry of Science and Technology of the People's Republic of China in 2006.

Reagents

For BrdU proliferation assay: Polyphosphate lysine (Fujian Maixin Company, China); Streptavidin-Peroxidase (SP) immunohistochemical (IHC) kit, DAB color-display reagent and Mice anti-BrdU monoclonal antibody (Beijing Zhongshan Biotech Co., China);

For RT – PCR: Primer and RT - PCR kit (TaKaRa Biotechnology(Dalian)Co., Ltd, China); Wnt1 (Santa Cruz Company, America); β-catenin, β-actin (Labvision Company, America);

For western blot: Sheep anti-rat polyclonal antibody (Wnt1) (Santa Cruz Company, America) ; Rabbit anti-rat polyclonal antibody(β-catenin) (Labvision Company, America);

rhG - CSF injection (Qilu Pharmaceutical Co Ltd, China).

BrdU cell proliferation assay

12 hours before execution, animals were injected intraperitoneally with BrdU (50mg/kg, every four hours). BrdU were incorporated into cellular DNA during cell proliferation. Then anti-BrdU antibody (1/100 dilution, mouse against rat) was used to detect the incorporated BrdU. Anti-mouse IgG, HRP-linked antibody is then used to recognize the bound antibody. The magnitude of the absorbance for the developed color is proportional to the quantity of BrdU incorporated into cells, which is a direct indication of cell proliferation. Metalnorph/EvolutionMP5.0 microscopic image analysis system was used to count the number of positive cells at the five same given areas of every part of each section.

RT - PCR

Total RNA from rat hippocampal tissue (0.5 g) was isolated using 1ml Trizol. First-strand cDNA was synthesized using random hexamers and Superscript II reverse transcriptase according to the manufacturer's instructions (Invitrogen), and subsequently diluted with nuclease-free water to 12.5 ng/μl cDNA. RT-PCR amplification mixtures (25 μl) contained 25 ng template cDNA, 2x PCR Master Mix buffer (12.5 μl) and 300 nM forward and reverse primer (table 1). PCR reaction conditions: Wnt1, β-catenin, β-actin, preheat denatured for 2 min at 94℃, 30 s at 95℃, 40 s at 52℃, 1 min at 72℃, and extend to 8 min at 72℃ after 30 cycles. Relative quantitative analysis was done using FluChemV2.0 image analysis software.

Western Blot Assay for the Determination of Wnt1 and β-catenin level.

8 μl of sample was mixed with 4 μl of 3 × sample loading buffer (6% SDS, 15% 2-mercaptoethanol, 30% glycerol, and 0.3 mg/ml bromphenol blue in 188 mM Tris-HCl, pH 6.8), heated at 100°C for 10 min, and separated by SDS-PAGE. Separated proteins in the gels were transferred onto nitrocellulose membrane. The blotted membrane was blocked with 5% skim milk in PBS containing 0.05% Tween 20 (PBS-T buffer) for 30 min. After washing the membrane with PBS-T, the first antibody diluted in PBS-T containing 0.25% BSA (Anti-Wnt1, 1/100 dilution; Anti-β-catenin l: 200 dilution), was added and incubated for overnight at 4°C. The bound antibodies were detected by horseradish peroxidase-conjugated anti-mouse IgG secondary antibody followed by ECL detection system according to the manufacturer's instruction. Band density was quantitated by densitometric analysis using FluochemV2.0 image analysis software.

Statistical analysis

Data were presented as means± SEM. SPSS 13.0 software was used to analyze the data..
Comparison among groups was conducted using single factor analysis of variance, and comparison between two groups using t-test. p <0.05 indicates statistical significance

Results

BrdU IHC test

Compared with the Sham group, I/R group showed more BrdU positive cells in the hippocampus. On 7d after reperfusion, the number of BrdU positive cells reached the highest. Compared with I/R group, the number of BrdU positive cells in ischemia hippocampus in I/R + rhG - CSF group was significantly increased (P < 0.05) (Figure.1) on 3d, 7d, 14d and 21d after reperfusion.

mRNA expression of Wnt1, β-catenin in the hippocampus at different date after reperfusion

Wnt1 mRNA expression in Sham group was rarely detected. β-catenin mRNA in Sham group had mild expression. In I/R Group, the expression of Wnt1 mRNA, β-catenin mRNA increased gradually. Both

Wnt1 mRNA and β-catenin mRNA levels reached a peak after reperfusion for 7d and declined after 14d. Both Wnt1 mRNA and β-catenin mRNA in I/R + rhG - CSF group were increased significantly compared with I/R group (P<0.05) (Figure.2).

Figure 1

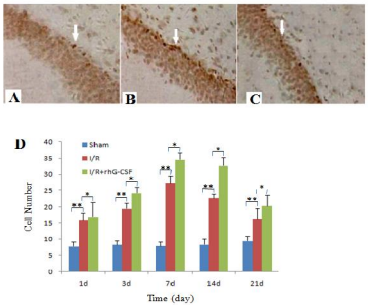

Figure 1. Immunohistochemistry for BrdU in the hippocampus of the Sham (**A**), I/R+rhG-CSF (**B**), and I/R (**C**) group on 7d after reperfusion. In the I/R+rhG-CSF group, BrdU positive nuclei (arrow) are markedly increased compared with the other groups. **D:** The mean number of BrdU positive nuclei per secion of each group (n=10 per group) at different date after reperfusion. * $P < 0.05$, significantly different from the Sham groups; ** $P < 0.01$, significantly different from the I/R groups. Error bars indicate SEM.

Figure 2

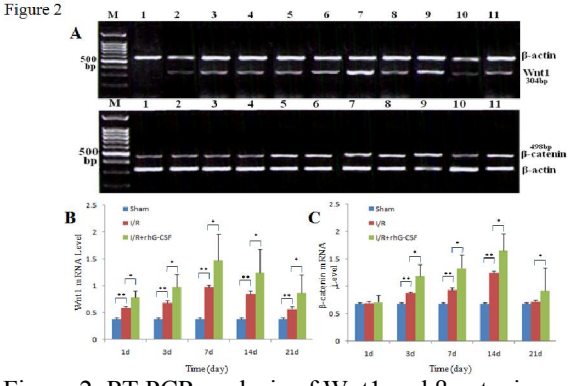

Figure 2. RT-PCR analysis of Wnt1 and β-catenin mRNA. β-actin was used as loading control (**A**). Lane M, marker; lane 1, Sham; lane 2, I/R 1d; lane 3, I/R+rhG-CSF 1d; lane 4, I/R 3d; lane 5, I/R+rhG-CSF 3d; lane 6, I/R 7d; lane 7, I/R+rhG-CSF 7d; lane 8, I/R 14d; lane 9, I/R+rhG-CSF 14d; lane 10, I/R 21d; lane 11, I/R+rhG-CSF 21d. The quantification (normalized by the β-actin levels) is shown below (B, C).* $P < 0.05$, significantly different from the Sham groups; ** $P < 0.01$, significantly different from the I/R groups. Error bars indicate SEM.

The protein expression of Wnt1, β-catenin in the hippocampus at different time point after reperfusion

The expression of Wnt1, β-catenin increased in I/R group with the extension of ischemia-reperfusion time. Wnt1 reached a peak after reperfusion for 7d and β-catenin reached a peak after reperfusion for 14d. Compared with Sham group, both proteins had statistically significant differences ($P < 0.01$). In I/R + rhG - CSF group, both Wnt1 and β-catenin were increased, which had statistically significant differences compared with I/R group (P<0.05) (Figure.3).

Figure 3

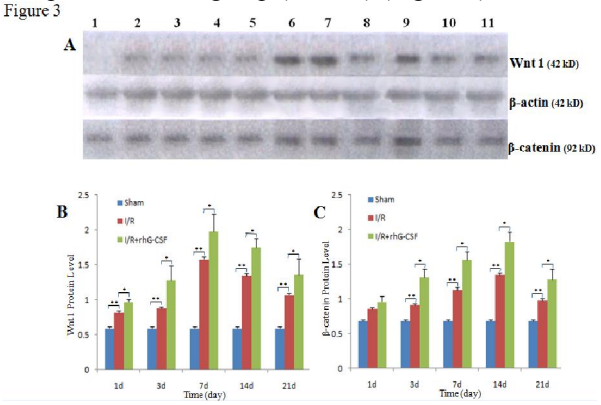

Figure 3. Representative western blot for Wnt1 and β-catenin. β-actin was used as loading control (**A**). Lane 1, Sham; lane 2, I/R 1d; lane 3, I/R+rhG-CSF 1d; lane 4, I/R 3d; lane 5, I/R+rhG-CSF 3d; lane 6, I/R 7d; lane 7, I/R+rhG-CSF 7d; lane 8, I/R 14d; lane 9, I/R+rhG-CSF 14d; lane 10, I/R 21d; lane 11, I/R+rhG-CSF 21d. The quantification (normalized by the β-actin levels) is shown below (B, C).* $P < 0.05$, significantly different from the Sham groups; ** $P < 0.01$, significantly different from the I/R groups. Error bars indicate SEM.

Discussion

The focus of this study was to observe whether there was NSCs hyperplasia in the hippocampus after cerebral ischemia reperfusion and whether rhG - CSF could influence endogenous NSCs proliferation.

Brdu assay has been used to study neuroregeneration, in situ mobilization and proliferation [19, 20]. Liu et al [21] established the gerbil whole cerebral ischemia reperfusion model, and their results showed that the number of Brdu positive cells in the damaged brain reached peak at 11th day. The numbers of stem cells in the damaged brains were 12 times more than that in the control group.

Our results showed BrdU positive cells in the hippocampus of rats of Sham group at each corresponding time point had little change, which indicated that the hippocampus, as the important neurogenesis area, its proliferation was in a stable state.

NSCs in the hippocampus of I/R group significantly increased, which suggest that NSC's proliferation may be relate to locally increased release of signal molecules such as nerve growth factor bFGF and Shh[22].

Wnt signal pathway plays an important role of regulating NSCs proliferation and differentiation [23-25]. β- catenin, the hub molecule of Wnt signal

pathway, can regulate the proliferation and differentiation of neurons [26, 27]. Other studies also confirmed that early proliferation of NSCs and Wnt1/β-catenin signal pathway are closely related [27, 28]. Further analysis found that there was a linear correlation between the proliferation of neural stem cells and the elevated expression of Wnt1 protein [29]. Further study is needed to clarify the mechanisms of regulation of Wnt/β- catenin signal pathway [30, 31] on the proliferation and differentiation of NSCs.

In our study, the change of the numbers of BrdU positive cells in I/R + rhG - CSF group was consistent with the expression of Wnt/β-catenin protein and gene. Increased BrdU positive cells in I/R + rhG - CSF group may also suggest that G-CSF may cause these stem cells to proliferate and migrate. Another possibility for the increased Brdu cells in the damaged brain area, is that the increased cytokines such as cell adhesion molecule and chemotactic factor strengthened the migration of the stem cells into the damaged area.

Cell microenvironment could induce the stem cells "nested" in the damaged area and vascular regeneration, which may contribute to the functional compensation of damaged hippocampus, and thus may benefit to the treatment of brain damage due to IR [32].

The neural stem cells in SVZ and the hippocampus have the capability of proliferation [10].

The induction of proliferation, migration and differentiation of endogenous NSCs *in-situ* and the related mechanisms after craniocerebral injury are not entirely clear [33]. There might be the regulation function of extracellular mesenchymal stem cells, the intercellular effect on the participation of stem cell growth and differentiation [34]. Using the biological potential of NSCs [35] to activate the therapeutic effect of NSCs in the treatment of brain damage will have a potential effect.

In conclusion, ischemic brain damage can induce NSCs proliferation in the hippocampal dentate gyrus of adult rats. rhG – CSF raise NSCs proliferation level in the area of ischemic brain. However, induction in-situ, proliferation, differentiation, migration about endogenous NSCs after the reperfusion injury of cerebral ischemia is not entirely clear. Further studies about how specific microenvironment affects NSCs are needed.

Acknowledgements

This study is supported by Chinese National Natural Science Foundation (31070938/C090202).

Table 1. Primers used in RT-PCR

Wnt1: Forward primer	5 '- AGGTGAAAGGGCAAGGAA - 3'
Wnt1: Reverse primer	5 '- CTGGCAGACAAGAGGAGTGA - 3' (Amplicon length: 304 bp)
β-catenin: Forward primer	5 '- CCCGGCTCTCAGATGGTGTC - 3'
β-catenin: Reverse primer	5 '-ACGATGGCCGGCTTGTTGC - 3 (Amplicon length: 498 bp)
β-actin: Forward primer 1	5 '- CCAAGGCCAACCGCGAGAAGATGAC - 3'
β-actin: Reverse primer 1	5 '- AGGGTACATGGTGGTGCCGCCAGAC - 3' (Amplicon length: 587 bp)
β-actin: Forward primer 2	5 '- ATCATGTTTGAGACCTTCAACA - 3'
β-actin: Reverse primer 2	5 '- GCGCTCCTGAGAATCTCCA - 3' (Amplicon length: 308 bp)

*Corresponding author : Department of Neurology, The First Affiliated Hospital of Zhengzhou University, No. 1, East Jian She Road, Zhengzhou, Henan Province 450052, China

References

[1] Fewel ME, Thompson BG, Jr., Hoff JT. Spontaneous intracerebral hemorrhage: a review. Neurosurg Focus. 2003 Oct 15;15(4):E1.

[2] Qureshi AI, Tuhrim S, Broderick JP, Batjer HH, Hondo H, Hanley DF. Spontaneous intracerebral hemorrhage. N Engl J Med. 2001 May 10;344(19):1450-1460.

[3] Yuan TF, Arias-Carrion O. Locally induced neural stem cells/pluripotent stem cells for in vivo cell replacement therapy. Int Arch Med. 2008;1(1):17.

[4] Seri B, Garcia-Verdugo JM, Collado-Morente L, McEwen BS, Alvarez-Buylla A. Cell types, lineage, and architecture of the germinal zone in the adult dentate gyrus. J Comp Neurol. 2004 Oct 25;478(4):359-378.

[5] Morrison SJ, Spradling AC. Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. Cell. 2008 Feb 22;132(4):598-611.

[6] Yu F, Morshead CM. Adult stem cells and bioengineering strategies for the treatment of cerebral ischemic stroke. Curr Stem Cell Res Ther.2011 Sep;6(3):190-207.

[7] Okano H. Adult neural stem cells and central nervous system repair. Ernst Schering Res Found Workshop. 2006; (60):215-228.

[8] Lindvall O, Kokaia Z. Stem cells for the treatment of neurological disorders. Nature. 2006;441(7097):1094-1096.

[9] Zhu Y, Wan S, Zhan R-y. Inducible pluripotent stem cells for the treatment of ischemic stroke: current status and problems. Reviews in the Neurosciences. 2012;23(4).

[10] Chen J, Boyle S, Zhao M, Su W, Takahashi K, Davis L, et al. Differential expression of the intermediate filament protein nestin during renal development and its localization in adult podocytes. J Am Soc Nephrol. 2006 May;17(5):1283-1291.

[11] Chiba S. Notch signaling in stem cell systems. Stem Cells. 2006 Nov;24(11):2437-2447.
[12] Bauer S, Patterson PH. Leukemia Inhibitory Factor Promotes Neural Stem Cell Self-Renewal in the Adult Brain. Journal of Neuroscience. 2006;26(46):12089-12099.
[13] Ripa RS. Granulocyte-colony stimulating factor therapy to induce neovascularization in ischemic heart disease. Dan Med J.2012 Mar;59(3):B4411.
[14] Lehrke S, Mazhari R, Durand DJ, Zheng M, Bedja D, Zimmet JM, et al. Aging impairs the beneficial effect of granulocyte colony-stimulating factor and stem cell factor on post-myocardial infarction remodeling. Circ Res. 2006 Sep 1;99(5):553-560.
[15] Kuethe F, Krack A, Fritzenwanger M, Herzau M, Opfermann T, Pachmann K, et al. Treatment with granulocyte-colony stimulating factor in patients with acute myocardial infarction. Evidence for a stimulation of neovascularization and improvement of myocardial perfusion. Pharmazie. 2006 Nov;61(11):957-961.
[16] Ueda K, Takano H, Hasegawa H, Niitsuma Y, Qin Y, Ohtsuka M, et al. Granulocyte colony stimulating factor directly inhibits myocardial ischemia-reperfusion injury through Akt-endothelial NO synthase pathway. Arterioscler Thromb Vasc Biol. 2006 Jun;26(6):e108-113.
[17] Longa EZ, Weinstein PR, Carlson S, Cummins R Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke.1989 Jan;20(1):84-91.
[18] Takano K, Tatlisumak T, Bergmann AG, Gibson DG, 3rd, Fisher M. Reproducibility and reliability of middle cerebral artery occlusion using a silicone-coated suture (Koizumi) in rats. J Neurol Sci. 1997 Dec 9;153(1):8-11.
[19] Hermann A, Maisel M, Wegner F, Liebau S, Kim DW, Gerlach M, et al. Multipotent neural stem cells from the adult tegmentum with dopaminergic potential develop essential properties of functional neurons. Stem Cells. 2006 Apr;24(4):949-964.
[20] Ishii K, Nakamura M, Dai H, Finn TP, Okano H, Toyama Y, et al. Neutralization of ciliary neurotrophic factor reduces astrocyte production from transplanted neural stem cells and promotes regeneration of corticospinal tract fibers in spinal cord injury. J Neurosci Res. 2006 Dec;84(8):1669-81.
[21] Liu J, Liu MC, Wang KK. Calpain in the CNS: from synaptic function to neurotoxicity. Sci Signal. 2008;1(14):re1.
[22] Cai J, Huang Y, Chen X, Xie H, Huang Y, Deng L. Regulation of sonic hedgehog on vascular endothelial growth factor, basic fibroblast growth factor expression and secretion in bone marrow mesenchymal stem cells. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi.2012 Jan;26(1):112-116.
[23] Machon O, Backman M, Machonova O, Kozmik Z, Vacik T, Andersen L, et al. A dynamic gradient of Wnt signaling controls initiation of neurogenesis in the mammalian cortex and cellular specification in the hippocampus. Dev Biol. 2007 Nov 1;311(1):223-237.
[24] Decaens T, Godard C, de Reynies A, Rickman DS, Tronche F, Couty JP, et al. Stabilization of beta-catenin affects mouse embryonic liver growth and hepatoblast fate. Hepatology. 2008 Jan;47(1):247-258.
[25] Grigoryan T, Wend P, Klaus A, Birchmeier W. Deciphering the function of canonical Wnt signals in development and disease: conditional loss- and gain-of-function mutations of beta-catenin in mice. Genes Dev. 2008 Sep 1;22(17):2308-2341.
[26] Nusse R. Wnt signaling and stem cell control. Cell Res. 2008 May;18(5):523-527.
[27] Wexler EM, Geschwind DH, Palmer TD. Lithium regulates adult hippocampal progenitor development through canonical Wnt pathway activation. Mol Psychiatry. 2008 Mar;13(3):285-292.
[28] Daneman R, Agalliu D, Zhou L, Kuhnert F, Kuo CJ, Barres BA. Wnt/beta-catenin signaling is required for CNS, but not non-CNS, angiogenesis. Proc Natl Acad Sci U S A. 2009 Jan 13;106(2):641-646.
[29] Wang XL, Yang YJ, Xie M, Yu XH, Liu CT, Wang X. Proliferation of neural stem cells correlates with Wnt-3 protein in hypoxic-ischemic neonate rats after hyperbaric oxygen therapy. Neuroreport. 2007 Oct 29;18(16):1753-1756.
[30] Kormish JD, Sinner D, Zorn AM. Interactions between SOX factors and Wnt/beta-catenin signaling in development and disease. Dev Dyn. 2010 Jan;239(1):56-68.
[31] Rabelo Fde S, da Mota LM, Lima RA, Lima FA, Barra GB, de Carvalho JF, et al. The Wnt signaling pathway and rheumatoid arthritis. Autoimmun Rev. 2010 Feb;9(4):207-210.
[32] Sato T, Suzuki H, Kusuyama T, Omori Y, Soda T, Tsunoda F, et al. G-CSF after myocardial infarction accelerates angiogenesis and reduces fibrosis in swine. Int J Cardiol. 2008 Jul 4;127(2):166-173.
[33] Itoh T, Satou T, Hashimoto S, Ito H. Isolation of neural stem cells from damaged rat cerebral cortex after traumatic brain injury. Neuroreport. 2005 Oct 17;16(15):1687-1691.
[34] Filipczyk AA, Passier R, Rochat A, Mummery CL. Regulation of cardiomyocyte differentiation of embryonic stem cells by extracellular signalling. Cell Mol Life Sci. 2007 Mar;64(6):704-718.
[35] Cheng L, Lai MD, Sanderson JE, Yu CM, Li M. Enhanced fusion of myoblasts with myofibers for efficient gene delivery induced by a partially purified protein fraction from rat muscle extract. Arch Biochem Biophys. 2005 Sep 15;441(2):141-150.

3/5/2013