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# Mesenchymal stem cells enhance the differentiation of c-kit$^+$ cardiac stem cells

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## 1. ABSTRACT

C-kit$^+$ cardiac stem cells (CSCs) were isolated from neonatal rat and tested for the expression of Nkx2.5 and GATA-4 genes which are important in cardiac development. C-kit$^+$ CSCs were plated into the well below the insert of transwell inserts and bone marrow mesenchymal stem cells (BMMSCs) were plated into the inserts. The expression of cardiac Troponin T (cTnT), p27, CDK2, transforming growth factor-β receptor II (TGF-β R II), and Smad2 protein in CSCs were tested by western blot. Expression of p27, TGF-β R II and Smad2 was found to be upregulated in the co-culture group. In contrast, the expression of CDK2 was downregulated. Our results suggest that BMMSCs could promote the differentiation of c-kit$^+$ CSCs, probably through paracrine activity via the TGF-β signaling pathway.

## 2. INTRODUCTION

It has been demonstrated that stem cells can be employed for tissue repair. However, there are a number of limitations to their practical application, including the lack of a suitable source of stem cells, the absence of an efficient mechanism to regulate stem cells, and the inability of stem cells to differentiate into the desired cell type. CSCs, a kind of endogenous stem cell, have been found in the adult human heart, which was previously believed to be a non-regenerative organ. Progenitor cardiac stem cells were capable of differentiating into cardiac myocytes *in vitro*.

It had been believed that after birth, mammalian cardiac myocytes were withdrawn from the cell cycle, and that cardiac repair was dependent on exogenous stem cells

such as BMMSCs or endothelial stem cells. Stem cells derived from the bone marrow mesenchyme can be effectively used to produce cardiac myocytes, since the BMMSCs exhibit good cell-to-cell contact, cell confluence, and trophic effects.

Recent findings have shown that cardiac-resident stem cells are capable of differentiating into mature cardiac myocytes (1). However, the molecular mechanisms that regulate their differentiation and proliferation remain to be explained. Other studies have recently revealed that exogenous stem cells may activate the resident cardiac stem cells by mediating the endogenous regeneration of these cells (2). The paracrine effects of BMMSCs could improve the proliferation of cardiac myocytes *in vivo*. Transforming growth factor-β (TGF-β) produced by BMMSCs could control cell growth and differentiation, and regulate processes as diverse as development, proliferation, wound healing, and tumor progression. TGF-β influences the architecture of the heart under both normal and pathological conditions (3). Therefore, members of the TGF-β superfamily might play important roles during the differentiation of c-kit$^+$ CSCs derived from neonatal rats.

We set up a coculture system of CSCs and BMMSCs in transwell plates in order to prevent cell fusion and transdifferentiation. We focused mainly on whether BMMSCs could promote the activation of resident cardiac stem cells by TGF-β signaling. The study of TGF-β mediators is essential for formulating future treatment regimes for ischemic cardiac disease.

## 3. MATERIALS AND METHODS

### 3.1. C-kit$^+$ CSC cultures

CSCs were isolated from 1-day-old to 3-day-old Sprague-Dawley (SD) rats. The hearts of neonatal rats were sliced into 1 mm-thick slices, and the explants were placed on poly-D-lysine-coated 10-cm plates containing complete explant medium (CEM). The plates were maintained in a humidified atmosphere of 5% $CO_2$ in air at 37°C (4). After about 7 d, most of the primary cultures from the explants attained 70% confluence. At this point, the cells were transferred into a centrifugation tube by 5 sequential 10-minute digestions with 0.1% trypsin in ethylenediaminetetraacetic acid (EDTA). After centrifugation at 400 × g for 10 min, the collected cells were transferred to a 24-well plate ($1\times10^5$ cells per well) (Corning Costar, USA) with differentiation medium CEM supplemented with 1% fetal bovine serum, 2% B27, 0.1 mmol/L 2-mercaptoethanol, 10 ng/mL epidermal growth factor (EGF), 20 ng/mL basic fibroblast growth factor (bFGF), 40 nmol/L cardiotrophin-1, 40 nmol/L thrombin, 100 IU/mL penicillin, 100 IU/mL streptomycin, and 100 μg/mL ascorbic acid (4). The number of cells was determined using a hemocytometer.

### 3.2. Identification of c-kit$^+$ CSCs

The passaged cells were incubated for 3 d on gelatin-coated slides and fed with CEM; subsequently, the cells were fixed with ice-cold 4% paraformaldehyde for 20 minutes, air dried, and blocked using 10% normal goat serum in blocking reagent (0.03% Triton X-100 and 1% bovine serum albumin (BSA)) at 37°C (5). The slides were incubated overnight at 4°C with a primary monoclonal antibody to c-kit (Santa Cruz Biotechnology). Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody (1:100; Jackson Laboratories) was applied to the slide for 1 h at room temperature. Next, the slide was incubated with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich Chemicals) for 10 min. An inverted fluorescence microscope (Nikon) was used to visualize the stained cells.

### 3.3. Isolation of BMMSCs

BMMSCs were isolated from the femoral and tibial bones of 6-week-old SD rats. Both ends of the bones were cut off and the marrow was flushed out using alpha modified Eagle's medium (α-MEM; Gibco BRL). The collected bone marrow was dissociated using a pipette, and mixed with chilled α-MEM. The cell suspension was loaded onto a 20–60% gradient of lymphocyte separation medium and centrifuged at 800 × g at 4°C for 10 min. Cells obtained from the bone marrow were washed 3 times with phosphate-buffered saline (PBS) and resuspended in α-MEM supplemented with 10% fetal bovine serum (FBS). BMMSCs were expanded up to the third passage in tissue culture flasks (6).

### 3.4. Coculture assay

A coculture system was established *in vitro* using transwell double-chamber wells (7). BMMSCs were grown as monolayers on collagen-coated transwells until they reached confluence. TGF-β R II kinase inhibitor was used at 1 μM and was added fresh at each refeeding, prepared from a 1000× concentrated stock solution in dimethylsulfoxide. The wells of a 24-well plate were divided into 3 groups. Group I comprised wells containing c-kit$^+$ CSCs. Group II comprised wells containing BMMSCs plated on polyester membrane inserts. Group III comprised wells containing BMMSCs. The TGF-β R II kinase inhibitor (SB-431542, Merck) was added to the medium at the time of plating.

### 3.5. Polymerase chain reaction gene array

Total RNA was extracted from the CSCs of the 3 groups at 24 h using Trizol reagent (Invitrogen) in keeping with the manufacturer's instructions. Complementary DNA (cDNA) was generated using a reverse transcription kit (Toyobo). Approximately 100 ng of total RNA was treated with DNase, and 5 μL of the solution obtained was used as a template for the PCR reaction. PCR was then performed on each sample of cDNA using the ReverTra Dash™ (Toyobo) in a Mastercycler gradient PCR machine (Eppendorf) under the following conditions: Initial denaturation at 94°C for 15 min, followed by 40 cycles at 94°C for 60 seconds; annealing at 55°C for 30 seconds; and final elongation at 74°C for 60 seconds, with an infinite hold at 74°C. The primers used in this study are summarized in table 1. The PCR products were size-fractionated by 2% agarose gel electrophoresis and stained using ethidium bromide.

**Table 1.** Primers used for real time polymerase chain reaction amplification

| Primer | Forward primer | Reverse primer |
|---|---|---|
| Nkx2.5 | 5′- CTTCAAGCAACAGCGGTACCT′ | 5′- CGCTGTCGCTTTCACTTGTA -3′ |
| GATA-4 | 5′- CTGTGCCAACTGCCAGACTA-3′ | 5′- AGATTCTTGGGCTTCCGTTT -3′ |
| cTnT | 5′- AGAGGACTCCAAACCCAAGC -3′ | 5′- ATTGCGAATACGCTGCTGTT -3′ |
| β-actin | 5′- GTAAAGACCTCTATGCCAACA -3′ | 5′- GGACTCATCGTACTCCTGCT -3′ |

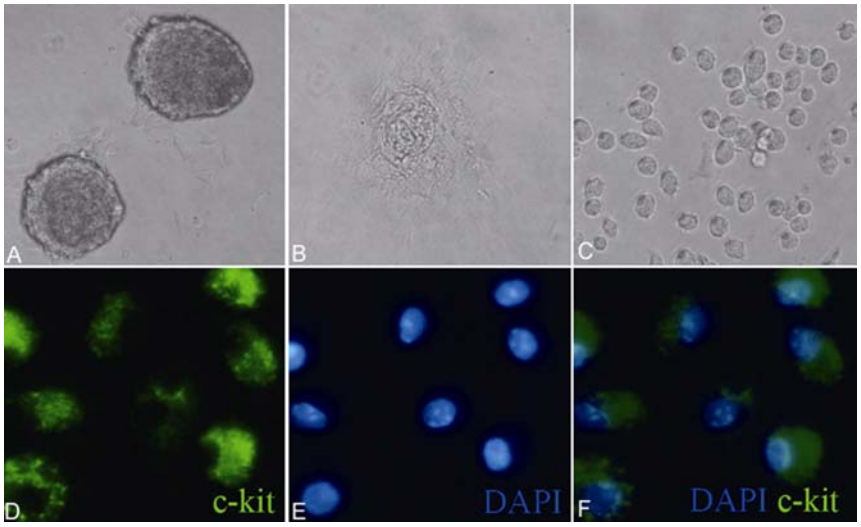


**Figure 1.** Histology and immunocytochemistry of CSCs. Explants of rat heart tissues were grown as separate clones on a plate (A, B). The cells were well distributed after the first passage (C). C-kit was expressed in third generation cells (D, E, F).

### 3.6. Western blot analysis

Cells were harvested, pelleted by centrifugation, and resuspended in lysis buffer. Equal amounts of protein (20μg) were loaded onto a 5% acrylamide stacking gel and separated by SDS-PAGE using a 10% separating gel. Following transfer of separated proteins, nitrocellulose membranes were blocked and probed overnight at 4°C with with primary antibodies (anti-cTnT mouse monoclonal antibody (mAb), anti-p27 mouse mAb, anti-cyclin-dependent kinase (CDK)-2 mouse mAb, anti-TGF-β R II goat polyclonal antibody, anti-Smad2 mAb (Santa Cruz), and anti-cTnT mouse monoclonal antibody (Abcam))

Next, the membranes were reincubated at 37°C with a horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson Laboratories), and the immunoreactivity was detected by chemiluminescence. To quantify the proteins expression, each band density was normalized to actin protein.

### 3.7. Data collection and statistical methods

All values are expressed as mean ± standard deviation (SD). The accepted level of significance was $p < 0.05$.

## 4. RESULTS

### 4.1. Explant culture of c-kit$^+$ CSCs and histology and immunocytochemistry of CSCs

The cloned cardiosphere-derived cells were found to express antigens or markers specific to CSC progenitors. Low-serum, B27, growth factors (EGF and bFGF), cardiotrophin-1 (CT-1), and thrombin supplied in the CEM promoted the formation of sphere-generating cells into cells that biochemically (but not phenotypically) resembled cardiac myocytes.

The explants of heart tissue from rats were grown as separate clones on a plate (Figure 1A, B). The cells were found to be well distributed after the first passage (Figure 1C). Some cells in the third generation were found to be positive for c-kit expression (Figure 1D, E, F).

### 4.2. The expression of Nkx2.5 and GATA-4

The tissue-restricted transcription factor, GATA-4, and the homeodomain protein, Nkx2.5, are the 2 early markers of pre-cardiac cells. Coexpression of GATA-4 and Nkx2.5 during organogenesis enhances the development of committed precursors into cardiac cells (9). Cardiac troponin T (cTnT) is considered as a specific marker for myocardial cells.

The findings of RT-PCR analyses indicate that c-kit$^+$ CSCs did not express Nkx2.5 and GATA-4 at first. Instead, they retained c-kit expression, as evident from the results of immunocytochemistry. However, following coculture with BMMSCs, the expression of Nkx2.5, GATA-4 and cTnT were increased in the c-kit$^+$ cardiac cells at 24 h. The RT-PCR findings confirmed the differentiation potential of c-kit$^+$ CSCs (Figure 2).

### 4.3. Western blot analysis

Quantitative protein analysis indicated that when c-kit$^+$ CSCs were cocultured with BMMSCs, expression of the cell cycle control protein p27 increased, while expression of cyclin-dependent kinase 2 (CDK2) was reduced, which may suggest that co-culture could affect the

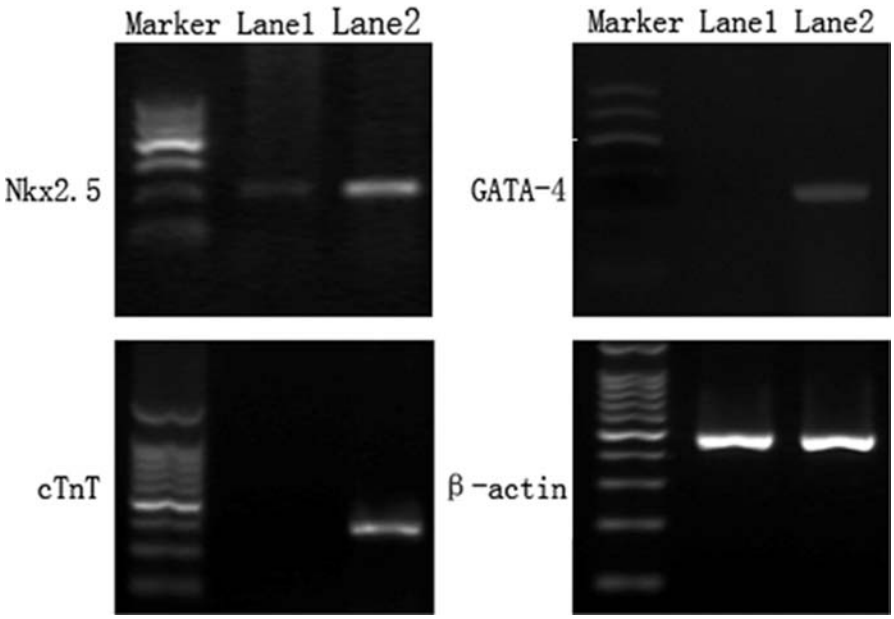


**Figure 2.** Expression of Nkx2.5 and GATA-4 mRNA in c-kit$^+$ CSCs cocultured with and without BMMSCs. Lane 1: c-kit$^+$ CSCs cocultured without BMMSCs did not express Nkx2.5 or GATA-4. Lane 2: c-kit$^+$ CSCs cocultured with BMMSCs showed increased expression of Nkx2.5 and GATA-4at 24 h

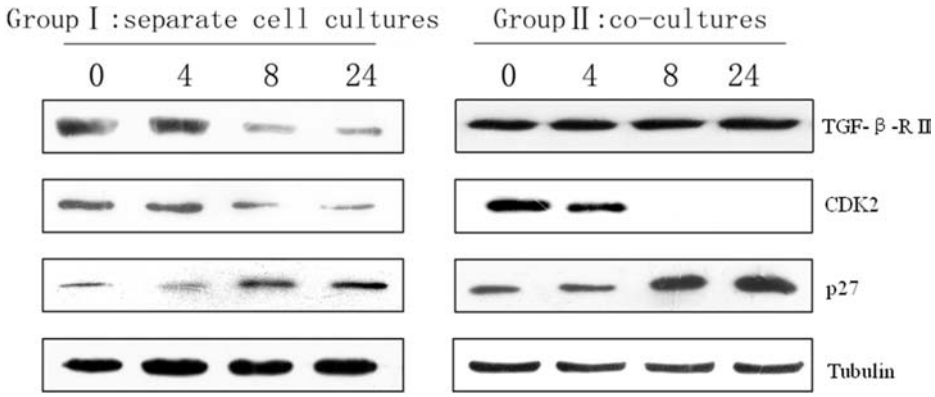


**Figure 3.** Expression of TGF-β R II, CDK2 and p27 in c-kit$^+$ CSCs cocultured with and without BMMSCs

cell cycle. Expression of TGF-β R II and its downstream protein Smad2 also increased (Figure 3), but when the TGF-β R II kinase inhibitor SB-431542 was added to the culture medium, expression of TGF-β R II and Smad2 was suppressed, while p27 and CDK2, which are regulated by Smad2, were inhibited or activated to regulate the cell cycle (Figure 4).

## 5. DISCUSSION

It has been previously reported that BMMSC-transplanted heart tissues demonstrated an upregulation in the expression of signaling factors, including anti-apoptotic factors and angiogenic cytokines (10). In addition, it has been reported that BMMSCs are able to secrete growth factors into the culture medium *in vitro*, reducing myocyte senescence and death (11). In the cell transplant model, BMMSCs might regulate progression of the cell cycle in resident cardiac cells by secreting the above-mentioned paracrine factors.

In resident stem cells, the balance between survival and death, quiescence and proliferation, self-renewal and differentiation, and active migration and passive localization is dependent on the local environment and the supporting cells in their niche. The resident CSCs, particularly those located in the atria and the apex, are surrounded by a mixed population of supporting cells. These CSCs produce compounds that are essential for maintaining tissue homeostasis and for nurturing endogenous stem cells (12). In humans and other animals, only a few cardiac resident stem cells are induced into differentiated progression after ischemic injury (4).

The transwell coculture system was able to prevent cell mixing and provided a stable environment to

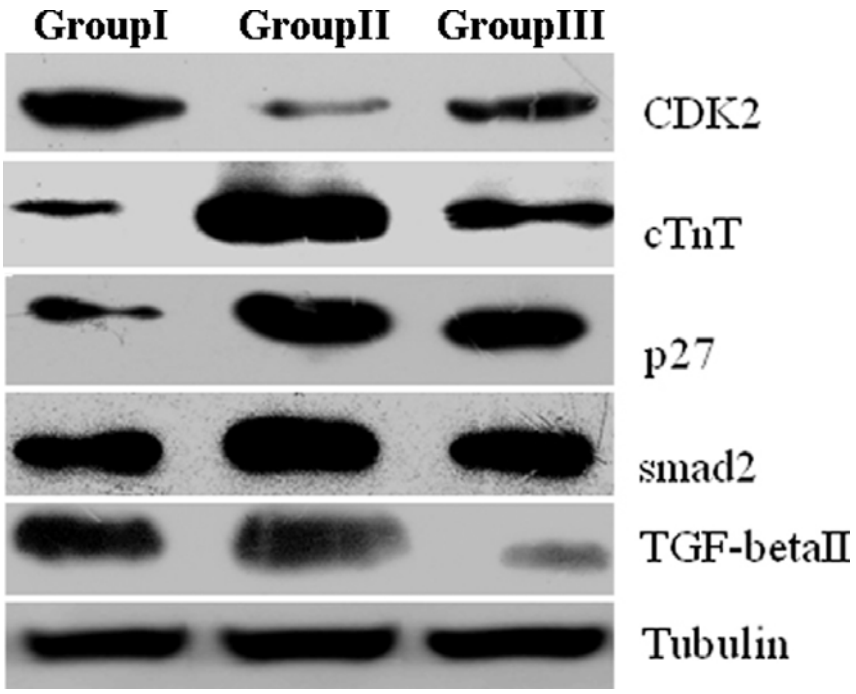


**Figure 4**. Expression of cTnT, CDK2, p27, TGF-β R II, and Smad2 protein in c-kit$^+$ CSCs cocultured with and without BMMSCs, and TGF-β R II kinase inhibitor group.

investigate the effects of paracrine signaling factors on c-kit$^+$ CSCs.

Differentiation is a common cellular process that is initiated by a cell cycle block. It is known that cell cycle-related proteins such as p27 and CDK2 play an important and direct role in driving cardiomyocyte differentiation as well as cell-cycle regulation (13). p27 is a powerful inhibitor of the cell cycle and may play a direct role in myocyte development in the fetal hearts of rats (14). CDK2 is associated with the proliferation and differentiation of cells and acts as a positive regulatory factor of the G1-S checkpoint (15). In human adult heart failure, the expression of cyclin dependent kinase inhibitors, such as p27, shows a gradual increase during differentiation in adulthood (16).

Extracellular TGF-β signals through a membrane-bound receptor complex of TGF-β type I and type II receptors. Ligand binding induces specific phosphorylation steps, resulting in the phosphorylation by TGF-β R II of receptor-regulated Smad2/3 at the C-terminus. SB-431542 is a synthetic molecule that inhibits the receptor TGF-β R II (17)

## 6. CONCLUSIONS

In our study, after coculturing CSCs with BMMSCs, the expression of p27, cTnT, TGF-β R II, and Smad2 increased in group II. SB-431542 treatment reduced the expression of p27, cTnT, TGF-β RII, and Smad2 in c-kit$^+$ CSCs. Coculture activates the TGF-β signaling pathway; it is this pathway that is required for differentiation and development. In addition, the expression of CDK2 was raised in Group II. We concluded that co-culture with BMMSCs facilitated differentiation of c-kit$^+$ CSCs.

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## 8. REFERENCES

1. Chamuleau SA, Vrijsen KR, Rokosh DG, Tang XL, Piek JJ, Bolli R: Cell therapy for ischaemic heart disease: focus on the role of resident cardiac stem cells. *Neth Heart J* 17(5), 199-207 (2009)

2. Gnecchi M, Zhang Z, Ni A, Dzau VJ: Paracrine mechanisms in adult stem cell signaling and therapy. *Circ Res* 103 (11), 1204-1219 (2008)

3. Ramos-Mondragon, R., Galindo C.A. and Avila G.: Role of TGF-beta on cardiac structural and electrical remodeling. *Vasc Health Risk Manag* 4(6), 1289-300 (2008)

4. Messina E, De Angelis L, Frati G, Morrone S, Chimenti S, Fiordaliso F, Salio M, Battaglia M, Latronico MV, Coletta M, Vivarelli E, Frati L, Cossu G, Giacomello A:

Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ Res* 95(9), 911-921 (2004)

5. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa P. Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell* 114(6), 763-76 (2003)

6. Pittenger M.F. and B.J. Martin: Mesenchymal stem cells and their potential as cardiac therapeutics. *Circ Res* 95(1), 9-20 (2004)

7. Tretiach M, Madigan MC, Wen L, Gillies MC. Effect of Muller cell co-culture on in vitro permeability of bovine retinal vascular endothelium in normoxic and hypoxic conditions. *Neurosci Lett* 378(3), 160-5 (2005)

8. Aghila Rani KG, Jayakumar K, Srinivas G, Nair RR, Kartha CC: Isolation of ckit-positive cardiosphere-forming cells from human atrial biopsy. *Asian Cardiovasc Thorac Ann* 16(1), 50-6 (2008)

9. Dentice M, Morisco C, Vitale M, Rossi G, Fenzi G, Salvatore D: The different cardiac expression of the type 2 iodothyronine deiodinase gene between human and rat is related to the differential response of the Dio2 genes to Nkx-2.5 and GATA-4 transcription factors. *Mol Endocrinol* 17(8), 1508-1521 (2003)

10. Tögel F, Weiss K, Yang Y, Hu Z, Zhang P, Westenfelder C: Vasculotropic, paracrine actions of infused mesenchymal stem cells are important to the recovery from acute kidney injury. *Am J Physiol Renal Physiol* 292(5), F1626-35 (2007)

11. Liu C.H. and Hwang S.M.: Cytokine interactions in mesenchymal stem cells from cord blood. *Cytokine* 32(6), 270-9 (2005)

12. Barile L, Messina E, Giacomello A, Marban E..: Endogenous cardiac stem cells. *Prog Cardiovasc Dis* 50(1), 31-48 (2007)

13. Martín A, Odajima J, Hunt SL, Dubus P, Ortega S, Malumbres M, Barbacid M: Cdk2 is dispensable for cell cycle inhibition and tumor suppression mediated by p27(Kip1) and p21(Cip1) *Cancer Cell* 7(6), 591-8 (2005)

14. Li J, Xie XY, Wang DM, Pei XT: (FRIL maintains quiescence of hematopoietic stem cells through regulation of cell cycle related factors) *Zhonghua Xue Ye Xue Za Zhi* 28(1), 37-40 (2007)

15. Zhang R., Banik N.L., and Ray S.K.: Combination of all-trans retinoic acid and interferon-gamma upregulated p27(kip1) and down regulated CDK2 to cause cell cycle arrest leading to differentiation and apoptosis in human glioblastoma LN18 (PTEN-proficient) and U87MG (PTEN-deficient) cells. *Cancer Chemother Pharmacol* 62(3), 407-16 (2008)

16. Horie N, Mori T, Asada H, Ishikawa A, Johnston PG, Takeishi K. Implication of CDK inhibitors p21 and p27 in the differentiation of HL-60 cells. *Biol Pharm Bull* 27(7), 992-7 (2004)

17. Callahan JF, Burgess JL, Fornwald JA, Gaster LM, Harling JD, Harrington FP, Heer J, Kwon C, Lehr R, Mathur A, Olson BA, Weinstock J, Laping NJ: Identification of novel inhibitors of the transforming growth factor beta1 (TGF-beta1) type 1 receptor (ALK5) *J Med Chem* 45(5), 999-1001 (2002)

18. Nagai T, Shiojima I, Matsuura K, Komuro I: Promotion of cardiac regeneration by cardiac stem cells. *Circ Res* 97(7), 615-7 (2005)

19. Schafer, R. and H. Northoff, Cardioprotection and cardiac regeneration by mesenchymal stem cells. *Panminerva Med* 50(1), 31-9 (2008)

**Abbreviations:** CSCs; C-kit$^+$ cardiac stem cells, cTnT; cardiac Troponin T, TGF-βR II, transforming growth factor-β receptor II, SD rat, Sprague-Dawley rats

**Key Words:** Cardiac Stem Cells, Mesenchymal Stem Cells, Differentiation, p27, TGF-βR

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