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Hypothermic Perfusion of Donor Heart With a Preservation Solution

Supplemented by Mesenchymal Stem Cells

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Running title: Mesenchymal stem cells conditioned medium and transplantation

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Abstract

BACKGROUND: Heart transplantation is the definitive treatment for end-stage heart failure. A shortage of donor hearts forced transplant programs to accept older donors and longer ischemic times. Previous studies have suggested that the administration of mesenchymal stem cells (MSCs) or their conditioned medium (CM) protects the heart against ischemia/reperfusion injury (IRI). We

hypothesized that the preservation of donor hearts with a CM would protect the graft from IRI after prolonged storage in 15-month-old rats and investigated mRNA-changes attributable to CM.

METHODS: Rat MSCs were isolated and cultured. The CM was used and characterized by a 90-antibody-array, revealing the presence of 28 factors involved in apoptosis, inflammation, and oxidative stress. Hearts from 15-month-old donor rats were explanted and continuously perfused for 5h with oxygenated, 4°C cardioplegic solution, supplemented with either regular cell culture medium (control-group) or CM. Then, the hearts were heterotopically transplanted. We evaluated *in vivo* left-ventricular graft function 1.5h after transplantation and the myocardial expression of 120-genes using PCR-arrays.

RESULTS: Systolic contractility and relaxation parameters were significantly reduced in 15-month-old rats compared to young ones. After transplantation, systolic function (dp/dt_{max} : 1197 ± 94 vs. 1825 ± 279 mmHg/s at $140 \mu\text{l}$, $p < 0.05$) and diastolic function (dp/dt_{min} : 737 ± 168 vs. 1200 ± 166 mmHg/s at $140 \mu\text{l}$, $p < 0.05$) were significantly improved in the CM-group compared to controls. Among the surveyed genes, the expression of 66 were altered. Genes of proinflammatory cytokines and interleukins were down-regulated, while the expression of antioxidant gene superoxide dismutase-2 was up-regulated in the CM-treated grafts compared to the control-group.

CONCLUSIONS: Perfusion of donor hearts with MSCs-CM protects against myocardial IRI in 15-month-old rats.

KEYWORDS: mesenchymal stem cells; 15-month-old rats; heart transplantation; cardioprotection

Heart transplantation is the preferred surgical treatment of patients with end-stage heart failure. Despite significant progresses, the shortage of available donor hearts and growing number of patients on the transplant waiting list remains an unsolved problem. Efforts have been made to expand the donor pool by accepting older hearts¹ and donor hearts that have been subjected to longer ischemic insults.² We previously showed that ageing is associated with decreased left-ventricular (LV) contractility and marked impairment of endothelial function.^{3, 4} Furthermore, we have demonstrated that the ischemic tolerance of older donor hearts is reduced after prolonged preservation time, and the endothelium is more vulnerable to ischemia/reperfusion injury (IRI).⁵ Myocardial IRI is inevitable during heart transplantation and contributes to short-term and long-term graft outcome. Key underlying subcellular factors include ATP depletion of cardiomyocytes, the increase of intracellular calcium concentration, the development of metabolic acidosis, the generation of reactive oxygen species (ROS) and inflammatory cytokines, and infiltrating neutrophils.⁶ These factors are known to lead to apoptosis⁷ and to organ dysfunction. Taken together, aged donor hearts that have been subjected to a prolonged ischemic time followed by warm reperfusion may have higher rates of primary graft failure than younger donors.⁸ Currently, organ preservation solutions permit a safe ischemic time of up to 4h for cardiac grafts regarding graft survival rates^{9, 10}, however preventing the adverse effects of myocardial IRI during the transplant procedure remains a problem.

Bone marrow-derived mesenchymal stem cells (MSCs), which are multipotent adult stem cells, are regarded as promising preventive agents against myocardial IRI.^{11, 12} Initially, the generation of cardiomyocytes, endothelial cells, and vascular smooth muscle cells from MSCs or cell-to-cell contact was proposed as the principal mechanism underlying the therapeutic action. However, the number of newly differentiated cells has been demonstrated to be too low to justify the functional therapeutic benefits seen after MSCs transplantation. Therefore, paracrine mechanisms, including the release of a combination of growth factors, chemokines and cytokines by MSCs¹³, have been shown to play an important role in cardioprotection.^{14, 15} Accordingly, conditioned medium from bone marrow-derived MSCs (MSCs-CM), added at the onset of reperfusion after ischemia, was found to significantly protect the heart in an *ex vivo* model of myocardial IRI.¹⁶ Potential paracrine mechanisms underlying the therapeutic benefits of MSCs include the activation of endogenous injured tissue repair,

the stimulation of angiogenesis, the attenuation of cardiac remodeling, and the reduction of apoptosis. This original strategy, i.e. administration of factors secreted by MSCs, may represent a new effective non-cell-based therapeutic option against myocardial IRI.

We hypothesized that the perfusion of donor hearts with MSCs-CM enriched preservation solution improves functional graft recovery after prolonged storage in 15-month-old rats. Furthermore, we evaluated the possible molecular mechanisms underlying the cardioprotective effect of MSCs-CM after transplantation. We sought its link to oxidative stress-, apoptosis-, inflammation-, and PI3K/Akt-signaling-related gene expression changes using mRNA expression profiling.

Methods

Animals

Male rats were purchased from Janvier Labs (Saint Berthevin, France), housed in a room at $22\pm 2^{\circ}\text{C}$ under 12-h light/dark cycles, and were fed standard laboratory diet *ad libitum*. All animals received humane care in compliance with the ‘Principles of Laboratory Animal Care’, formulated by the National Society for Medical Research, and with the ‘Guide for the Care and Use of Laboratory Animals’, prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996). This investigation was approved by the appropriate institutional review committees (G183/14).

Preparation of bone marrow derived MSCs-CM

Bone marrow-derived MSCs were prepared from young rats (8-12 week-old) and CM was obtained at Passage-3. As a control, Dulbecco’s modified Eagle’s medium was used.

Antibody array

RayBio® Biotin Label-based rat antibody array 1 (BioCat GmbH, Heidelberg, Germany) was used for the simultaneous detection of the relative expression levels of 90 target proteins in MSCs-CM, according to the manufacturer's instructions. Two CMs from passage-3 MSC cultures were tested.

3-month-old and 15-month-old rats

Experimental groups

Lewis rats were randomly assigned into two groups: (a) 3-month-old: animals were used as reference (young) and (b) 15-month-old: rats were purchased at 8 months of age and studied at the age of 15 months.

Left-ventricular cardiac function

LV pressure-volume (PV) analysis of cardiac function was performed as previously described¹⁷, using

a 2F microtip pressure-conductance catheter (SPR-838; Millar Instruments, Houston, TX).

Rat model of heterotopic heart transplantation

Experimental groups

Rats were randomly assigned to two groups. Hearts from 15-month-old donor rats were explanted and continuously perfused in an isolated Langendorff model for 5h with 10mL of oxygenated cold 4°C preservation solution (Custodiol) supplemented either with serum-free medium (medium-group, n=6-9 rats) or MSCs-CM (CM-group, n=6-9 rats). Five-hour continuous perfusion was selected to ensure better diffusion of CM through the coronary circulation into myocardial tissue. The protein concentration of the MSCs-CM was quantified with Bradford protein assay to ensure that equal concentrations (0.5g/mL) of CM was used to perfuse each of the hearts.

Surgical technique of heart transplantation

Transplantations were performed in an isogenic Lewis to Lewis rat strain model. The experimental model was described elsewhere.¹⁸ Cardiac arrest was induced by Custodiol (histidine-tryptophane-ketoglutarate) cardioplegic solution (Dr. Franz Köhler, Chemie GmbH, Bensheim, Germany). To minimize variability between experiments, the duration between explantation and reperfusion has been standardized to 1h in each and every procedure. After completion of the anastomoses, the hearts were heterotopically transplanted into recipients, and *in vivo* reperfusion lasted for 1.5h. The re-beating time (time to restoration of heartbeat after transplantation) was recorded.

Functional measurement of the graft

As previously reported¹⁹, 1.5h after transplantation a 3F latex balloon catheter (Edwards Lifesciences Corporation, Irvine, CA), connected to a precision-calibrated syringe for the administration and withdrawal of fluid, was introduced simultaneously with a Millar micromanometer catheter (Millar Instruments, Houston, TX) into the left ventricle via the apex. LV systolic pressure, LV end-diastolic pressure, the maximal slope of systolic pressure increment (dP/dt_{max}) and diastolic pressure decrement (dP/dt_{min}) were measured at different LV volumes (LVV). LVV were calculated as the volume of

saline injected into the balloon plus the volume of the empty balloon (0.02ml). Data for a complete pressure-volume curve were obtained through incremental increases in LVV by 0.03ml until a volume of 0.17ml was reached and were constructed using PVAN 3.6 software (Millar Instruments, Houston, TX).

Gene expression analysis in the graft

Using a Custom RT² ProfilerTM PCR Array (SABiosciences, Qiagen, Hilden, Germany), the expression of a total number of 120 target genes involved in oxidative stress, apoptosis, inflammatory response, and PI3K/Akt-signaling were profiled following heart transplantation (**Table 1**).

Statistical analysis

All data is expressed as mean±SEM. Statistical analyses of data were performed using GraphPad Prism 7.02 software (GraphPad Software, Inc., CA). Before statistical tests were applied, the Shapiro-Wilk normality test was used to assess normal distribution. For data with normal distribution, the two-sample Student's t-test was used to analyze the differences between medium and CM groups. In case of non-normal distribution, non-parametric Mann-Whitney test was applied. In case of the PCR-array gene expression, p values were calculated using a Student's t-test (two-tail distribution and equal or unequal variances between the two samples) with the average deltaCt value. A value of $p < 0.05$ was considered indicative of significance.

Results

Characterization of MSCs-CM

Table 2 shows factors detected in MSCs-CM. Large antibody arrays revealed the presence of 28 factors involved in either apoptosis, inflammation, oxidative stress, angiogenesis, cell proliferation, or immunomodulation (**Figure 1**).

Characterization of left-ventricular systolic and diastolic function in 15-month-old rats

15-month-old rats presented with a significant reduction of LV contractile function, including indices of load-dependent- (LV end-systolic pressure and dP/dt_{max}) and load-independent parameters (the slopes E_{es} and E_{max} of the end-systolic PV relationship, the slope of dP/dt_{max} /end-diastolic volume relationship, and maximal elastance) compared to the 3-month-old-group (**Figures 2A-F**). Additionally, ventricular relaxation was significantly impaired in the 15-month-old-group compared to young rats (decreased dP/dt_{min} and prolonged Tau-Weiss) (**Figures 2G-H**).

Effect of MSCs-CM on the transplanted heart

Re-beating time

Perfusion of 15-month-old donor hearts with MSCs-CM showed significantly reduced re-beating time compared to the medium-group (CM $46 \pm 15s$ vs Medium $87 \pm 7s$, $p < 0.05$).

Left-ventricular graft function after transplantation

After transplantation, LV systolic function (LV systolic pressure, developed pressure, and dP/dt_{max}) (**Figures 3A-C**) and diastolic function (dP/dt_{min} , Tau and LV end-diastolic pressure) (**Figure 4**) were significantly improved in the CM-treated grafts compared to medium-group. The rate pressure product, used to determine the myocardial workload, was significantly increased in the CM compared to the medium-group (**Figure 3D**).

Myocardial gene expression after transplantation

To identify molecular mechanisms behind the beneficial effects of CM on functional alterations, expression levels of 120 genes involved in oxidative stress, apoptosis, inflammation, and PI3K/Akt-pathways were assessed in the graft after transplantation. A volcano plot indicates the statistical significance of gene expression changes between CM-treated grafts and medium-group and the clustergrams create a heat map with dendrograms to indicate which genes are co-regulated (**Figure 5**). Among 120 genes, 64 genes were significantly down-regulated and 2 genes were up-regulated in the CM-treated grafts compared to the medium-group (**Table 3, Figure 5**). These include the upregulation of *sod2* and *selectin E*, and down regulation of genes related to oxidative stress (*Serpinb1b*, *Dnm2*, *Txnrd2*, *Epx*, *Gpx*, *Mpo*, *Dhcr24*, *Tpo*, *Nox4*, *Noxo1*, and *Aox1*), apoptosis (*Birc5*, *Cd40lg*, *Fadd*, *Bcl2l11*, *Casp2*, *Faslg*, *Bid*, *Casp8*, *Akt1*), down regulation of proinflammatory cytokine (*Tnf*, *Ccl11*) and interleukin (*Il9*, *Cd40lg*) genes, and genes involved in the PI3K/Akt-pathway (*Tcl1a*, *Akt1*, *Akt3*, *Mapk3*, *Pak1*, *Foxg1*, *Mtor*, *Adar*) (see **Table 1 for genes abbreviation**).

Discussion

In this study, we assessed whether hypothermic perfusion of donor hearts with a preservation solution supplemented by MSCs-CM protects the graft after prolonged storage in 15-month-old rats. To our knowledge, this is the first report of an experimental cardiac transplantation study suggesting that Custodiol-enriched with MSCs-released molecules results in the functional recovery of grafts after cardioplegic arrest, prolonged hypothermic storage, implantation and *in vivo* blood reperfusion in 15-month-old rats.

An approach to increase the donor shortage has been the utilization of marginal organs, especially hearts from older donors and hearts having undergone longer ischemic times.²⁰ Currently, only the hearts of brain-dead donors can be used for transplantation. Kumarasinghe et al have shown that older hearts were more susceptible to IRI induced by brain death and prolonged hypothermic storage.²¹ To distinguish the specific effects of age on IRI, we avoided additional factors which might influence our hypothesis. Therefore, 15-month-old donor animals were not subjected to brain death. Additionally, 15-month-old rats would be roughly equivalent to a 40-year-old human.²² In the present study, animal age was chosen based on alterations of cardiac function, which showed that rats at this age already presented a significant decline in LV contractile function and ventricular relaxation when compared to the 3-month-old group. These functional results are in accordance with our previous published study with 22-month-old rats.³ Preconditioning, with conservation solution, is known to be a powerful method in experimental studies to protect grafts against dysfunction induced by cold ischemia/warm reperfusion. Although a few studies suggest that *in vivo* administration of MSCs-CM protects the heart in murine and porcine models following myocardial infarction^{14, 23}, there is no experimental evidence after cardiac transplantation using allografts in 15-month-old donors. The clinical importance, and thus the translational aspect of our study is that transplantations were performed on hearts from 15-month-old donors, aiming to obtain further possible “druggable” mechanisms to ameliorate graft function. MSCs-CM contains a number of chemokines, cytokines, growth factors and other factors, which play a role in its protective effect. In the present study, factors identified in MSCs-CM, such as tissue inhibitor of metalloproteinase (TIMP)-1, growth hormone, prokineticin, vascular endothelial growth factor (VEGF), and activin A, may confer protection against

early IRI and contribute to improved functional outcome after transplantation. It has been demonstrated that the intramyocardial injection of VEGF₁₆₅ cDNA significantly improved cardiac performance and reduced apoptosis in a rat model of IR-induced myocardial infarction.²⁴ Additionally, treatment with recombinant activin A protein has been shown to protect cultured myocytes subjected to hypoxia/reoxygenation-induced apoptosis by upregulation of Bcl-2 protein expression and reduction of caspase activation and cellular apoptosis.²⁵ Urayama et al. have shown that prokineticin receptor-1 signaling elicits survival signal transduction in cardiomyocytes and reduces apoptotic cell death following hypoxia.²⁶ Furthermore, TIMP-1-based therapy has been suggested to improve cardiac function and to inhibit apoptosis in the ischemic myocardium of rats.²⁷ It has been reported that human multipotent MSCs-CM inhibited apoptosis on human endothelial cells exposed to hypoxia, and that the protection was partly related to the presence of angiogenic and anti-apoptotic factors such as IL-6, VEGF and MCP-1 in MSCs-CM.²⁸ Consistent with these results, our data suggest that the combined effects of a broad range of soluble factors identified in MSCs-CM may contribute to tissue preservation, thereby improving graft performance after heart transplantation. Further studies are needed to elucidate the role of other factors identified in MSCs-CM after transplantation following cold storage in 15-month-old rats.

Modulation of gene expression profile induced by MSCs-CM during long storage on graft function after transplantation in 15-month-old rats

The apoptosis, inflammation and oxidative stress response, are all the key factors involved in the pathogenesis of IRI, leading to tissue damage. During myocardial IRI, enhanced ROS generation induces cell death through apoptosis and activates neutrophils. In cardiac transplantation, a rapid recovery of myocardial function is essential and plays an important role in long-term outcomes. Severe hemodynamic changes occur during the early phase after transplantation, therefore we sought to discover the molecular mechanisms underlying the *in vivo* protective action of MSCs-CM 1.5h after reperfusion. Accordingly, we performed gene profiling to determine whether transcription levels were altered. We found that there are gene expression signatures regulated by MSCs-CM in the early stages (<9h). These include the upregulation of the anti-oxidant gene *Sod2* and downregulation of oxidative

stress response genes (*Serpinb1b*, *Dnm2*, *Txnrd2*, *Epx*, *Gpx*, *Mpo*, *Dhcr24*, *Tpo*, *Nox4*, *Noxo1*, and *Aox1*) which may, in turn, be responsible for the down regulation of pro-apoptotic genes (*Birc5*, *Cd40lg*, *Fadd*, *Bcl2l11*, *Casp2*, *Faslg*, *Bid*, *Casp8*, and *Akt1*) and for the down regulation of genes of proinflammatory cytokines (*Tnf*, *Ccl11*) and interleukins (*Il9*, *Cd40lg*), which might have resulted in the improvement LV graft function. Even though this study does not provide direct mechanistic evidence that factors, characterized from MSCs-CM are in fact responsible for the cardioprotective effect seen, it does show that the expression of genes involved in the pathogenesis of IRI were inversely regulated. Timmers et al. have shown that intravenous infusion before the onset of reperfusion followed by an intracoronary bolus of concentrated human MSCs-CM reduces myocardial infarct size, improves cardiac function, and attenuates myocardial apoptosis and oxidative stress in a porcine model of IRI.²⁹ Our results show that the substance extracted from MSCs culture, i.e. CM, might regulate genes (related to apoptosis, inflammation, and oxidative stress), that affect many major cellular processes and a number of participating pathways, thereby protecting 15-month-old donor hearts submitted to longer storage time and reperfusion injury. Additionally, *in vitro* studies of Angoulvant et al. suggested that the protection afforded by MSCs-CM may result from the activation of the PI3K-pathway in neonatal rat cardiomyocytes.¹⁶ However, in our *in vivo* study all the tested genes involved in the protective PI3K/Akt-pathway were significantly down-regulated in the MSCs-CM-treated graft compared to controls. It is tempting to speculate that the downregulation of PI3K/Akt-pathway genes is a compensatory response to the activation of this signaling pathway by MSCs-CM.

Some limitations exist in the current study. First, IRI is imposed upon one comorbidity, ageing, however, in the clinical setting, there are additional major risks for cardiovascular disease, which often coexist and were not considered in the present study.³⁰ Second, a heterotopic rat heart transplantation model was selected as a suitable model to evaluate IRI. However, the left ventricle is beating in an unloaded condition, and therefore it does not eject, allowing a faster recovery after IRI. An assessment of long-term effects was not possible in this model. Third, it would have been interesting to compare simple hypothermic storage to hypothermic perfusion for 5h. Lastly, we did not assess protein expression but confined the investigation to the mRNA patterns. Despite these

limitations, our study shows promising potential for MSCs-CM-based therapy against IRI occurring during heart transplantation. It may identify new aspects to expand the heart donor pool.

From the clinical point of view, due to the shortage of organs available for transplantation, there is an extreme necessity for a more optimized use of all available organs. The use of marginal donor hearts compared to standard grafts is associated with a higher sensitivity to IR damage. Additionally, if longer ischemic period time is added, in clinical transplantation, a more effective long-term preservation would be required. In the clinical setting, one promising method, among others, may be the supplementation of standard heart preservation solutions with cell-free MSC derived CM (rather than injections). MSC-CM may provide key advantages over living cells, as less complications relative to issues regarding transplantation and in terms of storage and handling may occur. However, before its clinical application several issues must be addressed, including standardization of MSC cultivation, collection, and preservation of the CM to establish the safety and efficacy profile. To the best of our knowledge, this is the first study to provide evidence that the presence of MSCs-CM in the preservation solution (rather than injections into animals) protects 15-month-old hearts and improves graft function in the setting of prolonged hypothermic perfusion in an experimental model of heart transplantation. Future elucidation of the exact mechanism of action of the factors secreted by MSCs and of the optimal methods for long-term preservation (simple cold storage vs hypothermic oxygenated perfusion vs normothermic perfusion with oxygenated blood) may have important implications for successful clinical translation of CM, as a novel and promising alternative to supplement cardioplegic solutions.

Disclosure statement

The authors have no conflicts of interest to disclose.

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Supplementary Data

Supplementary data related to this article can be found at Online Appendix.

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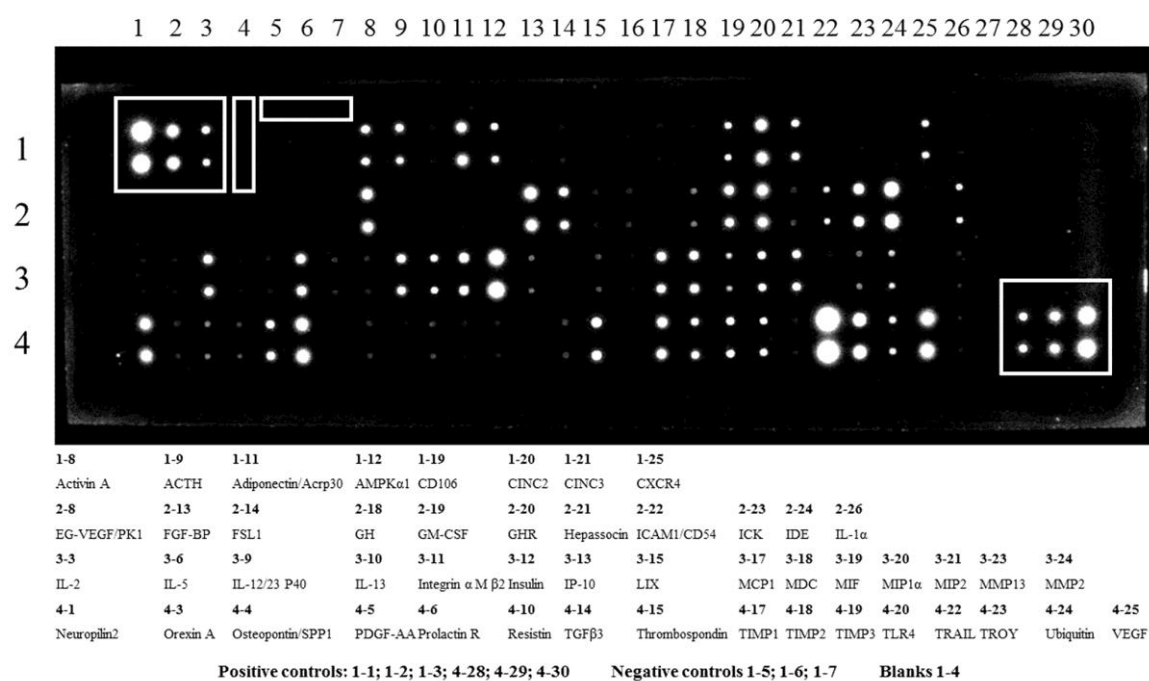


Figure 1 Characterization of conditioned medium from bone marrow-derived mesenchymal stem cells by an antibody assay.

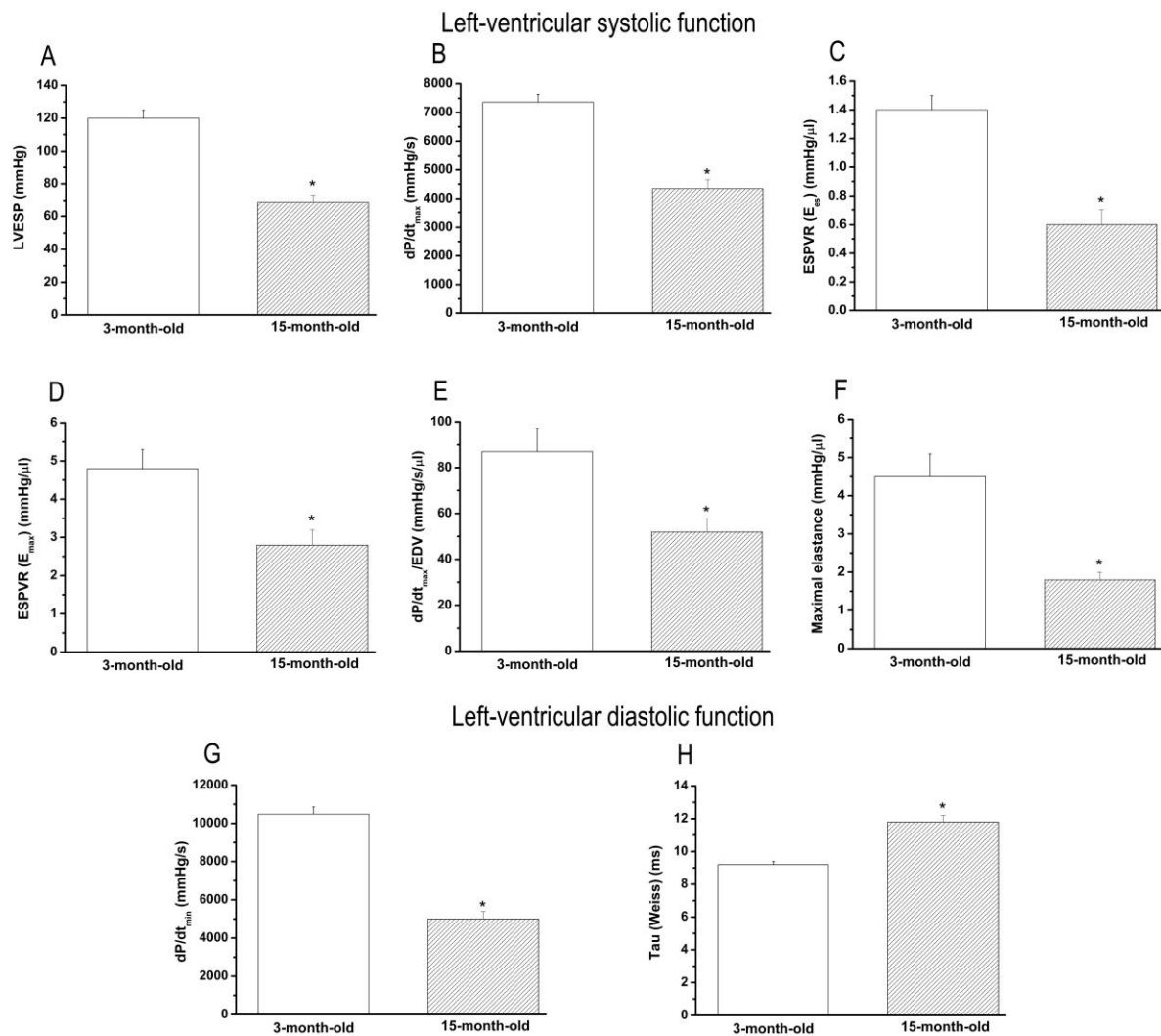


Figure 2 *In vivo* left-ventricular (LV) systolic and diastolic function in 3-month-old and 15-month-old healthy rats. (A) LV end-systolic pressure (LVESP); (B) maximal slope of systolic pressure increment (dP/dt_{max}); the slopes (C) E_{es} and (D) E_{max} of the LV end-systolic pressure-volume relationship; (E) $dP/dt_{max}/EDV$ (end-diastolic volume); (F) maximal elastance; (G) maximal slope of diastolic pressure decrement (dP/dt_{min}); and (H) Tau, time constant of the LV pressure decay. Data are represented as mean $\pm$ SEM. * $P<0.05$ versus 3-month-old.

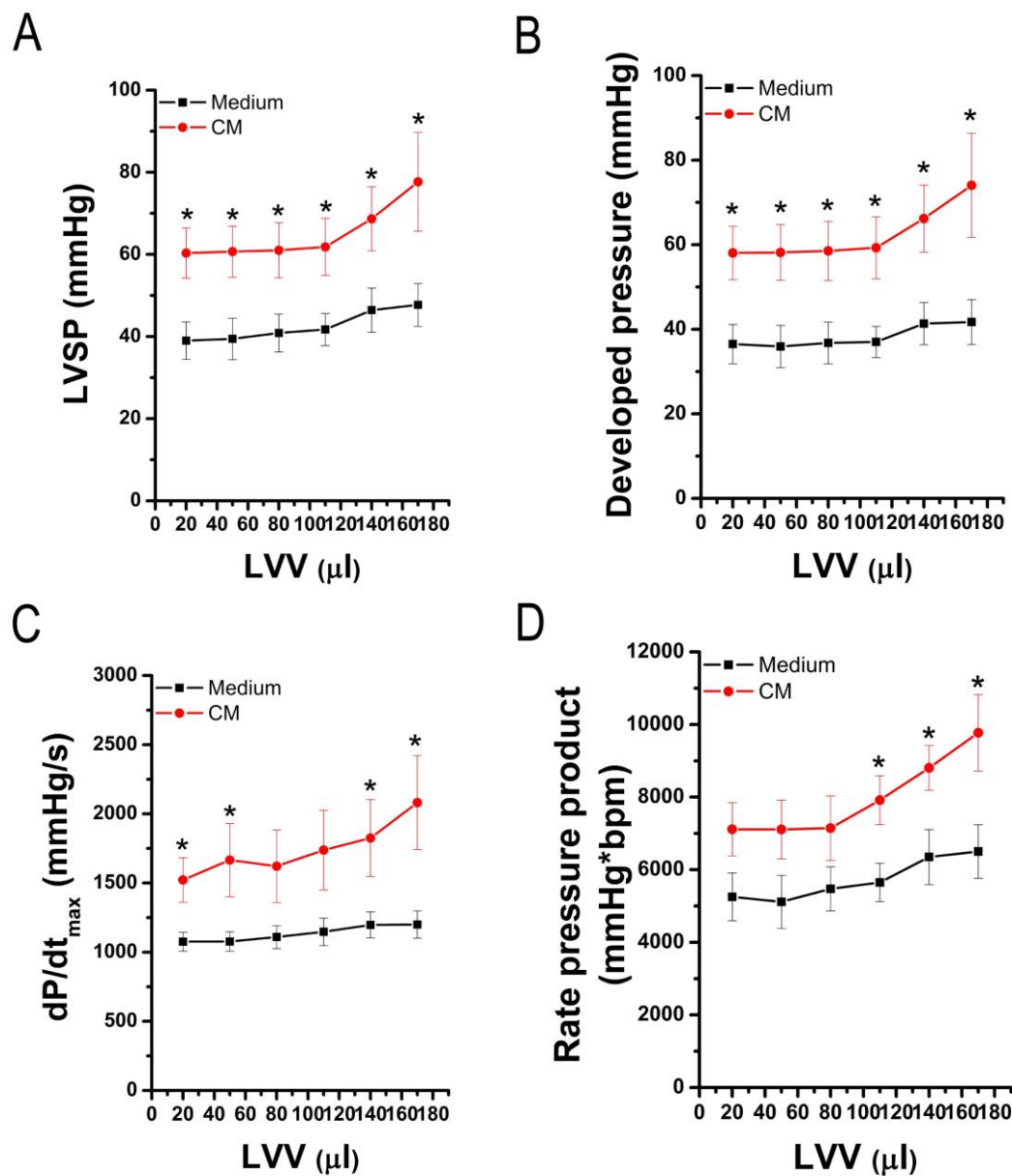


Figure 2 Effect of mesenchymal stem cells conditioned medium (CM) on left-ventricular (LV) graft systolic function after heart transplantation. (A) LV systolic pressure (LVSP), (B) developed pressure, (C), maximal slope of systolic pressure increment (dP/dt_{max}), and (D) rate pressure product. Data are represented as mean $\pm$ SEM. n=6-7 rats/group. *P<0.05 vs. CM.

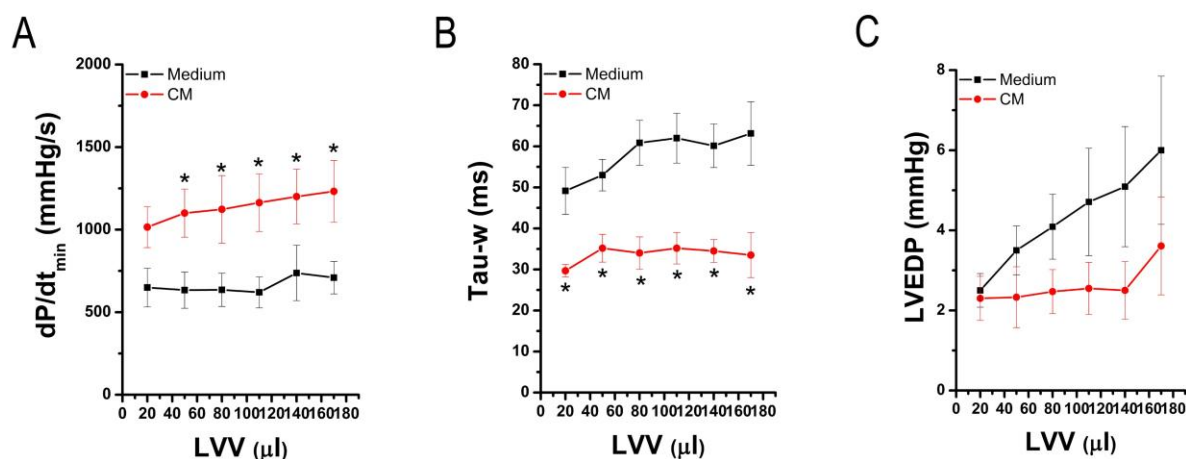
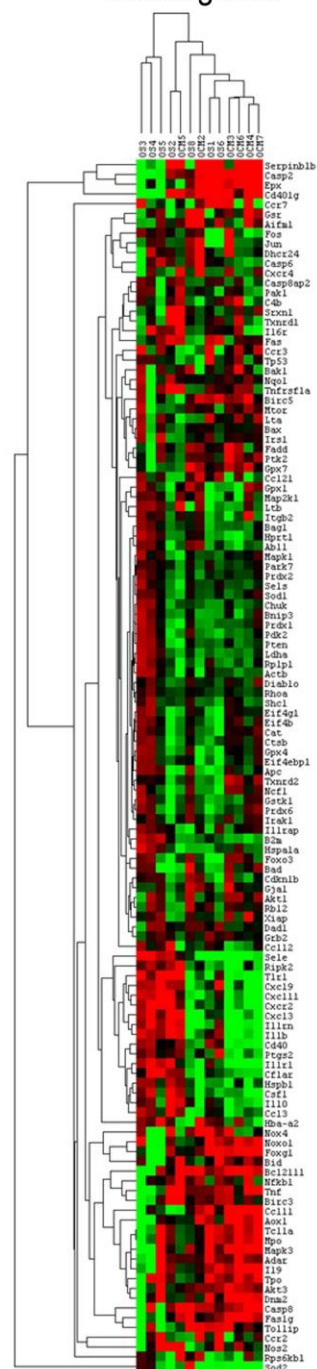


Figure 3 Effect of mesenchymal stem cells conditioned medium (CM) on left-ventricular (LV) graft diastolic function after heart transplantation. (A) maximal slope of diastolic pressure decrement (dP/dt_{min}), (B) time constant of the LV pressure decay Tau-w, and (C) LV end-diastolic pressure (LVEDP). Data are represented as mean $\pm$ SEM. n=6-7 rats/group. * $P < 0.05$ vs. CM.



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plots their p-value. (B) Clustergrams create a heat map with dendograms to indicate which genes are co-regulated. Degrees of red and green indicate relatively high and low expression of the corresponding gene, respectively, and black squares denote genes equally expressed. The x-axis indicates the rat's number [OS corresponds to medium (control) group, including seven rats (OS1, OS2, OS3, OS4, OS6, and OS8), and OCM to CM group, including six rats (OCM2, OCM3, OCM4, OCM5, OCM6, and OCM7)], and the y-axis indicates the genes. n=6-7 rats/group.

TABLES

Table 1 Genes Table

Official full name	Gene symbol
24-dehydrocholesterol reductase	Dhcr24
ABL proto-oncogene 1, non-receptor tyrosine kinase	Abl1
actin, beta	Actb
adenomatous polyposis coli	Apc
adenosine deaminase, RNA-specific	Adar
aldehyde oxidase 1	Aox1
apoptosis-inducing factor, mitochondrion-associated 1	Aifm1
baculoviral IAP repeat-containing 3	Birc3
baculoviral IAP repeat-containing 5	Birc5
BCL2/adenovirus E1B interacting protein 3	Bnip3
BCL2-antagonist/killer 1	Bak1
BCL2-associated agonist of cell death	Bad
BCL2-associated athanogene	Bag1
Bcl2-associated X protein	Bax
BCL2-like 11 (apoptosis facilitator)	Bcl2l11
beta-2 microglobulin	B2m
BH3 interacting domain death agonist	Bid
CASP8 and FADD-like apoptosis regulator	Cflar
Caspase-2	Casp2
Caspase-6	Casp6
Caspase-8	Casp8
Caspase-8 associated protein 2	Casp8ap2
catalase	Cat
cathepsin B	Ctsb
C-C motif chemokine receptor 2	Ccr2
CD40 ligand	Cd40lg
CD40 molecule, TNF receptor superfamily member 5	Cd40
chemokine (C-C motif) ligand 11	Ccl11
chemokine (C-C motif) ligand 12	Ccl12
chemokine (C-C motif) ligand 21	Ccl21
chemokine (C-C motif) ligand 3	Ccl3
chemokine (C-C motif) receptor 3	Ccr3
chemokine (C-C motif) receptor 7	Ccr7
chemokine (C-X-C motif) ligand 11	Cxcl11
chemokine (C-X-C motif) ligand 3	Cxcl3
chemokine (C-X-C motif) ligand 9	Cxcl9
chemokine (C-X-C motif) receptor 2	Cxcr2
chemokine (C-X-C motif) receptor 4	Cxcr4
colony stimulating factor 1 (macrophage)	Csf1
complement component 4A (Rodgers blood group)	C4a
conserved helix-loop-helix ubiquitous kinase	Chuk
cyclin-dependent kinase inhibitor 1B	Cdkn1b
defender against cell death 1	Dad1
diablo, IAP-binding mitochondrial protein	Diablo
dynammin 2	Dnm2
eosinophil peroxidase	Epx
eukaryotic translation initiation factor 4 gamma, 1	Eif4g1
eukaryotic translation initiation factor 4B	Eif4b
eukaryotic translation initiation factor 4E binding protein 1	Eif4ebp1
Fas (TNFRSF6)-associated via death domain	Fadd
Fas cell surface death receptor	Fas
Fas ligand (TNF superfamily, member 6)	Faslg
FBJ osteosarcoma oncogene	Fos
forkhead box G1	Foxg1
forkhead box O3	Foxo3
gap junction protein, alpha 1	Gja1

glutathione peroxidase 1	Gpx1
glutathione peroxidase 4	Gpx4
glutathione peroxidase 7	Gpx7
glutathione reductase	Gsr
glutathione S-transferase kappa 1	Gstk1
growth factor receptor bound protein 2	Grb2
heat shock 70kD protein 1A	Hspa1a
heat shock protein B1	Hspb1
hemoglobin, alpha 1	Hba1
hypoxanthine phosphoribosyltransferase 1	Hprt1
insulin receptor substrate 1	Irs1
integrin, beta 2	Itgb2
interleukin 1 beta	Il1b
interleukin 1 receptor accessory protein	Il1rap
interleukin 1 receptor antagonist	Il1rn
interleukin 1 receptor, type I	Il1r1
interleukin 10	Il10
interleukin 6 receptor	Il6r
interleukin 9	Il9
interleukin-1 receptor-associated kinase 1	Irak1
jun proto-oncogene	Jun
lactate dehydrogenase A	Ldha
lymphotoxin alpha	Lta
lymphotoxin beta (TNF superfamily, member 3)	Ltb
mechanistic target of rapamycin (serine/threonine kinase)	Mtor
mitogen activated protein kinase 1	Mapk1
mitogen activated protein kinase 3	Mapk3
mitogen activated protein kinase kinase 1	Map2k1
myeloperoxidase	Mpo
NAD(P)H dehydrogenase, quinone 1	Nqo1
NADPH oxidase 4	Nox4
NADPH oxidase organizer 1	Noxo1
neutrophil cytosolic factor 1	Ncf1
nitric oxide synthase 2, inducible	Nos2
nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	Nfkb1
p21 protein (Cdc42/Rac)-activated kinase 1	Pak1
parkinson protein 7	Park7
peroxiredoxin 1	Prdx1
peroxiredoxin 2	Prdx2
peroxiredoxin 6	Prdx6
phosphatase and tensin homolog	Pten
prostaglandin-endoperoxide synthase 2	Ptgs2
protein tyrosine kinase 2	Ptk2
pyruvate dehydrogenase kinase, isozyme 2	Pdk2
ras homolog family member A	Rhoa
receptor-interacting serine-threonine kinase 2	Ripk2
retinoblastoma-like 2	Rbl2
ribosomal protein S6 kinase, polypeptide 1	Rps6kb1
ribosomal protein, large, P1	Rplp1
selectin E	Sele
serine (or cysteine) peptidase inhibitor, clade B, member 1b	Serpinb1b
SHC (Src homology 2 domain containing) transforming protein 1	Shc1
sulfiredoxin 1	Srxn1
superoxide dismutase 1, soluble	Sod1
superoxide dismutase 2, mitochondrial	Sod2
T-cell leukemia/lymphoma 1A	Tcl1a
thioredoxin reductase 1	Txnrd1
thioredoxin reductase 2	Txnrd2
thyroid peroxidase	Tpo
toll interacting protein	Tollip
toll-like receptor 1	Tlr1

tumor necrosis factor	Tnf
tumor necrosis factor receptor superfamily, member 1a	Tnfrsf1a
tumor protein p53	Tp53
v-akt murine thymoma viral oncogene homolog 1	Akt1
v-akt murine thymoma viral oncogene homolog 3	Akt3
VCP-interacting membrane protein	Vimp
X-linked inhibitor of apoptosis	Xiap

ACCEPTED MANUSCRIPT

Table 2 Factors detected in conditioned medium from bone marrow-derived mesenchymal stem cells

Phenomena	Specific properties	Factors
Apoptosis	Anti-apoptotic	TIMP1, Growth Hormone/Growth Hormone Receptor, EG-VEGF (PK1), VEGF, Activin A
	Pro-apoptotic	TRAIL, Thrombospondin, TROY
Angiogenesis	Pro-angiogenesis	EG-VEGF (PK1), VEGF, MMP2, FGF-BP, Neuropilin2, PDGF-AA, MCP1
	Anti-angiogenesis	TIMP2, Thrombospondin, IP-10
Stress	Protection from oxidative stress	FGF-BP
Inflammation	Pro-inflammatory	CINC2/3, FSL1, MCP1, MDC, MIF, MIP1 α , Osteopontin/SPP1
	Anti-inflammatory	MDC
	Immunomodulator	Osteopontin, GM-CSF, IP-10, LIX
Others	Cell proliferation	TIMP1
	Metabolism	Growth Hormone/Growth Hormone Receptor, IDE
	ECM modulation	TIMP1, TIMP3, MMP-13

CINC2 indicates cytokine induced neutrophil chemoattractant 2; EG-VEGF, endocrine gland-derived vascular endothelial growth factor, also called prokineticin 1 (PK1); FGF-BP, fibroblast growth factor-binding protein; FSL1, fibroblast-stimulating lipopeptide; GM-CSF, granulocyte-macrophage colony-stimulating factor; IDE, insulin degrading enzyme; IP-10, interferon gamma-induced protein; LIX, lipopolysaccharide-induced chemokine; MCP1, monocyte chemoattractant protein-1; MDC, macrophage-derived chemokine; MIF, macrophage migration inhibitory factor; MIP1 α , macrophage inflammatory proteins 1 α ; MMP2 metalloproteinase 2; PDGF-AA, platelet-derived growth factor-AA; SPP1 secreted phosphoprotein 1; TIMP1, tissue inhibitor of metalloproteinase 1; VEGF, vascular endothelial growth factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand).

Table 3 List of significantly altered genes with fold regulation in the graft after heart transplantation. Two genes were significantly upregulated and 64 genes were downregulated in conditioned medium-treated hearts compared to the medium-treated group.

Phenomena	Specific properties	Gene Symbol	Fold Regulation
Oxidative stress	Anti-oxidant	Sod2	2.176715456
Inflammation	Regulation of the inflammatory response	Sele	2.124068693
Oxidative stress	Anti-oxidant	Serpinb1b	-10.00455488
	Anti-oxidant	Gsr	-3.898247706
	Anti-oxidant	Srxn1	-3.071917267
	Anti-oxidant	C4b	-2.250495747
	Oxygen transporter	Dnm2	-4.666131473
	Oxidative stress responsible gene	Txnrd1	-2.647492418
	Oxidative stress responsible gene	Txnrd2	-2.424458249
	Oxidative stress responsible gene	Epx	-6.674738159
	Oxidative stress responsible gene	Gpx7	-3.02358587
	Oxidative stress responsible gene	Mpo	-3.790129262
	Oxidative stress responsible gene	Nqo1	-2.298149569
	Oxidative stress responsible gene	Dhcr24	-3.296673214
	Oxidative stress responsible gene	Tpo	-3.357593945
	Superoxide metabolism gene	Nox4	-2.334473859
	Superoxide metabolism gene	Noxo1	-3.719301296
	Reactive oxygen species metabolism gene	Aox1	-8.960473858
Apoptosis	Anti-apoptotic	Birc5	-2.555977225
	Anti-apoptotic	Birc3	-4.480816949
	Anti-apoptotic	Cd40lg	-14.01746259
	Anti-apoptotic	Xiap	-2.035322231
	Induction of apoptosis	Bak1	-2.012517381
	Induction of apoptosis	Casp6	-2.41750115
	Induction of apoptosis	Fadd	-2.857269887
	Induction of apoptosis	Bcl2l11	-10.45191047
	Induction of apoptosis	Casp2	-11.4445
	Induction of apoptosis	Faslg	-5.153037276
	Induction of apoptosis	Bid	-2.970165191
	Induction of apoptosis	Casp8	-7.49090044
	Induction of apoptosis	Akt1	-2.372417837
	Death domain receptor	Tnfrsf1a	-2.536703096
	Caspases activation	Aifm1	-2.922174656
Inflammation	Cytokine	Tnf	-6.356547683
	Cytokine	Ccl11	-6.901894899
	Interleukin	Ltb	-2.090503567
	Interleukin	Il9	-4.531051117
	Interleukin	Cd40lg	-14.01746259
	Cytokine receptor	Ccr3	-2.028027673
	Chemokine receptor	Ccr2	-2.359231913
	Chemokine receptor	Cxcr4	-5.691451936
	Regulation of the inflammatory response	Nfkb1	-3.826431698
	Regulation of the inflammatory response	Nos2	-2.431910521
	Regulation of the inflammatory response	Tollip	-3.252940874
PI3K-AKT	AKT & PI3K family members & their regulators	Tcl1a	-3.903335656
	AKT & PI3K family members & their Regulators	Akt3	-4.110498824
	AKT & PI3K family members & their Regulators	Akt1	-2.372417837

IGF-1 signaling	Mapk3	-4.301430006
IGF-1 signaling	Jun	-3.485382358
IGF-1 signaling	Map2k1	-2.028032392
Cytoskeleton regulator	Pak1	-2.110082121
Akt signaling gene	Foxg1	-5.889388178
Regulation of eIF4e & p70 S6 kinase	Mapk3	-4.301430006
Regulation of eIF4e & p70 S6 kinase	Rps6kb1	-3.552263085
Regulation of eIF4e & p70 S6 kinase	Akt1	-2.372417837
mTOR signaling	Mtor	-2.403397959
mTOR Signaling	Akt1	-2.372417837
mTOR Signaling	Rps6kb1	-3.552263085
PTEN-dependent cell cycle arrest & apoptosis	Foxo3	-2.006180527
PTEN-dependent cell cycle arrest & apoptosis	Akt1	-2.372417837
PTEN-dependent cell cycle arrest & apoptosis	Mapk3	-4.301430006
PTEN-dependent cell cycle arrest & apoptosis	Ptk2	-3.074762505
GSK3 inactivation & β -catenin accumulation	Adar	-3.520150648
GSK3 inactivation & β -catenin accumulation	Akt1	-2.372417837
GSK3 inactivation & β -catenin accumulation	Gja1	-2.615255865
GSK3 inactivation & β -catenin accumulation	Irak1	-2.093682005

See Table 1 for genes abbreviation. PI3K indicates phosphoinositide 3-kinase; IGF, insulin like growth factor; eIF, eukaryotic translation initiation factor; mTOR, mechanistic target of rapamycin; PTEN, Phosphatase and tensin homolog; GSK3, Glycogen synthase kinase-3.