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Recipient Adipose-Derived Stem Cells Enhance Recipient Cell Engraftment and Prolong Allotransplant Survival in a Miniature Swine Hind-Limb Model

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Abstract

Donor mesenchymal stem cells (MSCs) could prolong vascularized composite allotransplantation (VCA) survival in our previous studies. However, recipient adipose tissue is easier to harvest than donor tissue for preconditioning modulation. Hence, this study investigated the efficacy of recipient autologous adipose-derived stem cells (rADSCs) for VCA survival. The heterotopic hind-limb transplantation from female donor to male recipient was performed in outbred miniature swine. Group I ($n = 6$) was untreated controls. Group II ($n = 4$) obtained rADSCs infusions (given on weeks 0, +1, +2, and +3). Group III ($n = 4$) obtained tacrolimus (FK506, weeks 0 to +4). Group IV ($n = 8$) received irradiation (IR; day −1), FK506 (weeks 0 to +4), and rADSC infusions (weeks 0, +1, +2, and +3). The results revealed treatment with multiple injections of rADSCs along with IR and FK506 resulted in a statistically significant increase in allograft survival. The percentage of $CD4^+/CD25^+/Foxp3^+$ regulatory T cells were significantly increased in the rADSC-IR-FK506 group as compared to controls. Analysis of recipient peripheral blood revealed that transforming growth factor β1 (TGFβ1) was significantly increased in the rADSC-IR-FK506 group. The polymerase chain reaction (PCR) analysis and immunohistochemical staining showed recipient sex-determining region of Y (SRY) chromosome gene expression existed in donor allotissues in the rADSC-IR-FK506 group. These results indicate that rADSCs in addition to IR and transient immunosuppressant could prolong allotransplant survival, modulate T-cell regulation, and enhance recipient cell engraftment into the allotransplant tissues.

Keywords

adipose-derived stem cells, regulatory T cell, vascularized composite allotransplantation, graft rejection

Introduction

Vascularized composite allotransplantation (VCA) has become a clinical reality as demonstrated by the recent success in composite hand and face allotransplantations.[1,2] However, the clinical practice of VCA is currently limited by the risks of the side effects of the therapeutic immunosuppression that is used to prevent graft failure.[3] Inducing tolerance could potentially eliminate graft rejection without the long term use of immunosuppressants. There is an abundance of experimental tolerance protocols in animal models that are indispensable for the development of clinical tolerance strategies.[4,5]

Mesenchymal stem cells (MSCs) are pluripotent cells that are present in multiple tissues, including bone marrow (BM), adipose tissue, skin, muscle, blood, and placenta, and can be isolated and expanded ex vivo.[6,7] Studies revealed that

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MSCs do not express immunogenic costimulatory molecules such as B7-1, B7-2, or CD40.[8–10] Therefore, they are most likely unable to stimulate alloreactive T cells. Immunomodulatory effects of MSCs have indicated that MSCs can regulate T-cell activities.[9,11,12] Our previous studies showed donor BM-derived MSCs (BM-MSCs) combined with preoperative irradiation (IR) and short-term cyclosporine A (CsA) could prolong allograft survival in a miniature swine.[13–15]

Recently, it was reported that adipose tissue is a rich source of stem cells as compared to BM. Studies also indicated that BM-MSCs and adipose-derived stem cells (ADSCs) are equally capable of differentiating into various lineages of the mesenchyme and rendering immunosuppressive and immune modulatory effects in vitro and in vivo.[16,17] Our previous studies have demonstrated that multiple donor allogeneic ADSCs have immunomodulatory effects to prolong allograft survival and increase the percentages of $CD4^+/CD25^+/Foxp3^+$ regulatory T-cell populations in a rodent hind-limb model.[18] Although we have demonstrated the beneficial effect of donor MSCs as a posttransplant immunosuppressive therapy, the donor allogeneic MSCs revealed a low percentage of engraftment. Additionally, no obvious migration of recipient BM cells into donor allograft tissue was detected.

In contrast, recipient adipose tissue is easier to harvest than donor tissue and could modify the theoretical protocol for preconditioning immune modulation. A recent study revealed that recipient syngeneic ADSCs have the effect of modulating the immune response and prolonging rodent allograft survival.[19] Therefore, in this study, we investigated whether the recipient adipose-derived stem cell (rADSC) therapy combined with short-term immunosuppressant FK506 (tacrolimus) in addition to IR treatment could prolong allotransplant survival and increase the recipient cell engraftment and regeneration to donor tissue in a heterotopic miniature swine hind-limb VCA model.

Materials and Methods

Animals

Thirty-four outbred domestic miniature swine purchased form Taitung Animal Propagation Station, Taitung, Taiwan (Lan-Yu strain; age: 3 mo; weight: 18 to 25 kg) were included in the study. The Lan-Yu strain is an indigenous breed from Lau-Yu Island, southeast of Taiwan, with the genotype of GPI (glucose-6-phosphate isomerase)-BB subtype, and the genotype of PGD (glucose-6-phosphate dehydrogenase)-AA subtype.[13,14] The inherited differences in donors and recipients from the original parental generation are identified and reported by the National Tai-Tung Veterinary Research Institute, Taiwan. This study was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health, Bethesda, Maryland, USA. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Kaohsiung Chang Gung Memorial Hospital.

Heterotopic Hind-Limb Swine Model

Heterotopic hind-limb transplantation was performed as previously described.[13,20] In brief, animals were premedicated with an intramuscular injection of ketamine (10 mg/kg; Sigma-Aldrich, St Louis, MO, USA) and xylazine (1.5 mg/kg; Tai Yu Chemical & Pharmaceutical Co., Hsinchu, Taiwan.) Inhalational anesthesia was maintained with isoflurane (0.5% to 3% inhaled, Forane; AbbVie, Taipei, Taiwan) and oxygen throughout the procedure. A composite tissue consisting of tissues from the tibia, fibula, knee joint, distal femur, and surrounding muscle was harvested from the donor swine. A skin paddle, measuring approximately 8×8 cm^2, was preserved on the medial aspect of the knee supplied by the superficial femoral vessels. The tibia and fibula were divided at approximately 5 cm below the knee, and the femur was divided 5 cm above the knee. The thigh muscles were divided at the mid-femur level. Upon division of the vascular pedicle, heparinized saline (BD Pharmingen, San Diego, CA, USA) was flushed through the femoral artery. The recipient swine were prepared in a similar manner as described for the donor swine. A subcutaneous pocket was created in the anterolateral abdominal wall, and the limb graft was placed into the pocket. The vessels were anastomosed in an end-to-end fashion onto the host femoral vessels with a 9-0 nylon interrupted suture using microscopic magnification. A defect was created in the skin of the host's flank, and the skin paddle was sutured in place on the donor limb.

Culture of the rADSCs

The groin fat pad from recipients was harvested, washed, and digested with 0.2% type I collagenase (Sigma-Aldrich) for 30 min at 37 °C. After centrifugation (260*g*, 5 min), the pellet was resuspended in phosphate-buffered saline (PBS), following the red blood cell (RBC) lysis process with ammonium chloride lysis buffer (155 mM ammonium chloride, 0.1 mM ethylenediaminetetraacetic acid [EDTA], and 10 mM sodium bicarbonate, pH 7.2; all from Sigma-Aldrich). After being washed and centrifuged, the rADSC-containing pellet was resuspended in low-glucose Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY, USA) and plated in a 75T flask. The isolated primary culture (P0) rADSCs were cultured in low-glucose DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (both from Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified incubator with 5% CO_2. The rADSCs were expanded up to passage 5 and characterized by flow cytometry (Becton Dickinson, San Jose, CA, USA) following positive surface staining for CD44, CD90, major histocompatibility complex (MHC) class I, and CD106, but not for CD45, MHC class II, and CD80/B7-1. Before experimental use, the multilineage differentiation ability of rADSCs toward adipocytes, osteoblasts, and chondrocytes was tested. Osteoblasts were identified by von Kossa staining, Oil Red O was used for lipid droplet staining, and

chondrogenic differentiation was visualized by Alcian blue staining[16] (all from Abcam, Taipei, Taiwan).

Experimental Design

Miniature swine underwent heterotopic hind-limb transplantation. Twenty-two male swine were recipients and 12 female were donors; 2 hind limbs harvested from a single donor were given to 2 different recipients. Group I ($n = 6$) was the control cohort and hence did not undergo immunosuppressive therapy. Group II ($n = 4$) received the rADSC treatment (1×10^6 cells/kg/dose, given on weeks 0, +1, +2, and +3). Group III ($n = 4$) received oral administration of FK506 (tacrolimus; Sigma-Aldrich) for 4 wk (weeks +1 to +4; 1 mg/kg/d for 2 wk followed by 0.5 mg/kg/d for 2 wk). Group IV ($n = 8$) received preconditioning IR (day −1; 150 cGy for total body IR and 700 cGy for intrathymus IR), FK506 for 4 wk (same protocol as group III; weeks +1 to +4), and rADSCs (1×10^6 cells/kg/dose, weeks 0, +1, +2, and +3). The timing of the immunosuppressant was that used in a previous study.[14] The dosage and timing of ADSC injections were similar to our previous donor ADSC infusion protocol.[18] The clinical experimental end point was defined as desquamation and necrosis of the entire area of donor skin. The definition of immune tolerance for the allograft induced by recipient cells was allograft survival for more than 15 wk posttransplantation without any signs of rejection.

Histological Evaluation of Graft Rejection

The transplanted limb was observed daily for signs of rejection as defined by the well-characterized sequence of epidermolysis, dyskeratosis, and necrosis. Full-thickness 3-mm biopsies of donor skin and muscle were obtained +2 and +6 postoperative weeks. The harvested tissues for biopsies were provided around 0.5×0.5 cm^2 by punch. The biopsy specimens were fixed and embedded in paraffin blocks for hematoxylin and eosin (H&E; Sigma-Aldrich) and immunohistochemical (IHC) staining. Rejection grades were scored using Banff classification, according to the severity of pathological changes.[21]

Evaluation of $CD25^+$/Forkhead Box p3 $(Foxp3)^+$ T-Cell Expression

Frozen sections were obtained from the alloskin of the grafted limbs on +2 and +6 weeks posttransplantation. To assess the effects of regulatory T cells after transplantation, alloskin samples were subjected to IHC staining. A horseradish peroxidase–diaminobenzidine (HRP-DAB) kit (Bio SB, Santa Barbara, CA, USA) was used in all immunostaining experiments to visualize the expression profiles. Tissue sections were stained with mouse antiporcine CD25 (Serotec, Oxford, UK) or with mouse antirat Foxp3 (Serotec) antibodies. The reacted sections were incubated with biotinylated antimouse antibody (BD Pharmingen) as a secondary antibody. After counterstaining with H&E, the tissue sections were mounted, cleared, and coverslipped.

Tissue sections obtained from 6 individual specimens were analyzed. For immunostaining quantification, sections were analyzed using a Zeiss Axioskop 2 plus Microscope (Carl Zeiss, Göttingen, Germany). Areas (3 mm^2) containing $Foxp3^+$ T cells were calculated. Four areas were randomly captured at 400× magnification by a Cool CCD camera (SNAP-Pro cf Digital Kit; Media Cybernetics, Silver Spring, MD, USA). Images were analyzed using Image-Pro Plus image analysis software (version 7) (Media Cybernetics Rockville, Maryland, USA).

Flow Cytometric Assessment of $CD4^+$/$CD25^+$/$Foxp3^+$ T Cells

Flow cytometric analyses were performed on the peripheral blood samples of recipients collected on weeks +2, +6, and +15 posttransplant. The whole blood was incubated for 20 to 30 min in the dark (room temperature) with 5 μL of mouse antiporcine CD25-fluorescein isothiocyanate (BD Pharmingen) and combinations of mouse antipig CD4-phycoerythrin (BD Pharmingen) and antimouse/rat Foxp3 PerCP-Cyanine5.5 (eBiosciences, San Diego, CA, USA). After incubation, RBCs were lysed (fluorescent-activated cell sorting (FACs) lysing solution; BD Pharmingen), and the remaining cells were centrifuged twice at 500*g* for 5 min. Cells were then analyzed by flow cytometry (FACScan; Becton Dickinson). Data were analyzed using the BD FACScan™ program (Becton Dickinson).

Engraftment Was Measured by Labeling rADSCs with 5-bromo-2'-deoxyuridine (BrdU)

In vitro BrdU-incorporated rADSCs were used to analyze rADSCs' engraftment into the donor allograft. rADSCs were labeled with 2 rounds of 7.5 μg/mL BrdU in culture medium (−3 and −1 days before single injection). Multiple injections of BrdU-labeled ADSCs were performed intravenously (+0, +1, +2, and +3 wk). After incorporation, a 1×10^7 cells/mL suspension was intravenously injected into the recipient animals in order to assess the rADSC population in the donor transplanted skin tissue. Biopsy bilateral samples of recipient tissue in the IR/rADSC/FK (FK506 [tacrolimus]) group were harvested at weeks +2, +6, and +15 after injection. Biopsy tissue sections were IHC stained with goat antimouse BrdU antibody (Sigma-Aldrich), and the signals were visualized using an HRP-DAB staining kit (BioGeneX Laboratories, Fremont, CA, USA).

Expression of Transforming Growth Factor β1 (TGF-β1) and Tumor Necrosis Factor α (TNF-α) in Peripheral Blood of Recipients

The expression of the soluble form of TGF-β1 and TNF-α in peripheral blood was determined by an enzyme-linked

immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA). Blood serum samples were collected at predetermined specific days posttransplantation (+2, +6, and +15 wk).

Polymerase Chain Reaction (PCR) Amplification of Sex-Determining Region of Y Chromosome (SRY)

The punched biopsy skin and muscle from the donor graft (female) and recipient tissues in the IR/rASC/FK group (male) were cut into small pieces, and around 1 mg of tissues was transferred to a 1.5 mL Eppendorf tube containing 100 μL of lysis buffer (10 mM Tris–HCl, pH 7.6, 10 mM KCl, 2 mM EDTA, 4 mM $MgCl_2$; all from Sigma-Aldrich) for genomic DNA extraction. The male-specific *SRY* gene to the donor allograft was amplified using the following forward primer set: *Sus scrofa SRY*_F (5′-CTG GGA TGC AAG TGG AAA AT-3′) and *Sus scrofa SRY*_R (5′-GGC TTT CTG TTC CTG AGC AC-3′). The *SRY* primers were designed from a sequence available on GenBank assembly ID: GCA_000003025.4. PCR amplification was performed under the following conditions: an initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 20 s, annealing at 65 °C for 20 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 7 min in a thermal cycler (GeneAmp 9700; PerkinElmer, Foster City, CA, USA). All the amplified DNA samples were electrophoresed in a 1.5% agarose gel (Merck KGaA, Darmstadt, Germany) and visualized by ethidium bromide staining (Sigma-Aldrich) and photographed. The *SRY* protein profiles in donor allografts were determined by using IHC staining (Santa Cruz Biotechnology, Dallas, TX, USA).

Data Management and Statistical Analysis

Student's *t* test or analysis of variance (ANOVA) was utilized to assess the change and to compare the difference between these groups from normal distributions. Log transformation was performed prior to variance analysis and Student's *t* tests to reconfirm normality and equal variances for flow cytometry data. Graft survival was compared between the different groups of transplanted animals using Kaplan–Meier analysis and the log-rank test. A P value < 0.05 was considered statistically significant.

Results

rADSCs Combined with Short-Term Immunosuppressants Prolonged Allograft Survival

The results showed the progressive rejection of the swine hind-limb allograft by weeks 1.1 to 2.3 with a mean of 1.6 wk in the control group. Allograft recipients treated with multiple autologous rADSC injections alone in the absence of immunosuppressant treatment revealed a statistical increase in allograft survival as compared to controls (weeks 2.5 to 3.3 with a mean of 2.93 wk, $P = 0.003$). Allograft transplantation along with short-term FK506 treatment resulted in delayed rejection (weeks 6 to 10 with a mean of 8.3 wk, $P = 0.001$). However, treatment with multiple rounds of rADSCs in addition to IR and short-term FK506 resulted in significant prolongation of allotransplant survival (>28 wk), as compared to other experimental groups ($P < 0.001$; Fig. 1).

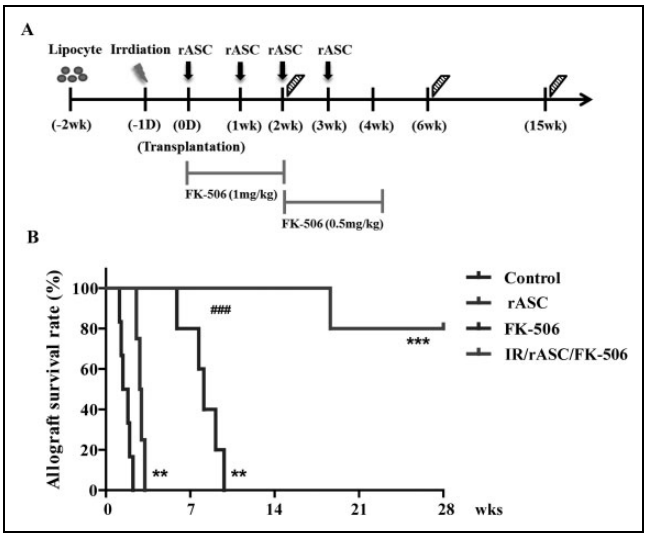

Fig. 1. Recipient adipose-derived stem cells (rADSCs) in combination with irradiation (IR) and transient FK506 (tacrolimus) therapy prolonged allotransplant survival. (A) Flowchart for the treatment protocol used in this study. Recipient pig received preconditioning IR (day −1; 150 cGy for total body IR and 700 cGy for intrathymus IR), FK506 (tacrolimus) for 4 wk, and rADSCs (1×10^6 cells/kg/dose, weeks 0, +1, +2, and +3). (B) Kaplan–Meier vascularized composite allotransplant survival curves for control and experimental groups. The results show early rejection of the swine hind-limb allograft by weeks 1.1 to 2.3 in the control group. Recipients treated with multiple autologous rADSCs alone revealed a statistical increase in allograft survival as compared to controls ($P = 0.003$). Allotransplant treated with short-term FK506 resulting in delayed rejection ($P = 0.0014$). Treatment with rADSCs/IR/FK506 showed significant prolongation of allotransplant survival (>28 wk), as compared to other experimental groups ($P < 0.001$). **$P < 0.01$; ###$P < 0.001$; ***$P < 0.001$.

rADSCs in Combination with Transient Immunosuppressant Therapy Suppressed Allograft Rejection

Histopathological evaluation of the allograft biopsies revealed severe rejection signs (grades III to IV) and mononuclear infiltration in the alloskin and muscle biopsies of the control group at 2 wk posttransplantation. Histological examination of the allograft biopsies of the rADSC groups revealed less inflammatory cell infiltration in the interstitial muscle layers as compared to that in controls at 2 wk posttransplantation (grade II to III). However, lymphocyte infiltration was nonetheless observed in the alloskin and interstitial muscle layers in group IV with

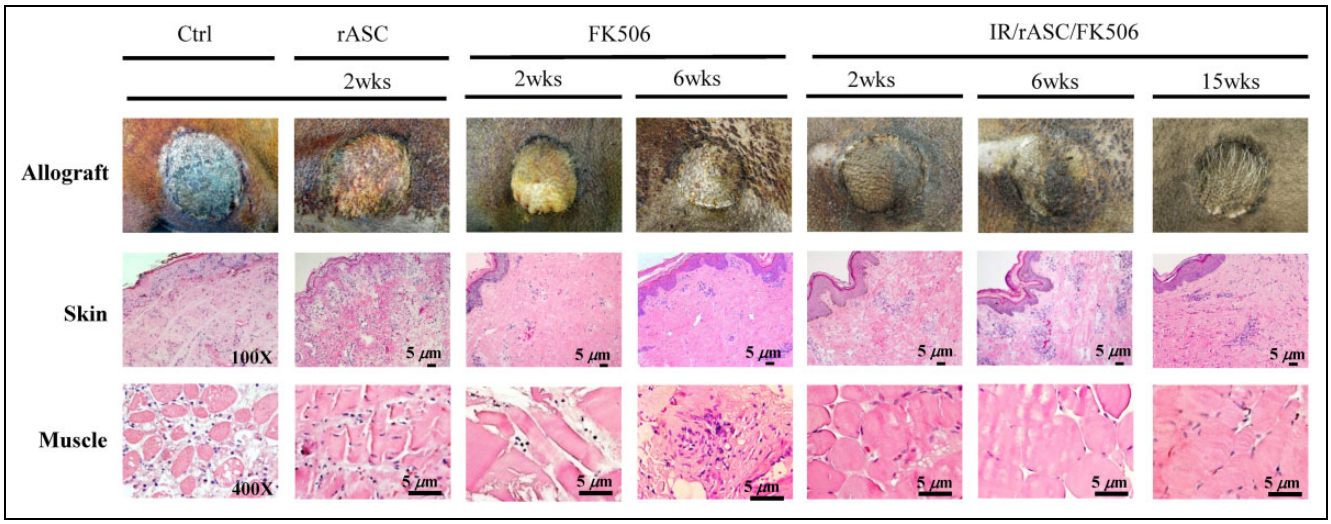

Fig. 2. Recipient adipose-derived stem cells (rADSCs) in combination with irradiation (IR) and transient FK506 (tacrolimus) therapy suppressed allograft rejection. Histopathological evaluation revealed severe rejection (grades III and IV) and mononuclear infiltration in the alloskin and muscle biopsies of the control group at 2 wk posttransplantation. The groups with allograft biopsies of rADSCs alone revealed less inflammatory cell infiltration in the interstitial muscle layers as compared to controls (grades II and III). Lymphocyte infiltration was nonetheless observed in the alloskin and muscle layers in group IV with rADSC/IR/FK506 treatment. At 15 wk posttransplantation, the allotransplant biopsy of group IV revealed rare mononuclear infiltration in the alloskin and interstitial muscle layers as compared to that in the other groups (grades 0 and I). Ctrl, control.

rADSC-IR-FK506 treatment at 2 and 6 wk posttransplantation (grades 0 and I; Fig. 2).

rADSC Modulated Regulatory T-Cell Expression in Circulating Blood and Allotissues

IHC staining of graft skin tissue biopsies revealed significant numbers of Foxp3+ T cells ($P < 0.001$) in the subcutaneous and dermis layers in animals treated with rADSCs, IR, and FK506, as compared to other groups at 2 and 6 wk posttransplantation (Fig. 3A). In parallel, flow cytometric analysis of recipient peripheral blood revealed that $CD4^+/CD25^+/Foxp3^+$ regulatory-like T-cell populations had a significant increase in the rADSC-IR-FK506 group at 6 wk posttransplantation as compared to other treatment groups (Fig. 3B). The populations of $CD4^+/CD25^+/Foxp3^+$ T cells were equal to normal in animals treated with rADSCs-IR-FK506 at 15 wk posttransplant. These results demonstrated that treatment with rADSCs combined with a short-term immunosuppressant regimen could increase regulatory T-cell populations.

rADSCs and Transient Immunosuppressive Therapy Regulated the Pro-Inflammatory and Anti-Inflammatory Cytokines

The concentrations of TGF-βl and TNF-α in peripheral blood were determined by ELISA after the various treatments. Analysis of recipient peripheral serum revealed that TGF-βl was apparently increased at 6 and 15 wk posttransplantation in animals treated with rADSCs, IR, and FK506, as compared to controls (Fig. 4A). The TNF-α level revealed a statistically significant decrease in the group treated with rADSCs, FK506, and IR at 2 and 6 wk posttransplantation, as compared to controls (Fig. 4B).

BrdU-Labeled Recipient ADSCs Engrafted into the Allotransplant Tissue

BrdU-labeled rADSCs were intravenously injected into the recipient swine to investigate the BrdU-rADSC engraftment to donor tissue. IHC staining of allotransplant tissue showed the population of BrdU-positive cells in the subcutaneous layers of alloskin was apparently expressed early (2 and 6 wk posttransplantation) (2.62% and 3.06%, respectively; Fig. 5). However, BrdU-positive cells could not be detected in alloskin 15 wk posttransplantation.

rADSCs Increased Recipient SRY-Positive Expression in Cells in Allotransplant Tissue

To detect the engraftment of the recipient cells to the donor transplant tissue, donor cells from the hind limbs of female swine were transplanted into male recipients. A recipient male-specific *SRY* gene situated in the Y chromosome sex-determination region was detected in allotransplanted tissue. The IHC staining for recipient *SRY* protein expressions in alloskin revealed significant expression in donor allotissue at 6 and 15 wk posttransplantation (Fig. 6A and B). In contrast, PCR analysis revealed that the recipient *SRY* gene had strong expressions in the donor alloskin and muscle at 6 wk posttransplantation in the group with rADSC-IR-FK506 treatment (Fig. 6C).

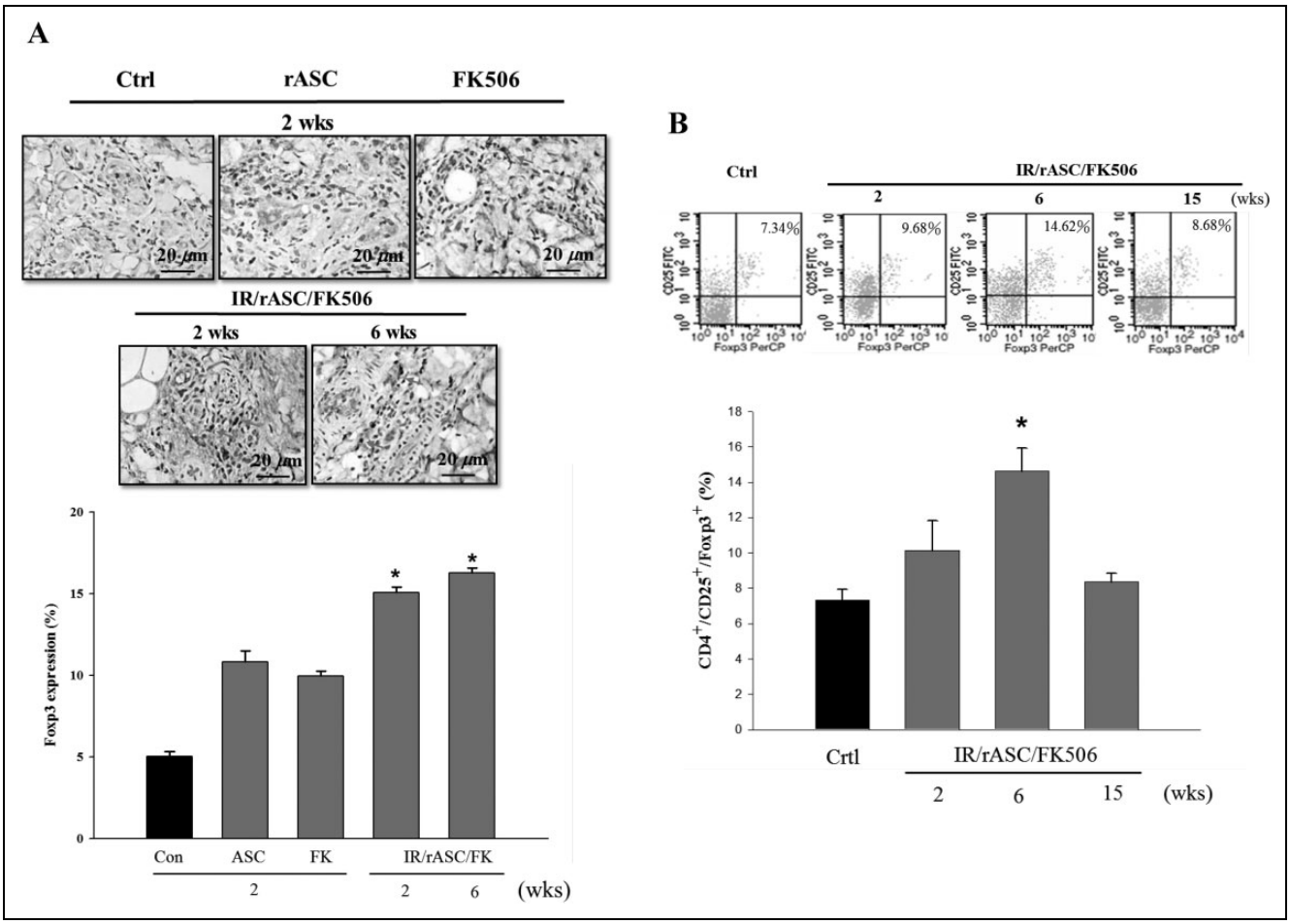

Fig. 3. Recipient adipose-derived stem cells (rADSCs) modulated regulatory T-cell expression in allotissues and circulating blood. (A) Immunohistochemical (IHC) staining of graft skin tissue biopsies revealed significant numbers of $CD25^+$ T cells and forkhead box p3 (Foxp3)+ T cells (*$P < 0.05$) in the subcutaneous and dermis layers in animals treated with rADSCs/irradiation/FK506 (tacrolimus), as compared to other groups at 2 and 6 wk posttransplantation. (B) Flow cytometric analysis of recipient peripheral blood revealed that $CD4^+$/$CD25^+$/$Foxp3^+$ T-cell populations have significant increase in rADSC/IR/FK506 treatment group at 6 wk posttransplantation as compared to that in other groups, but skewing down at 15 wk posttransplantation (*$P < 0.05$). Ctrl or con, control.

Discussion

VCAs are not routinely performed for tissue reconstruction because of the potentially adverse effects associated with lifelong administration of immunosuppressive agents, which is required for highly antigenic tissue. Therefore, the development of novel and nontoxic strategies that circumvent the long-term use of immunosuppressants is critical.[5] Our previous study revealed that the infusion of donor BM-MSCs or donor ADSCs could prolong allotransplant survival in a rodent or miniature swine hind-limb VCA model.[14,18] In addition, we showed that the presence of donor MSCs/ADSCs correlated with increases in regulatory T-cell populations in a hind-limb VCA model and induced mixed chimerism, resulting in long-term graft acceptance.[14,17,18]

Although we have demonstrated the beneficial effects of donor MSCs/ADSCs as a posttransplant immunosuppressive therapy, the donor allogeneic MSCs/ADSCs revealed a low percentage of engraftment of recipient cells into donor tissue to enhance allotissue regeneration. Moreover, in vivo immunogenicity of donor-derived MSCs/ADSCs raises uncertainty over their future clinical applicability. In contrast, recipient adipose tissue is easier to harvest than donor tissue without time limitation for preconditioning immune modulation. Given that human VCA donors are deceased, recipient origin ADSCs have a major advantage over any form of donor-derived MSCs in that they can be harvested from the recipient and stored in advance of VCA in an optimized form. Therefore, this study investigated whether the recipient ADSC therapy combined with IR and transient tacrolimus (FK506) treatment could prolong hind-limb VCA survival and enhance the degree of recipient cell engraftment and tissue regeneration into donor allotransplant.

Our past study has demonstrated that donor MSC combined with short-term cyclosporin A (CsA) could prolong allograft survival but still encountered rejection around 38 to 87 d posttransplantation.[15] The immunomodulatory

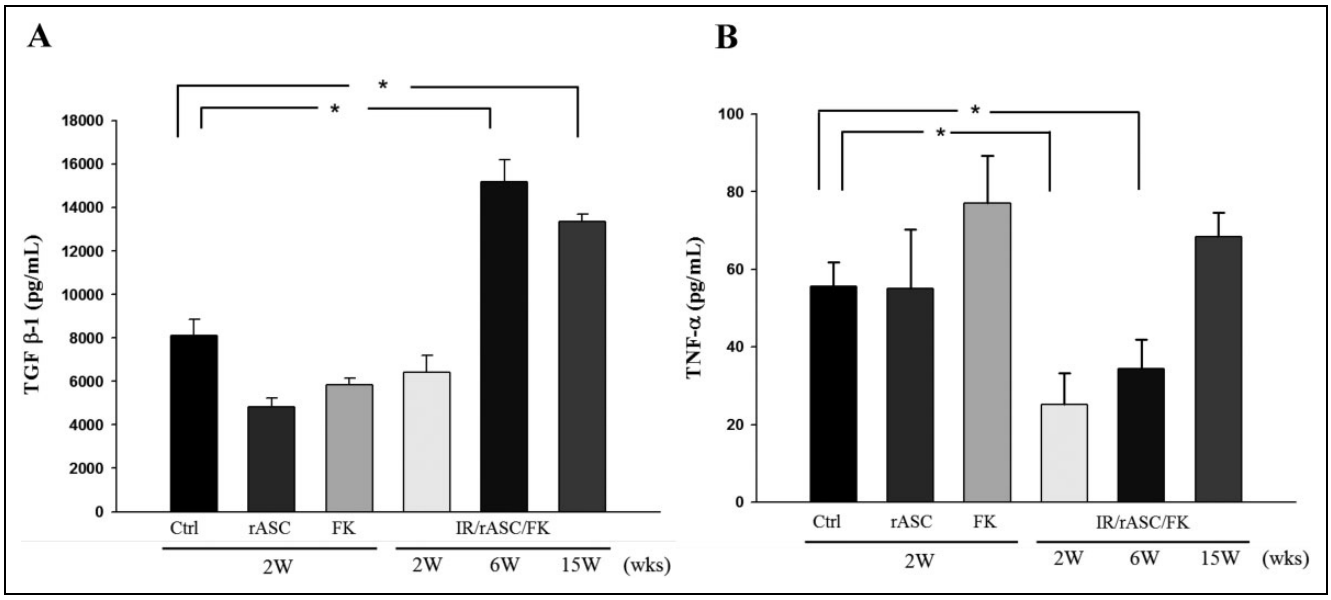

Fig. 4. Recipient adipose-derived stem cells (rADSCs) and transient immunosuppressive therapy that regulated the pro-inflammatory and anti-inflammatory cytokines were determined by enzyme-linked immunosorbent assay (ELISA) after the various treatments. (A) Analysis of recipient peripheral serum revealed that transforming growth factor βI was significantly increased at 6 and 15 wk posttransplantation in animals treated with rADSCs/irradiation (IR)/FK506 (tacrolimus), as compared to controls (*$P < 0.05$). (B) The tumor necrosis factor (TNFα) level revealed a statistically significant decrease in rADSCs/IR/FK506 group at 2 and 6 wk posttransplant, as compared to controls (ctrl; *$P < 0.05$), but there was no significant difference at 15 wk posttransplant.

cytokine expression also showed an increase in interleukin-10 (IL-10) and decrease in TNF-α expressions. However, the group treated with MSCs combined with CsA still demonstrated rejection and did not develop immune tolerance. Therefore, in this study, we focused on whether rADSC/FK506 therapy combined with IR could increase regulatory T-cell expression, reduce inflammatory cytokines, and promote immune tolerance. Our results revealed that the treatment of recipients with multiple rADSC injections in addition to IR and short-term FK506, similar to our previous donor ADSCs and short-term immunosuppressant treatment protocol, resulted in significant prolongation of allotransplant survival in comparison to other experimental groups. Histopathological analysis of biopsy tissue revealed that recipients receiving rADSCs/IR/FK506 therapy demonstrated no signs of rejection in either grafted skin or muscle biopsies at 2 and 6 wk posttransplantation. These data indicate the synergistic effect of rADSC injections and transient immunosuppressant administration increase the allotransplant survival.

Studies indicated that ADSCs may modulate immune responses through the induction of regulatory T cells.[18,22] Our IHC staining experiment revealed the proportion of $CD25^+$ and Foxp3+ regulatory T-cells' expressions in the alloskin tissues increased significantly in animals treated with rADSCs/IR/FK506, as compared to other groups. In contrast, the population of $CD4^+/CD25^+/Foxp3^+$ regulatory-like T cells in circulating blood at 6 wk posttransplantation significantly increased in animals treated with rADSCs/IR/FK506 compared to that in other groups. These data indicated that rADSCs combined with short-term immunosuppressant treatment, and compatible with donor ADSCs, have similar effects in modulating T-cell regulation.

Our study of soluble cytokines revealed expression of TNF-α, an inflammation-related cytokine, which significantly decreased at an early time point (2 and 6 wk posttransplantation). This indicated that rADSCs have an anti-inflammatory effect that affects allotransplant survival. Furthermore, we demonstrated that TGF-β, a T-cell regulation cytokine, had a statistically significant increase in expression in the rADSC/IR/FK506 group at 6 and 15 wk posttransplantation. These results indicate that rADSCs could indeed constitute a novel strategy to substantially prolong allotransplant survival and induce T-cell regulation. The rADSC-treated group showed a reduction in inflammatory cytokines compared to the control group. Additionally, our previous study demonstrated that donor ADSCs could modulate the regulatory T-cell population and inflammatory cytokine expression (increase TGF-β and suppressed TNF-α) in an in vitro study and a rodent in vivo model.[18] In this large animal study, we found that the rADSC-treated group displayed a reduction in inflammatory cytokines. Hence, ADSC has a correlated effect with the modulation of inflammatory cytokines.

To trace the engraftment of injected rADSCs, the BrdU-labeled rADSCs were intravenously injected into the recipient swine tissue. IHC staining of allotransplant tissue showed the population of BrdU-positive cells in the subcutaneous layers of alloskin was apparently increased at 2 and 6 wk posttransplantation. However, BrdU-positive cells

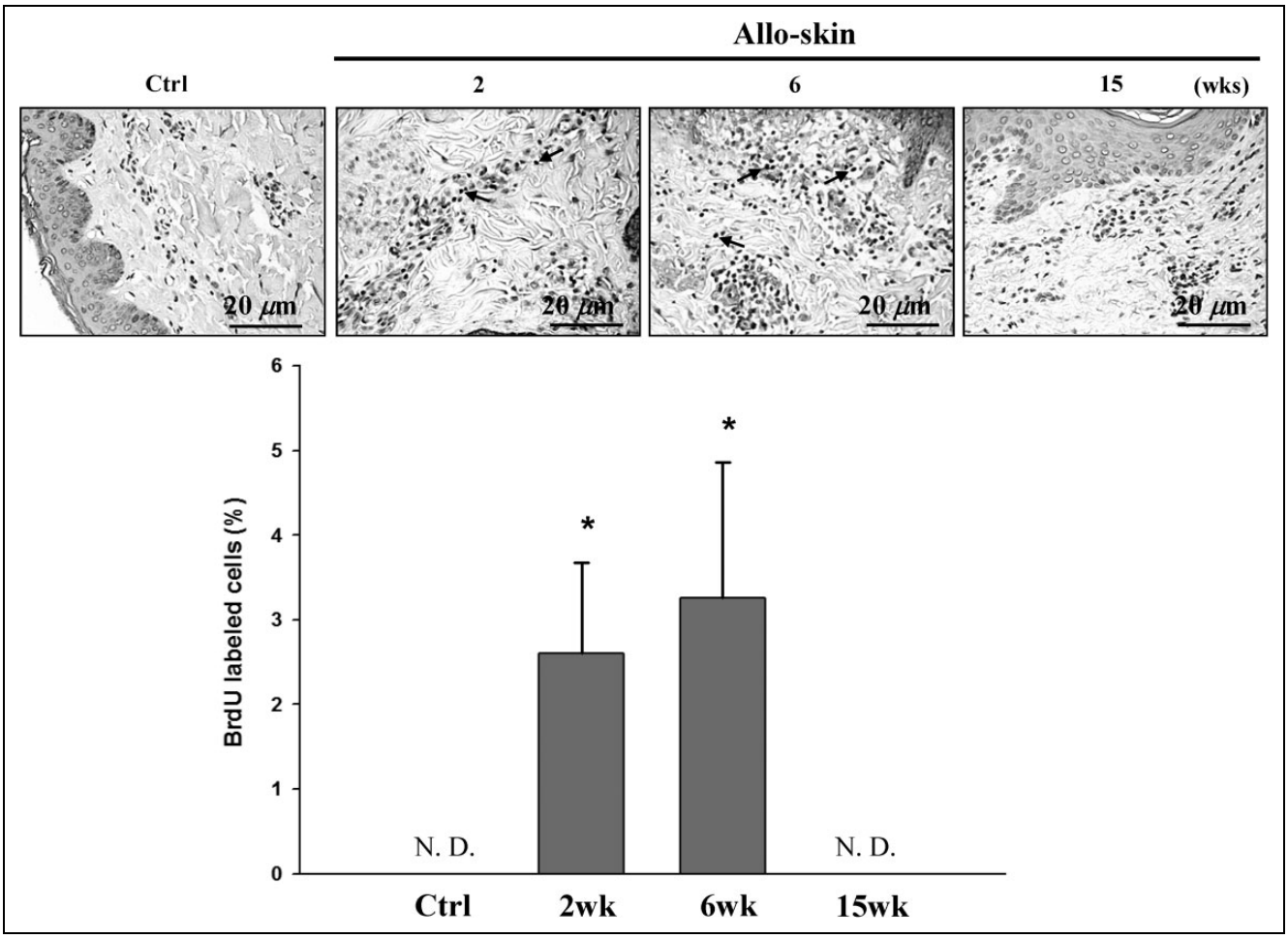

Fig. 5. Bromodeoxyuridine (BrdU)-labeled recipient adipose-derived stem cells (rADSCs) engrafted into the allotransplant tissue. Immunohistochemical (IHC) staining of allotransplant tissue showed the population of BrdU-positive rADSCs in the subcutaneous layers of alloskin was apparently increased at 2 and 6 wk posttransplantation (2.62% and 3.06%, respectively; $^*P < 0.05$). However, BrdU-positive ADSCs could not be detected in alloskin at 15 wk posttransplantation. Ctrl means negative control without BrdU-labeling normal skin tissue.

could not be detected in alloskin 15 wk posttransplantation. The similar result revealed in our formal study that the donor MSCs/ADSCs could be transient exist and correlate with modulation of allograft rejection. These results indicate the rADSCs could exist in the short term in allotransplant tissue and are involved in the modulation of T-cell regulation to prevent allograft rejection.

Although our previous studies have demonstrated the beneficial effect of donor MSCs/ADSCs as an immunomodulatory therapy,[13,15,18] no obvious migration of recipient cells into donor allograft tissue was detected to enhance allotissue regeneration. The present study detected whether the rADSCs combined with transient immunosuppressants could enhance the engraftment of recipient cells into the donor allotissue. Tissue from the hind-limbs of female miniature swine donors were transplanted into male recipient swine. Our results in the IHC staining of donor allotissue showed the recipient *SRY* protein was not only expressed at 6 wk posttransplantation but still constantly expressed at 15 wk posttransplantation. In contrast, PCR analysis revealed strong expressions of recipient male-specific *SRY* genes in the alloskin and muscle of female donors at 6 wk posttransplantation in the group with rADSC-IR-FK506 treatment. These results indicate that rADSCs could be effective in enhancing long-term engraftment of recipient cells into donor allograft and gradually replace the donor cells.

rADSCs provide a distinct advantage because they are easily accessible by liposuction and amenable to isolation, storage, and optimization to intensify their therapeutic benefits prior to transplantation. Additionally, VCA recipients could be pretreated with autologous ADSCs because they are not donor dependent. These factors as well as the strong effects that rADSC infusion exerted in this study suggest that this protocol could induce recipient cell regeneration, and toleration of allotransplanted tissues may have an important role in future clinical VCA application.

In summary, recipient ADSCs and IR in addition to short-term FK506 treatment could prolong allotransplant survival, modulate T-cell regulation, and enhance recipient cell engraftment into the allotransplant tissues. This immunomodulatory

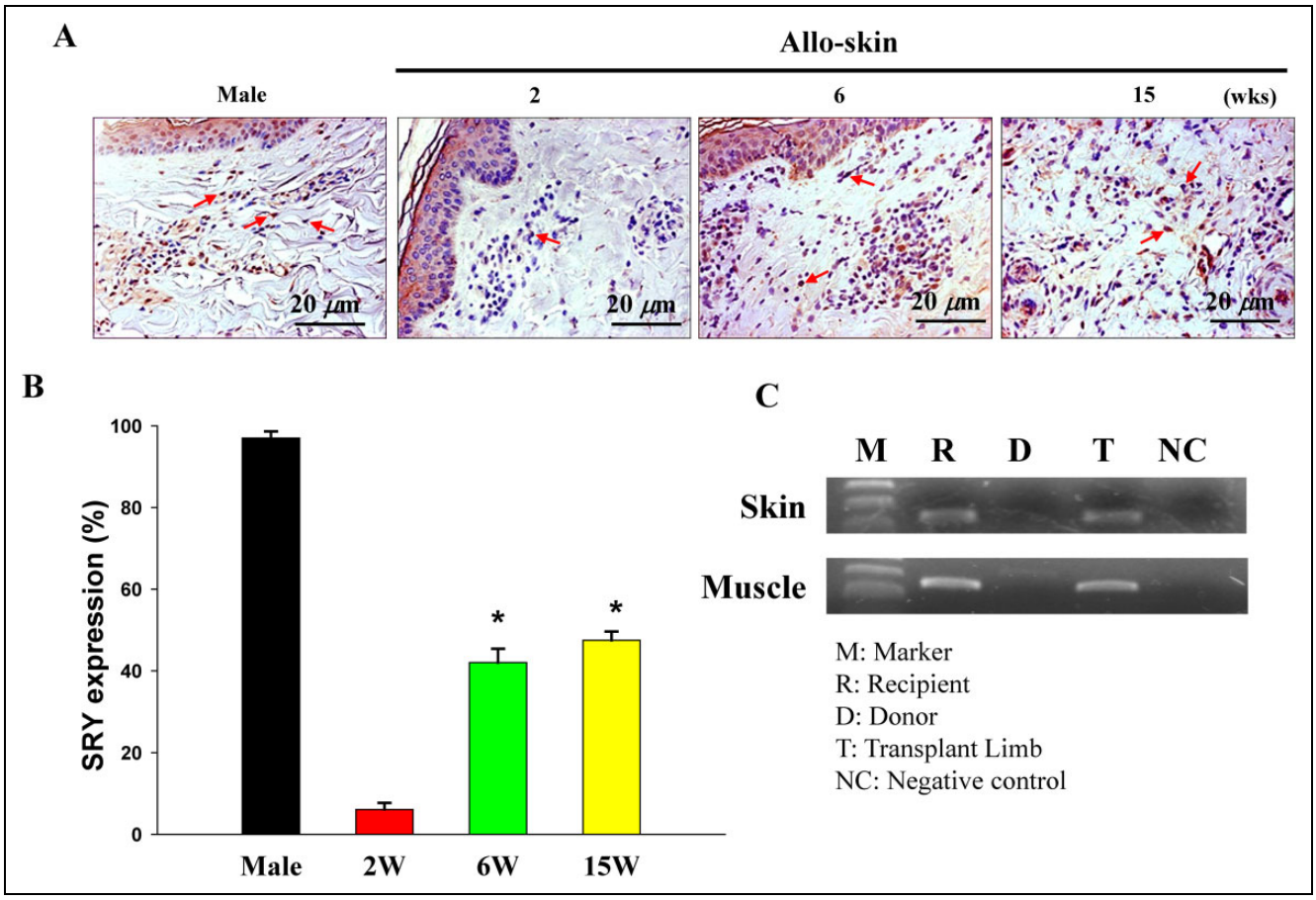

Fig. 6. Recipient adipose-derived stem cells (rADSCs) increased the recipient sex-determining region of Y chromosome (*SRY*)-positive cells in allotransplant tissue. Donor hind limbs from female swine were transplanted into male recipient swine (A and B) Immunohistochemical (IHC) staining for the recipient *SRY* protein expressions in alloskin revealed significant expression in donor alloskin at 6 and 15 wk posttransplantation (*$P < 0.05$). (C) Polymerase chain reaction (PCR) analysis revealed that the recipient *SRY* gene exhibited strong expressions in the donor alloskin and muscle at 6 wk posttransplantation in the group with rADSC/irradiation/FK506 (tacrolimus) treatment.

strategy might be more suitable for future clinical application and result in allotissue regeneration.

Author Contributions

Yur-Ren Kuo and Chien-Chang Chen contributed equally to this work.

Ethical Approval

The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Kaohsiung Chang Gung Memorial Hospital.

Statement of Human and Animal Rights

This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health, Bethesda, Maryland, USA.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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