EXPERIMENTAL STUDY

Cardiac Nestin+ Cells Derived from Early Stage of Dilated Cardiomyopathy Enhanced the Survival of the Doxorubicin-Injured Cardiac Muscle HL-1 Cells

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Summary

Dilated cardiomyopathy (DCM), as one of the common cardiomyopathies, is a disease of the heart muscle; however, the etiology and pathogenesis of DCM were still poorly understood. Nestin has been reported a special marker of stem/progenitor cells in various tissues, and the tissue resident Nestin+ cells could promote the wound healing and tissue remodeling. However, it remains unclear whether Nestin+ cells participate in the protection of cardiomyocytes during the pathogenesis of DCM. Here the model of mice DCM was induced by doxorubicin (DOX) intraperitoneal injection and observed heart failure and ventricular enlargement via echocardiography and histologic analysis, respectively. During DCM pathogenesis, the number of Nestin+ cells showed a significant peak on day 6 after DOX treatment, which then gradually decreases to lower than normal levels after day 30 in the total population of the heart. Furthermore, we found that the isolated increased heart-derived Nestin+ cells are mesenchymal property and could protect DOX-induced HL-1 cells toxicity in vitro by promoting their proliferation and inhibiting their apoptosis. Collectively, our results showed that Nestin+ cells increased during DCM pathogenesis and played an important role in protecting against the DOX-induced HL-1 cells loss via regulating proliferation and apoptosis. Thus, the loss of Nestin+ cells might be an etiology to DCM pathogenesis, and these cells could be a promising candidate cell source for study and treatment of DCM patients.

Key words: Etiology, Proliferation, Apoptosis

Dilated cardiomyopathy (DCM) is the most common cardiomyopathy worldwide and has many causes, including genetic and acquired elements.¹-³ DCM is characterized mainly by left ventricular (LV) systolic dysfunction, following with an associated increase in mass and volume.⁴,⁵ Treatments in clinic always focus on improvement of cardiac efficiency and reduction of mechanical stress that only contributes to relieve manifestations.⁶ Despite progress over the past 10 years, outcomes need to be improved.

The etiology of DCM underlying was unknown and complicated, although there are some possible triggers such as toxic, infectious, or metabolic agents and gene mutations associated with cardiac-related proteins.⁷ Increased evidence support that stem cell therapy may represent a new therapy option for DCM.⁸,¹² Especially, several studies have declared that heart resident stem cells are decreasing during the process of DCM.¹² Therefore, the loss of stem cells might contribute to the pathogenesis of DCM, yet the cellular type for cardiac protection needs to be further clarified.

Nestin, a class VI intermediate filament protein, is widely known as a marker of neural progenitors/stem cells residing in the central nervous system.¹⁵,¹⁶ Except for neural stem cells, Nestin also have been reported to be expressed in many other tissues, such as the bone marrow, kidney, testis, and hair follicle of the skin, which participate in cell renewal, proliferation, differentiation, and tissue regeneration as well as repair.¹⁵-¹⁷ Importantly, previous studies have reported that Nestin was up-regulated in the infarcted myocardium within 48 hours, and further indicated that Nestin+ cells participate in the wound healing...
after myocardial injury. However, whether Nestin+ cells participated in the pathogenesis of the DCM has not been reported yet.

In this study, we will investigate the Nestin expression following DCM period and confirm whether the isolated Nestin+ cells contribute to HL-1 cells protection from doxorubicin (DOX) injury in vitro. All together, these results support a critical role of Nestin+ cells as etiology and regulator for cardiac pathogenesis in DCM.

Methods

Animals and experimental protocols: Homozygous Nestin green fluorescent protein (Nestin-GFP) transgenic mice (4-week-old, 15 g) on C57BL/6 genetic background was provided by Yamaguchi and bred individually in cages at constant temperature (22 ± 1°C) and humidity (60%). To establish the mouse model of DCM, mice (6-week-old) were intraperitoneally injected with DOX (Sigma-Aldrich, St., Louis, MO, USA) at a dose of 2.5 mg/kg every other day for 2 weeks that cumulated a total dose of 15 mg/kg. From the beginning, mice were sacrificed under 4% (wt/vol) chloral hydras at days 0, 3, 6, 10, and 30, and all hearts were harvested and subjected to further analysis. All of the animal studies were carried out in accordance with the guidelines of the Sun Yat-Sen University Institutional Animal Care and Use Committee.

Histologic analysis: The heart tissues harvested at days 0 and 30 were fixed in paraformaldehyde (PFA) (n = 3 per group), then embedded with paraffin for sectioning at a thickness of 5 μm. Sections were conducted Masson’s trichrome staining to evaluate the size of the right ventricular (RV), LV, and interventricular septum (IVS). Images were obtained by a color image analyzer (BX-51/ Image-Pro plus, Olympus, Tokyo) and analyzed using the Image J software. All analyses were performed in a blinded manner.

Ventricular function evaluation: To assess the ventricular function, conventional echocardiography (at time of days 0 and 30 after DOX exposed, n = 3 per group) was performed with a mouse echocardiography system (Vevo2100 Imaging System, VisualSonics, Toronto, Canada), equipped with a 30 MHz phased transducer. Both two-dimensional and targeted M-mode tracings were obtained. In the short axis, LV end-diastolic volumes (LVEDV) were determined. LV fractional shortening (LVFS) and LV dimension were normalized with respect to those of GAPDH.

RNA extraction and quantitative polymerase chain reaction (Q-PCR): The total RNA of heart tissues at each time point were extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instruction. Total 1 μg of RNA was reverse transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The cDNA obtained was subjected to real-time polymerase chain reactions (Q-PCR) with the SYBR Green reagent (Roche, Indianapolis, USA) using the mouse primers Nestin, forward: 5’-CCCAAGAGAAGCTGGGAACT-3’, reverse: 5’-AGATCGCTCAGTCCGGA-3’; Collagen IV, forward: 5’-CCTCAAGGTATTGGCTGACAAC-3’, reverse: 5’-CAGAGGACCTTTGGTGCAGTAGG-3’; Collagen III, forward: 5’-GACCAAAAGGTTAGTGCTGGACAG-3’, reverse: 5’-CAAGACTCTGTGCCAGT-3’; Collagen IV, forward: 5’-ATGGCCTTGCCGAGAGATA-3’, reverse: 5’-TGGATTGGCCTTGGTGATCAG-3’; and GAPDH, forward: 5’-CATCCTGCGCCACAGAGACT-3’, reverse: 5’-TGGCAATGGTCGTCGTTAAGAG-3’. The relative mRNA abundance was calculated using the ΔΔCt methods, and gene expression levels were normalized with respect to those of GAPDH.

Isolation and culture of cardiac resident Nestin+ cells: The hearts of Nestin-GFP mice with 6 days’ DOX treatment and non-treated C57BL/6 mice (blank control) were harvested to homogenization and incubated with 5 mL HBSS digestive solution including Type II collagenase (300 U/mL; Gibco, USA) and DNase I (100 U/mL; Sigma-Aldrich, USA) at 37°C for 30 minutes with shaking every 10 minutes. Subsequently, the cell suspensions were passed through a 40 μm cell strainer to yield a single cell. GFP-positive cells were freshly sorted by flow cytometry (Influx, BD, USA) and cultured in Dulbecco’s Modified Eagle Media (DMEM/F12, Gibco, USA) medium with 2% fetal bovine serum, 20 ng/mL EGF (Peprotech, USA), 10 ng/mL bFGF (Peprotech, USA), 1% N2 (Invitrogen, USA), 2% B27 (Invitrogen, USA), and 100 IU/mL penicillin/streptomycin (Invitrogen, USA). The cells were cultured at 37°C under 5% CO2 and propagated every 3 days. Nestin+ cells were used for experiments when they were amplified to the third generation (passage 3).

Flow cytometry analysis: Flow cytometry analysis was performed on fluorescence-activated cell sorting (FACS) Caliber flow cytometer (BD, USA). The following anti-mouse antibodies were used: Anti-Mouse PDGFRα/CD140a (1:100, 14-1401-81, eBioscience); Sca-1/Ly-6A/E antibody (1:100, sc-52601, Santa Cruz); Human/Mouse CD117/C-kit antibody (1:100, AF1356, R&D systems), and anti-alpha-actinin (sarcomorphic) (α-SA) (1:500, A7811, Sigma-Aldrich). All images were obtained by LSM-800 confocal microscope (Zeiss). We randomly observed five fields in each whole heart tissue section or cell slices under the corresponding magnification in the figures and analyzed using the Image J software.
Figure 1. Echocardiography and histological evaluation of doxorubicin (DOX)-induced dilated cardiomyopathy (DCM) model in Nestin-GFP mice at days 0 and 30. A: M-mode imaging of the left ventricular (LV) displays dimensions of the ventricular walls, LV cavity, and cardiac function measurements. B: Echocardiographic parameters [LV fractional shortening (LVFS), LV ejection fraction (LVEF), LV end-diastolic volume (LVEDV)] were analyzed in anesthetized mice at days 0 and 30 during the course of the experiment. LVFS and LVEF at day 30 were significantly lower than that at day 0. C: For investigating the structure of the heart, heart sections were stained with Masson trichrome. D: Histological wall thickness parameters of LV, right ventricular (RV), and intraventricular septum (IVS). All data are presented as mean ± SEM. **P < 0.01; ns indicates not significant. Scale bar = 2 mm.

Isolation and culture of cardiac fibroblasts: The hearts of C57BL/6 mice (7-week-old) were digested as described before, and incubate the centrifugal cells into DMEM, with 4.5 g/L glucose, 10% fetal bovine serum, and 1% penicillin/streptomycin for 1 hour at 37°C. Next, remove the unattached cells, wash for two times carefully and culture with DMEM, with 1 g/L glucose, 10% fetal bovine serum, and 1% penicillin/streptomycin in the same condition with Nestin+ cells.

Co-culture of Nestin+ cells and DOX-induced HL-1 cells in vitro: The HL-1 cells (a cardiac muscle cell line) are derived from the AT-1 mouse atrial cardiomyocyte tumor lineage that could be used as cardiomyocyte. HL-1 cells were purchased from ATCC and maintained in standard cell culture conditions; then, the cells were seeded at 1 × 10⁵ cells/well in the lower chamber of 24-well flat-bottom plates and incubated for 24 hours, then following with DOX treatment (DOX group) in the wells at a concentration of 1 μM for 24 hours, whereas the control
Nestin expressions in the hearts of the Nestin-GFP transgenic mice were compensatory increased during the early stage of dilated cardiomyopathy (DCM), and then significantly decreased following DCM progression. A: Heart sections at days 0, 3, 6, 10, and 30 after doxorubicin (DOX) induction were analyzed by immunofluorescence staining. Nuclei were detected by 4', 6-diamidino-2-phenylindole (DAPI) staining; high magnification of the areas were outlined, and the arrows indicate Nestin-GFP cells. Scale bar = 200 μm (left column in A), scale bar = 100 μm (right column in A). B: The density of Nestin-GFP-positive cells at high magnification was calculated as the number of Nestin-GFP cells per mm². C: Cardiac Nestin mRNA levels were analyzed by quantitative real-time polymerase chain reaction at days 0, 3, 6, 10, and 30 after DOX induction and normalized with the expression of GAPDH, respectively.

Cardiomyocytes proliferation determination: At the beginning of co-culture described above, 5-ethynyl-29-deoxyuridine (EDU) was administered into the lower chamber along with Nestin+ cells at a concentration of 10 mM for 24 hours. Then, EDU incorporation in HL-1 cells was detected using Click-iT Alexa Fluor 594 Image Kit as per the manufacturer’s instruction (Invitrogen). All the results were analyzed by three independent experiments.

Cardiomyocytes apoptosis assay: After co-culture, cells were harvested and labeled with FITC-conjugated Annexin V antibody and propidium iodide (BD Biosciences) according to the manufacturer’s instruction. Apoptotic cells were assessed as Annexin V positive staining using FACS (Beckman Coulter, Fullerton, CA, USA); data were analyzed by Kaluza software (Beckman Coulter). All the results were analyzed by three independent experiments.

Statistical analysis: All results were performed for three independent experiments and were denoted as means ± SEM. All of the statistical comparisons were made using a two-tailed Student’s t-test (for two group comparisons) or a one-way ANOVA (for multigroup comparisons). P < 0.05 was considered to be statistically significant. Analysis and graph were performed using GraphPad Prism software 6.01 (San Diego, CA, USA).

Results
Echocardiography and histologic analysis after DCM:
The mice DCM induced by intraperitoneal injection by DOX and their heart function were examined by echocardiography and histologic analysis. Similar to the report of Zang,29) during the experiment, myocardium injury induced by DOX treatment were aggravated in mice, but there were no signs of over-toxicity or deaths that happened. M-mode image of the LV displays the ventricular walls, LV cavity, and cardiac function measurements (Figure 1A). At day 30 post-DOX implication, LVFS and LVEF were significantly reduced compared with day 0 (27.91% ± 0.6354% versus 36.66% ± 0.4538% $P < 0.01$; 57.53% ± 1.171% versus 67.92% ± 0.4869% $P < 0.05$, respectively), whereas LVEDV (28.89 ± 0.6834 μL versus 31.42 ± 1.560 μL $P > 0.05$) change did not reach statistical significance. Masson’s trichrome staining shows that the myocardial wall thickness of RV and IVS significantly reduced at day 30 compared with day 0 (0.7203 ± 0.09107 mm versus 0.2857 ± 0.01438 mm $P < 0.05$; 1.197 ± 0.02028 mm versus 0.6151 ± 0.01183 mm $P < 0.01$, respectively); however, the wall thickness of LV was not changed obviously (1.506 ± 0.2955 mm versus 0.7900 ± 0.1137 mm, $P > 0.05$). These data indicated that heart failure and ventricular enlargement occurred after DOX treatment.

**Nestin expression in animal models of DCM:** It has been reported that Nestin is expressed in adult hearts and up-regulated in the damaged hearts.30) To systemically assess the expression of Nestin during DCM process, we used Nestin-GFP transgenic mice; cells containing with GFP immunoreactivity were expressed Nestin; thus, we could analyze Nestin expression during DCM pathogenesis by detecting GFP under fluorescence microscope. The result of immunofluorescence staining showed that myocardial Nestin-GFP+ cells were markedly up-regulated early in the animal model of DCM (from day 0 to day 6) and then significantly decreased lately (from day 6 to day 30) (Figure 2A), and the statistics data showed that Nestin-GFP cells in a field size of mm² on day 6 (126.4 ± 6.635) increased to a large extent compared with day 0 (11.49 ± 6.634) and then reduced gradually (day 30 data show 3.83 ± 3.83) (Figure 2B). Furthermore, extensive evidence suggested that Nestin mRNA induction was observed using quantitative real-time PCR in heart tissues in the early stage and shown in similar expression phenomenon with histological analyses (Figure 2C). These data demonstrated that the increase of Nestin activity exists at the early time of DCM progression, but rapidly decreased flowing DCM development.

**Characterization of Nestin in heart tissues and isolated cells by immunofluorescence and flow cytometry:** To characterize the Nestin+ cell population in heart tissue, the expressions of several surface markers in the Nestin-GFP transgenic mice were analyzed by confocal microscopy. For the heart tissue sections after 6 days’ DOX-induced, most of the Nestin-GFP-positive cells co-stained with PDGFRα, a marker of mesenchymal cells, and only a few of them were Sca-1 positive, but never expressed C-kit (a cardiac stem cell marker) (Figure 4A). Furthermore, we
Figure 4. Characterization of Nestin in heart tissues and isolated cells analyzed by immunofluorescence and flow cytometry. A: Co-expression of Nestin-GFP-positive cells in the transgenic mice after 6 days’ doxorubicin (DOX) induced. PDGFRα (red) were highly co-expressing, but a few Sca-1 and not C-kit. Bar = 20 μm. B: Analysis of Nestin by flow cytometry. Histogram showed that the freshly isolated Nestin+ cells expressing high levels of PDGFRα, but rarely Sca-1 and not C-kit. The same phenomenon was found when Nestin+ cells were cultured to passage 4.

also confirmed the expression of these three markers on freshly isolated and expanded Nestin+ cells by flow cytometry (Figure 4B). Additionally, markers of cardiomyocytes (α-SA) and vascular cells (CD31) have been co-stained in the heart sections to understand the localization of the Nestin+ cells in the heart. The results showed that Nestin-GFP+ cells in heart sections rarely co-stained with α-SA, but were partly co-stained with CD31 (Supplementary Figure 1).

Nestin+ cells treatment increased the proliferation of DOX-induced HL-1 cells: To examine whether the Nestin+ cells participated in myocardial regeneration, the
cardiac Nestin+ cells were isolated from Nestin-GFP mice after 6 days’ DOX induction by FACS. The percentages of Nestin-GFP+ cells in the mouse heart of DCM are approximately 6% (Figure 3A). All sorted Nestin-GFP+ cells were seeded to a flask for adherent culture, after 7 days culturing, cells displayed fibroblast-like morphology (Figure 3B) and then dispersed when density reached 90%. Meanwhile, cardiac fibroblasts were isolated from the C57BL/6 mice at the same age as a control cell type. The shape of adherent cultured cardiac fibroblasts was shown under phase contrast microscope (Supplemental Figure 2A) and the mRNA levels of collagens I, III, and IV associated with cardiac fibroblast were highly evaluated by Q-PCR. (Supplemental Figure 2B).31)

To imitate the roles of Nestin+ cells on the cardiomyocytes during DCM, we performed co-culture experiment in vitro. To investigate the proliferation of DOX-induced HL-1 cells after co-culture with Nestin+ cells, HL-1 cells were analyzed by EDU expression (Figure 5 A). Importantly, statistical significance of proliferative ability in Nestin+ cells co-culture group was obtained when compared with the DOX-induced HL-1 cells (45.26% ± 4.82% versus 23.40% ± 1.52%, P < 0.05). Most importantly, Nestin+ cells co-culture group showed that the proliferation of DOX-induced HL-1 cells was indeed increased compared with that in the cardiac fibroblasts co-culture group (45.26% ± 4.82% versus 27.07% ± 4.09%, P < 0.05). And there was no significant difference between cardiac fibroblasts co-culture group and DOX-induced HL-1 cells (Figure 5B).

Nestin+ cells treatment decreased the apoptosis of DOX-induced HL-1 cells: To further investigate the role of Nestin+ cells on the apoptosis of cardiomyocyte, we co-cultured DOX-induced HL-1 cells with Nestin+ cells and cardiac fibroblasts in vitro. The apoptosis of HL-1 cells was evaluated by Annexin V/PI staining assay.32) Data are shown in Figure 6A. Interestingly, incubation of DOX-induced HL-1 cells with Nestin+ cells led to decreased apoptotic rate of approximately 14% after 24 hours’ co-culture compared with the fibroblast co-culture group (32.03% ± 0.69% versus 40.03% ± 1.05%, P < 0.01), and it also reduced compared with DOX-treated group (32.03% ± 0.69% versus 50.17% ± 4.54%, P < 0.05). However, Statistical differences do not exist in the fibroblast co-culture group and DOX-treated group (40.03% ± 1.05% versus 50.17% ± 4.54%, P > 0.05) (Figure 6B).

Discussion

In the present study, we demonstrate that in response to DCM process, the heart resident Nestin+ cells, characterized by co-expressing with PDGFRα but not Sca-1 and C-kit, significantly increased at the early stage, and gradual reduction was observed later, along with the heart dysfunction. We isolated Nestin+ cells from Nestin-GFP transgenic mice via FACS and co-cultured with DOX-induced
HL-1 cells that produced cardiomyocytes protection effects through promoting proliferation and inhibiting apoptosis. Thus, protection of cardiomyocytes by the increased cardiac resident Nestin+ cells during DCM provided significant evidence for the research of pathogenesis and therapeutic strategy to DCM.

DCM is the most common cardiomyopathy worldwide; however, the effective therapy still remains debated because of the unclear causes and pathogenesis. Because the mechanism underlying the progression needs to be elucidated, various models of DCM have been critically implemented, which can be characterized by virus infectious agent (Coxsackie virus B3), autoimmune material (myosin, troponin I, and DOX), genetical modification (Sod2 knockout), and pacing induction. As for mice, DOX-induced DCM model are widely used for research because of their advantages such as convenient utilizations, high efficiency, and rare over-toxicity or deaths. Consistent with prior studies, our model showed the same DCM characteristics described by thickness of ventricular walls, enlargement cavity, and deterioration of heart function.

It is convinced that patients with DCM have continuous heart tissue degeneration and irreversible cardiomyocytes apoptosis that result to ventricle abnormalities. Previous studies have showed that stem cell-deficient mice develop age-dependent DCM, and the transplantation of autologous and allogenic stem cells, such as skeletal myoblast and bone marrow stem cells, is effective against progressive DCM, and some reported that cardiac resident stem cells favor better than others in therapy. Several types of stem cells have been reported up-regulated in heart tissue after myocardial infarction that play an important role to histologically and functionally reverse ventricular remodeling. Thus, other types of the resident cardiac cells might be involved in the development and progression of the DCM.

Nestin, first identified in multipotent neural stem cell, is also expressed in several mature tissues like the pancreas, liver, and gastrointestinal tract and markedly up-
Nestin+ cells are a crucial cell source for DCM development and protective potent of Nestin + cells to DOX-induced HL-1 cardiomyocytes was markedly increased in vitro. Thus, this study suggested that cardiac Nestin+ cells would protect cardiomyocytes from death and decrease cardiac remodeling in DCM. The underlying mechanism against myocytes loss might be associated with inhibiting cardiomyocyte depletion. This increasing Nestin+ cells rarely express several known cardiac stem cell markers such as Sca-1 and C-kit but highly express PDGFRα and partly express CD31, suggesting that they might be mesenchymal and endothelial cell derived. Unfortunately, the expression of Nestin was still reduced significantly with the development of the DCM.

The progressive deterioration of myocardial function in DCM is reportedly due to an ongoing myocytes dropout or apoptosis. Nestin+ cells could reduce apoptosis to ameliorate kidney tissue damage. Therefore, we hypothesized that cardiac Nestin+ cells would protect cardiomyocytes from death and decrease cardiac remodeling in the process of DCM. To test the hypothesis, we firstly isolated cardiac Nestin+ cells from Nestin-GFP transgenic mice via FACS and cultured in vitro, and then co-culture system was used to investigate the role of Nestin+ cells on the cardiomyocytes. We found that the proliferation of DOX-induced cardiomyocytes was markedly increased and the apoptosis was obviously reduced after co-culture with Nestin+ cells. This could indicate that Nestin+ cells control proliferation and apoptosis of cardiomyocyte in DCM. The underlying mechanism against myocytes loss might be related to paracrine factors or cell differentiation. Therefore, additional experiments are required to further investigate the relationship between the Nestin+ cells and DCM.

In summary, we have showed the early up-regulation and late reduction of cardiac Nestin+ cells during the pathogenesis of DCM in vivo and further identified the protective potent of Nestin+ cells to DOX-induced HL-1 cells in vitro. Thus, this study suggested that cardiac Nestin+ cells are a crucial cell source for DCM development, which may assist in future therapeutic strategies to treat DCM.

**Disclosures**

**Conflicts of interest:** None.

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