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In vitro promotion of SDF-1α on the migration and proliferation of C-kit$^+$ cardiac stem cells in neonatal mice

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Abstract: The aim of this study was to explore the *In Vitro* effects of stromal-derived factor-1α (SDF-1α) on the migration and proliferation of c-kit$^+$ cardiac stem cells. The lentivirus containing SDF-1α (LV-SDF-1α) was constructed. Primary myocardial fibroblasts were transfected by LV-SDF-1α, followed by primary culture of cardiac tissue cells and separation of c-kit$^+$ cardiac stem cells with a flow cytometer, in order to investigate the effects of SDF-1α on the migration and proliferation of c-kit$^+$ cardiac stem cells using cell co-culture, immunofluorescence and EdU tracing technologies. The results showed that myocardial fibroblasts could secrete SDF-1α after the transfection with LV-SDF-1α. High-purity c-kit$^+$ cardiac stem cells were obtained through flow cytometry sorting and the positive rate was about 40%. The c-kit$^+$ cardiac stem cells cultured *In Vitro* could be differentiated into cTnT positive cardiomyocyte-like cells. After co-culture of myocardial fibroblasts and c-kit$^+$ cardiac stem cells transfected with lentivirus, SDF-1α might increase the migration of c-kit$^+$ cardiac stem cells, but SDF-1α did not promote the proliferation of c-kit$^+$ cardiac stem cells. In conclusion, the myocardial fibroblasts transfected with lentivirus can highly express SDF-1α, c-kit$^+$ cardiac stem cells can be differentiated into cTnT positive cardiomyocyte-like cells and SDF-1α can effectively enhance the migration of c-kit$^+$ cardiac stem cells but fails to stimulate the proliferation.

Key words: SDF-1α, cardiac stem cells, migration, proliferation

INTRODUCTION

Cardiovascular diseases are the most common causes of death in the world. About 17,300,000 people die of cardiovascular diseases throughout the year, accounting for 32% of total death, with nearly 40% dying from acute myocardial infarction (Benjamin *et al*. 2017). The treatment measures for myocardial infarction include drug therapy, percutaneous coronary intervention and coronary artery bypass grafting. Although the scope of myocardial infarction has been reduced to a certain extent, it is helpless to the necrotic myocardial cells. After myocardial infarction, ischemic necrotic cells are not regenerated in a timely and efficient way, but they are replaced by fibrous scar tissues, leading to ventricular remodeling and further heart failure (Peet *et al*. 2020). Meanwhile, after myocardial injury and necrosis, parenchymal cells cannot be produced quickly and efficiently and fibroblasts secrete the stroma to form fibrous scars, thus limiting the regeneration of damaged myocardial tissues (Cai *et al*. 2015).

Heart is always considered as the terminally differentiated organ. After birth, myocardial cells stop karyomitosis without proliferation ability any longer. In recent years, it has been found that myocardial cells are found to mitosis in the cardiac muscles of mice, rats and human beings. The discovery of *in situ* cardiac stem cells (CSCs) demonstrated that myocardium has some regenerative capacity (Bhartiya *et al*. 2021; Hou *et al*. 2016; Tariq *et al*. 2022). According to research findings, adult cardiac stem cells are mainly exist in stem cell niches such as cardiac apex and atrium (Mannino *et al*. 2022). Afterwards, cardiac stem cells similar to c-kit$^+$ are discovered in adults' cardiac muscles. Human cardiac stem cells are injected into the infracted cardiac muscles of rodents to form cardiac chimeras containing human myocardial cells and coronary vessels and thus both heart structure and function are improved (Bearzi *et al*. 2007). There are a variety of stem cells in the local myocardial microenvironment that can differentiate into cardiomyocytes, such as Sca-1$^+$ cardiac stem cells, isl1$^+$ cardiac stem cells and c-kit$^+$ cardiac stem cells and can also differentiate into endothelial cells and smooth muscle cells (Beltrami *et al*. 2001). Exogenous stem cell transplantation has a certain effect on myocardial regeneration, *e.g*. induced pluripotent stem cells have aroused extensive attention by virtue of convenient source without rejection. When clinically applied, they can be differentiated into functional myocardial cells (Yoshida and Yamanaka 2017). Mesenchymal stem cells, multipotent stem cells, embryonic stem cells and adipose-derived stem cells from bone marrows have important effects in the restoration of myocardial infarction, which can increase the blood vessel density in the infracted area to different degrees, reduce the area of myocardial infarction and improve myocardial functions (Argentati *et al*. 2018; Duelen and Sampaolesi 2017; Menasche *et al*. 2015). However, endogenous or exogenous stem cells, if intervening, can improve cardiac functions and remodel myocardial structures, but their effect is mainly

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manifested in repairing fibrous connective tissues. Moreover, cardiac stem cells can migrate into the site of myocardial injury and differentiate into functional cells, but the recovery rate is much slower than the rate of injury (Cai *et al*. 2015).

At present, a certain research progress has been achieved in paracrine promoting non-cardiac stem cells to repair myocardial infarction. Stromal-derived factor-1α (SDF-1α), a member of CXC family, is a key cellular chemokine in living organisms. As a receptor of SDF-1α, CXCR4 is expressed on the surface of multiple stem/progenitor cells and can couple with SDF-1α to mediate their migration. Research findings show that myocardial ischemia and vascular intimal injury can up-regulate the expression of SDF-1α, mediate the homing of multiple stem cells in new vessels and restore the damaged endothelium (Jiang *et al*. 2022). In our previous study, we found that the expression of SDF-1α was elevated in infarction region and the number of c-kit$^+$, Nonag$^+$ and CD34$^+$ cells was increased in the infarction region, accompanied by the reduction of infarction area (Cai *et al*. 2019). Cardiac stem cells can be divided into several subpopulations, among which c-kit$^+$ cardiac stem cells account for a large proportion with strong differentiation and proliferation abilities. How to rapidly drive the proliferation and migration of cardiogenic and noncardiogenic stem cells has become a focus of attention. In the present study, the c-kit$^+$ cardiac stem cells of newborn mice were sorted through a flow cytometer and the myocardial fibroblasts of newborn mice transfected with LV-SDF-1α were co-cultured to simulate the *In Vivo* paracrine-mediated migration, further to explore the effects of SDF-1α on the migration and proliferation of c-kit$^+$ cardiac stem cells.

MATERIALS AND METHODS

Viral plasmid construction

SDF-1α gene sequence was retrieved from GenBank, the gene name is SDF-1α (NM-001033883), the species is mouse. The CDS region of this gene was 360bp in length. Taking GV287 as the vector, its component sequence was Ubi-MCS-3FLAG-SV40-EGFP and the cloning site was AgeI/AgeI. Primers were designed to acquire the target gene segments (SDF-1α): P1: GAGGATCCCCGGGTA CCGGTCGCCACCATGGACGCCAAGGTCGTC, P2: TCCTTGTAGTCCATACCGTTTTTCTTTTTCTGGGC. Recombinant SDF-1α lentiviral vector (LV-SDF-1α), negative control virus CON145 and enhanced infection solution (Eni.S) were synthesized and provided by Jikai Gene Co., Shanghai, China.

Primary culture of myocardial fibroblasts

The newborn mice at the age of 1-2 d were provided from the Experimental Animal Center of Xinxiang Medical University and the animals were approved from the Ethics Committee of Xinxiang Medical University. Iscove's Modified Dulbecco Medium (IMDM), Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone, Logan, Utah, USA. After the digestion of cardiac muscle tissue blocks with 0.125% trypsin (Solarbio, Beijing, China), the cells were collected, filtered using 200-mesh filter screen, transferred into a 25 cm^2 culture flask and cultured in an incubator at 37°C with 5% CO_2 for 60 min. Then, isolated culture of myocardial fibroblasts was performed using the differential attachment method and the solution was replaced every 2d. Next, the vimentin (Cat no. ab45939; Abcam, Cambridge, UK) of myocardial fibroblasts was labeled through the immunofluorescence method for identification.

Lentiviral transfection experiment on myocardial fibroblasts

One day before the experiment, myocardial fibroblasts (5×10^4/mL) were inoculated into 12 wells of a 96-well culture plate with the volume of 90μL. LV-SDF-1α was diluted to three titers using Eni.S: 1×10^8 TU/mL (Group A), 1×10^7 TU/mL (Group B) and 1×10^6 TU/mL (Group C). Next, 10μL of solution was taken from each group and then blended for continuous culture. 8-12 h later, the cell state was observed and the culture medium was replaced. After transfection for 3-4 d, the fluorescent expressions were observed, the optimal infection conditions of myocardial fibroblasts were determined.

Detection of SDF-1α expression through Dot-blot method

In the group with the best transfection effect of LV-SDF-1α on myocardial fibroblasts, the supernatant of cell culture medium cultured for 1, 2, 3, 4, 6 and 8 d was respectively collected and dropped onto the methanol-pretreated PVD transfer film in groups for 3 times (8μL each time). After natural drying, 5% skimmed milk powders were added, followed by blocking and then shaking for 2 h; anti-rabbit SDF-1α antibody (1: 200; Cat no. ab6721; Abcam, Cambridge, UK) was added for incubation at a constant temperature of 37℃ for 1 h; the solution was rinsed using phosphate buffered saline (PBS) for 3 times and horse radish peroxidase-labeled goat anti-rabbit IgG (H+L) (1: 10000; Cat no. ab6721; Abcam, Cambridge, UK) was added for incubation under 37°C for 1 h, followed by developing in a dark room through BeyoECL Plus (Cat no. P0018M; hypersensitive ECL chemiluminescence kit; Beyotime, Shanghai, China). Next, the developing results were scanned by a scanner. Afterwards, the optical density (OD) of developing films was analyzed *via* Image J software (Bethesda, MD, USA) and the mean OD values of different groups were quantitatively compared to determine the optimal co-culture time.

Culture of primary cardiac stem cells through tissue block culture method

Myocardial tissues were cut into 1mm^3 tissue blocks, rinsed using sterile PBS for 3 times and transferred into a

15mL sterile centrifugal tube. Next, 1mL of 0.125% trypsin (Solarbio, Beijing, China) was added, the mixture was then gently blown and blended and digested at 37℃ for 1 min. Afterwards, an equal amount of IMDM culture medium containing 10% FBS was added for neutralization and blowing, the mixture was centrifuged at 1,200rpm for 5min and a small amount of medium was added to resuspend. Then, the tissue blocks were inoculated into a 25 cm^2 culture flask and evenly spread to make them as few as possible. The culture flask was obliquely placed in the 5% CO_2 incubator at 37°C for 30min and a small amount of culture medium was added until rightly immersing the tissue blocks. After culturing for 24h, the culture medium was added until reaching a full dose and the solution was replaced every 3d.

Sorting of c-kit$^+$ cardiac stem cells through flow cytometry

The isolation and culture of cardiac stem cells were carried out according to the improved method (Zhu *et al*. 2014). 500uL of PBS was resuspended in a 1.5 mL EP tube (cell suspension with the concentration of 1×10^5 cells/mL). Each tube was added with 5μL of c-kit PE-cy7 monoclonal antibody (1: 200; Cat no. 558163; BD Biosciences, New Jersey, USA) for incubation. Another tube of cells was taken and added with an equal amount of PBS solution as the negative control, incubate at 37°C for 40 min in the dark, centrifugation, resuspension and filtering with a 200-mesh filter screen. The mixture was then placed in a flow cytometry sampling tube and sorted by a flow cytometer. The obtained cells were inoculated in a 12-well plate and cultured through the conventional method and the morphological changes of the cultured cells were observed under a microscope (Olympus, Tokyo, Japan).

Immunofluorescence assay of c-kit$^+$ cardiac stem cell differentiation

The sorted cells were cultured for 20 d, rinsed gently with PBS for 3 times (5 min each time); fixed with paraformaldehyde for 30 min; rinsed gently with PBS for 3 times (5 min each time); hyalinized with 0.3% TritonX-100 for 15 min; rinsed as above mentioned; blocked with goat serum at 37°C for 30 min; rabbit anti-Nkx2.5 (1: 200; Abcam, Cambridge, UK) and mouse anti-cTnT (1: 200; Cat no. sc8697; Santa-Cruz, CA, USA) were added and incubated overnight at 4°C; on the next day, it was rewarmed for 30 min and conventionally rinsed and goat anti-rat cy3 and goat anti-rabbit FITC second antibodies (1:10000; Cat no. A1521, 65-6111; Invitrogen, Carlsbad, CA, USA) were added, followed by incubation away from light at 37°C for 1h and conventional rinsing; DAPI nucleus staining for 10 min, conventional rinsing and blocking with anti-quenching mounting medium. Images were observed and collected under FV-1000 confocal scanning microscope (Olympus, Tokyo, Japan). Then, the antibodies were replaced by PBS and a negative control was set in the experiment.

Detection of c-kit$^+$ cardiac stem cell migration

Virus-transfected fibroblasts and c-kit$^+$ cardiac stem cells were co-cultured *via* Transwell and then the migration of c-kit$^+$ cardiac stem cells was detected. The experiment was divided into CON145 transfection group, LV-SDF-1α transfection group and LV-SDF-1α transaction + AMD3100 blocking group (Cat no. S3013; Selleck, Houston, TX, USA). The myocardial fibroblasts transfected according to the optimal transfection conditions were inoculated in a 24-well plate and cultured in an incubator at 37°C for 4 d. Next, a Transwell (pore size: 8.0μm) chamber was set up on the 24-well plate and the c-kit$^+$ cardiac stem cells purified by flow cytometry were inoculated in the upper chamber with a cell count of 2×10^4/mL. Before sampling in AMD3100 group, the c-kit$^+$ cardiac stem cells inoculated into the upper chamber were incubated with AMB3100 for 1 h until reaching AMD3100 concentration of 10μg/mL, followed by incubation in the incubator at 37°C for 1h. After co-culturing for 24 h, the Transwell chamber was taken out and the culture medium in the lower chamber was collected and cryopreserved at -70°C to detect the concentration of SDF-1α in the supernatant. Next, carefully wipe off the cells on the upper side of the chamber with a cotton swab, rinse gently with PBS, fix with 75% ethanol for 10s and rinse again. Then, the Transwell chamber was erected on the discarded 24-well plate and 10μg/mL DAPI dyeing liquor was added for incubation at 37°C for 15 min. Afterwards, the cell count in each group was detected under a fluorescence microscope, a total of 5 high-power fields were randomly selected and the average value of the number of migrated cells in each group was taken.

Detection of c-kit$^+$ cardiac stem cell proliferation through EdU tracing

The experiments were divided into CON145 empty vector group and LV-SDF-1α transfection group. The transfection was performed through the steps ditto under the optimal conditions. The cells were cultured in an incubator at 37°C for 4d, the Transwell (pore size: 0.4μm) chamber was erected on a 24-well plate and the c-kit$^+$ cardiac stem cells purified through a flow cytometer were inoculated in the upper chamber with a cell count of $2x10^4$/mL. Next, the cells were labeled by EdU (Cat no. C10084; Invitrogen, Carlsbad, CA, USA) and cultured for 10d. Then, take out the Transwell chamber, wipe off the cells in the inner wall of the chamber with a cotton swab, rinse gently with PBS, fix with 75% ethanol for 10s and rinse with PBS again; the Transwell chamber was erected on the discarded 24-well plate, fixed using 4% paraformaldehyde for 15 min, rinsed with PBS twice, hyalinized using 5% Triton and rinsed with PBS twice. Afterwards, staining reaction liquid was prepared following the EdU kit protocol and 500μL of such liquid was added into each well, followed by incubation in a

decolorization shaking table away from light at room temperature for 30 min, rinsing with PBS for 3 times, preservation at 4°C. Images were observed and collected under FV-1000 confocal scanning microscope (Olympus, Tokyo, Japan).

Detection of SDF-1α content in culture medium through ELISA

The collected culture medium was centrifuged at a low temperature and a rate of 3,000 rpm for 30 min to remove the cell debris. This operation was performed according to ELISA kit protocol (Jianglai Biotechnology, Shanghai, China) of rat SDF-1α (SDF-1α/CXCL12). Then, the culture medium stood at room temperature for 30 min, working cleaning solution was prepared and 100μL of standard solution was successively added into 7 wells of the ELISA plate and standard curves were drawn with 100μL of cleaning solution as the blank control. The collected culture supernatant was added into empty wells (100μL in each well) and 50μL of enzyme-labeled solution was respectively added into the standard substance well and sample well, followed by incubation and reaction in a water batch at 37°C for 60 min. Next, the liquid in each well was absorbed and each well was filled with cleaning solution and spun-dry after 10 s, which was repeated for 5 times. 50μL of color developing agents A and B was respectively added into each well; incubation in a water bath away from light at 37°C for 15 min; 50μL of stopping solution was added into each well. Zero setting was performed for the ELISA instrument (wavelength: 450 nm) and then the OD value of each well was determined. Data were processed as follows: With the linear fitting function used, the logarithm was taken from the concentration of standard substance as X and the logarithm was taken, as Y, from the product obtained by deducting the corresponding OD value with the OD value in the blank control well, followed by linear fitting. Then, the concentration of each sample to be detected is calculated on the fitted line.

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS17.0 software (SPSS Inc, Chicago, IL, USA). Experimental data were expressed as mean±SD, one-way analysis of variance (ANOVA) was adopted for intergroup comparisons and LSD analysis for intragroup comparisons. $P<0.05$ meant statistically significant differences.

RESULTS

Primary culture of myocardial fibroblasts

Myocardial fibroblasts were acquired by combining enzyme digestion and differential adhesion method. After inoculated culture, it was observed under an inverted phase contrast microscope that the myocardial fibroblasts grew fast, presented a fusion state at 2-3d and were arranged tightly while some were overlapped. In addition, most myocardial fibroblasts were triangular or irregularly rounded with large cell bodies and transparent cytoplasm. The cell nuclei were large and oval and generally containing 1-2 nuclei. Vimentin labeled by the immunofluorescence was positively expressed (fig. 1).

The multiplicity of infection (MOI) detection of virus transfected fibroblasts

A small number of green fluorescent proteins (GFPs) were expressed on the first day of transfection. Cells were cultured until 4 days after virus transfection, the myocardial fibroblasts were observed under an inverted fluorescence microscope. GFPs were strongly expressed in both groups A and B (fig. 2A, B) while not expressed in group C (fig. 2C) and the cells in each group grew well under the white light channel (fig. 2A1, B1, C1).

Detection of SDF-1α expression in cell supernatant through Dot-blot method

It could be seen that SDF-1α was positively expressed in all groups (fig. 3A). Through Image J software analysis, the average OD value in each group (fig. 3B) continued to increase at 1-4 d, then gradually declined and intergroup comparative differences were statistically significant ($P<0.05$).

Culture of primary cardiac tissue cells through tissue block method

After the adhesion of tissue blocks for 24 h, long fusiform or irregular polygonal cells migrated from the edges of tissue blocks. At 48 h, the number of cells increased significantly and a small number of round and bright small cells migrated from the edges of tissue blocks. Above the long fusiform or irregular polygonal cell layer, these cells were small and spheroidal with a high refractive index. At about 5 d, a large number of small, circular and bright cells migrated from the edges of tissue blocks above the long fusiform or irregular polygonal cell layer. At about 20 d, these small, circular and bright cells overgrew above the long fusiform or irregular polygonal cell layer in the whole culture flask (fig. 4).

Sorting of c-kit$^+$ cardiac stem cells

The rate of c-kit$^+$ cardiac stem cells sorted by a flow cytometer reached about 40% (fig. 5A, B). Then, the sorted c-kit$^+$ cardiac stem cells were inoculated in a culture flask, they adhered well the next day and all the adhered cells presented c-kit positive expression as observed under a confocal scanning fluorescence microscope (fig. 5C, D).

Detection of c-kit$^+$ cardiac stem cell differentiation

The c-kit$^+$ cardiac stem cells sorted by flow cytometry were cultured for 20d and then the cells gradually grew into long fusiform or irregular oval shape. As detected by the double-labeling immunofluorescence, cells were negative for Nkx2.5 and positive for cTnT (fig. 6).

In Vitro migration assay on c-kit$^+$ cardiac stem cells

According to the pre-test results, primary myocardial fibroblasts were chosen and cultured for 4d, followed by virus transfection at MOI value of 10. Afterwards, it could be observed under a microscope that the cells grew well and showed strong positive expression of GFPs (fig. 7). Cells in all groups migrated to the back face of microporous membrane in the upper chamber of Transwell culture dish (fig. 8). Compared with the LV-SDF-1α group, the difference was statistically significant (P<0.05).

Detection of SDF-1α content in culture medium

The concentration of SDF-1α in the supernatant was detected via ELISA. The expression of SDF-1α in both LV-SDF-1α and AMD3100 groups was higher than that in CON145 group, the difference was statistically significant (P<0.05) (fig. 9).

Detection of c-kit$^+$ cardiac stem cells through EdU tracing

After EdU tracing, no fluorescent expression of Alexa-514 was observed and no proliferation of c-kit$^+$ cardiac stem cells was detected (fig. 10).

DISCUSSION

At present, about over a half of patients with cardiovascular diseases worldwide die of myocardial infarction. After myocardial infarction, a large number of necrotic myocardial cells fail to be regenerated and are replaced by scar tissues, thus causing left ventricular reconstruction and chamber expansion, finally leading to heart failure. In recent years, the research and discovery of cardiac stem cells have opened up a new way for the treatment of myocardial infarction. Following the myocardial damage and necrosis, many myocardial cells cannot be generated quickly and efficiently and fibroblasts are proliferated fast and secrete the matrix to form fibrous scars, which will restrict the regeneration of damaged myocardial tissues and impede the migration of cardiac stem cells towards the damage-induced necrosis region.

In 1994, Soonpaa *et al*. found that transplantation of mouse embryonic stem cells to the site of heart infarction in adult mice could inhibit scar repair and prevent the occurrence of heart failure after myocardial infarction (Soonpaa *et al*. 1994). The study found that patients dying of myocardial infarction, the mitosis rate of myocardial cells at the edge of myocardial infarction focus was 0.08% and the mitotic rate of cardiomyocytes away from the edge of myocardial infarction was 0.03%, indicating that a small number of regenerable myocardial cells are still contained in adult hearts, the heart is not the traditional thought of terminal differentiation organ and adult hearts still have regenerative potential (Beltrami *et al*. 2001). As proved by this research group, in the isoprenaline-induced myocardial infarction model, c-kit$^+$, Nanog$^+$ and CD34$^+$ cells existed in the infarction region and immature myocardial cells were generated in this area, hinting that adult rats are of self-repairing capability. Unfortunately, it is not possible to directly track the differentiation of c-kit$^+$ cells into immature myocardial cells (Cai *et al*. 2015).

Cardiac stem cells are divided into several subpopulations according to membrane surface associated antigens, such as c-kit, MDR1, lateral cell population, Sca-1, Flk-1 and islet-1 (Ling *et al*. 2018; Yellamilli *et al*. 2020; Phillips *et al*. 2022). The differentiation ability and myocardial repair ability of different subpopulations of cells have not been determined. *In Vitro* experiments show that although these stem cells can be differentiated into myocardial cells, the growth potential of c-kit$^+$cardiac stem cells is greater than that of Sca-1$^+$ cells or MDR1 cells. Preliminary clinical test results indicate that after myocardial infarction, the left ventricular systolic function can be effectively improved and the area of myocardial infarction can be reduced by coronary artery injection of autologous c-kit$^+$cardiac stem cells, manifesting the broad prospects of c-kit$^+$ stem cells in treating myocardial infarction (Bolli*et al*. 2011). Despite *in situ* cardiac stem cells in heart, their quantity is not enough to repair the damage caused by ischemia, anoxia and poisoning (Ebrahimi 2018; Hensley *et al*. 2017; Li *et al*. 2017; Sayed *et al*. 2022), the *In Vitro* proliferation, differentiation and migration abilities of c-kit$^+$cardiac stem cells are crucial for stem cells to repair the myocardium.

In recent years, cardiac stem cells have been acquired by combining cardiac muscle tissue block method with immunomagnetic beads or digestive enzyme passage method. In this study, small, circular and bright cells with a high refraction were observed to climb out from the edge of tissue blocks at 4d and then filled the bottom of culture flask at about 20d, which is consistent with the related literature reports. By using flow cytometry, the sorting rate of c-kit$^+$ cardiac stem cells in these cells could reach about 40%, which was much higher than 8% reported in other literatures and the reason needs to be further explored. As these cells were inoculated and continuously cultured, the adherent growth was good and the cells showed c-kit positive expressions. After continuous culturing, the cells turned into long fusiform or irregular oval shape and the myocardial specific marker cTnT was positive, indicating that these cells had been transformed into cardiomyocyte-like cells. The marker Nkx2.5 of cardiac precursor cell differentiation was not expressed, which may be related to the detection time or the non-expression of Nkx2.5 during the differentiation of the cells. The specific mechanism needed to be further explored.

SDF-1α is a chemokine secreted by stromal cells of tissues, a member of CXC chemokine subfamily and its only receptor is CXCR4.

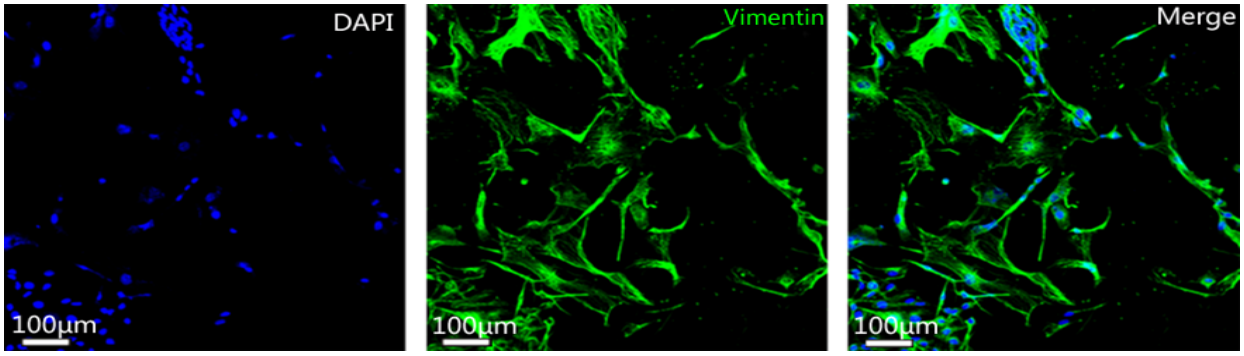
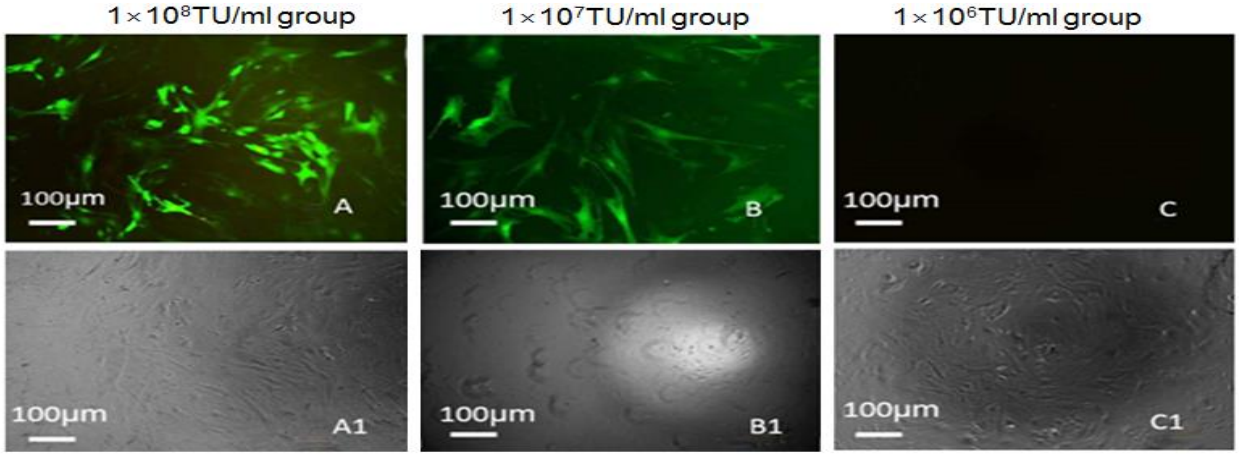
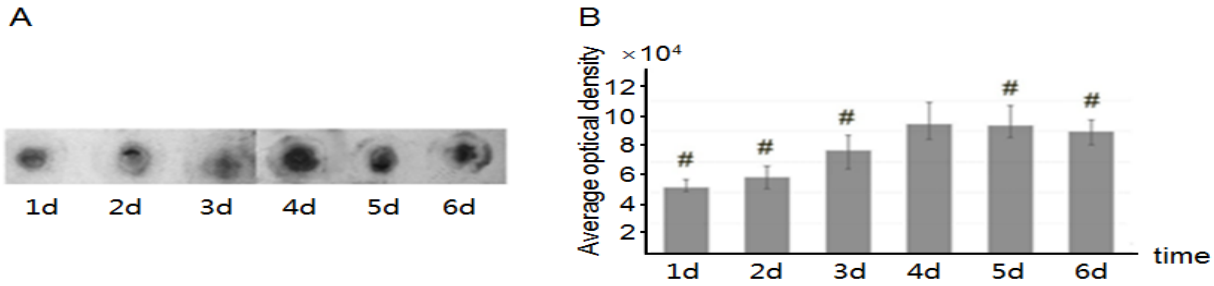


Fig. 1: The identification of the isolated fibroblasts with vimentin.



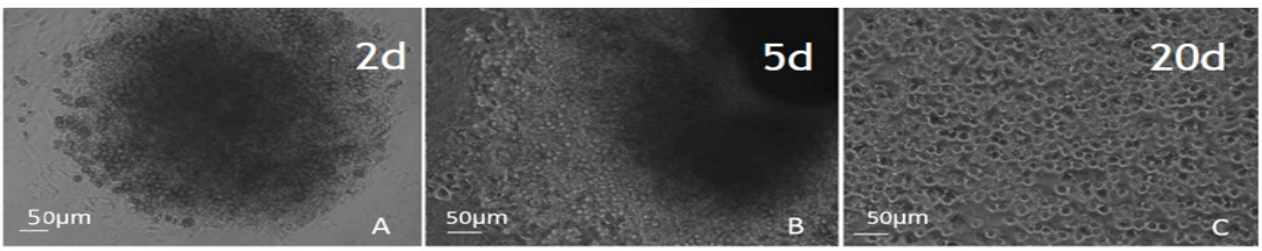
A, B, C. The GFP expression in lentivirus transfection fibroblasts with different doses. A1, B1, C1. The fibroblasts morphological feature in transmission channel.

Fig. 2: The detectionof the efficiency of lentivirus transfection into fibroblasts.



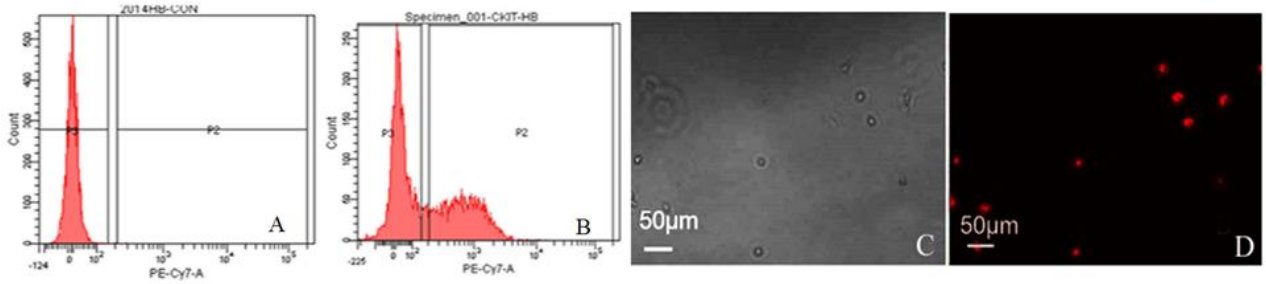
A. The expression levels of SDF-1α in LV-SDF-1α transfected cardiac fibroblasts at different times detected by Dot blot; B. Quantitative analysis of Dot blot results showed that SDF-1α protein expression continued to increase from 1 to 4 days, reaching the peak on day 4 and then gradually decreased, $^{\#}P<0.05$.

Fig. 3: The detection of SDF-1α expression at different times.



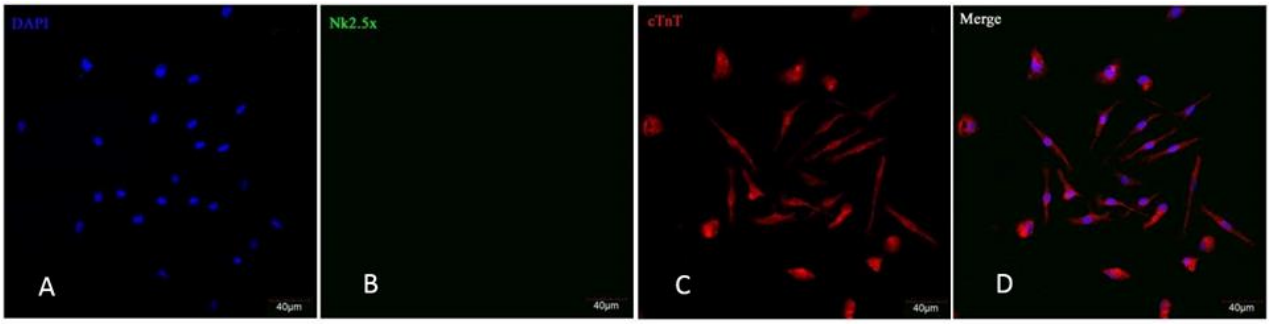
At about 20 d, those small, circular and bright cells overgrew above the long fusiform or irregular polygonal cell layer, which were filled with the whole culture flask at 20 days.

Fig. 4: Primary culture cardiac stem cells at different times.



A. Negative control; B. Flow cytometry sorting of c-kit$^+$ cardiac stem cells and the sorting rate was 40%; C. The morphology of c-kit$^+$ cardiac stem cells after adherence observed under white light channel. D. PE-Cy7- labeled c-kit$^+$ cardiac stem cells observed under fluorescence channel.

Fig. 5: Flow cytometry assay of c-kit$^+$ cardiac stem cells and observation under confocal scanning fluorescence microscopy.



The immunofluorescence assay indicated that the c-kit$^+$ cardiac stem cells presented Nkx2.5 negative and cTnT positive expressions.

Fig. 6: The detection of c-kit$^+$ cardiac stem cells differentiation via immunofluorescence.

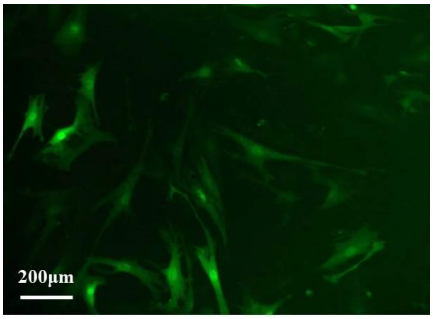
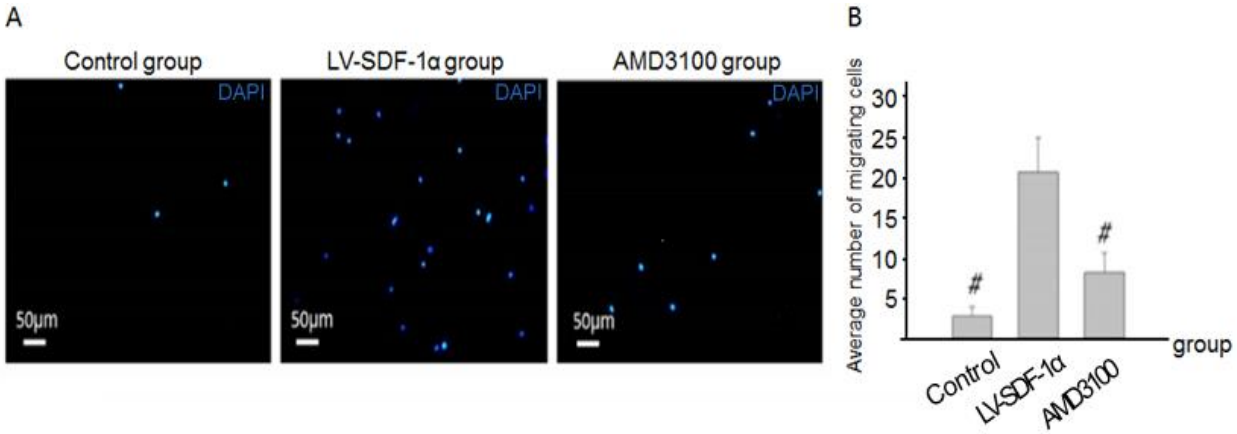
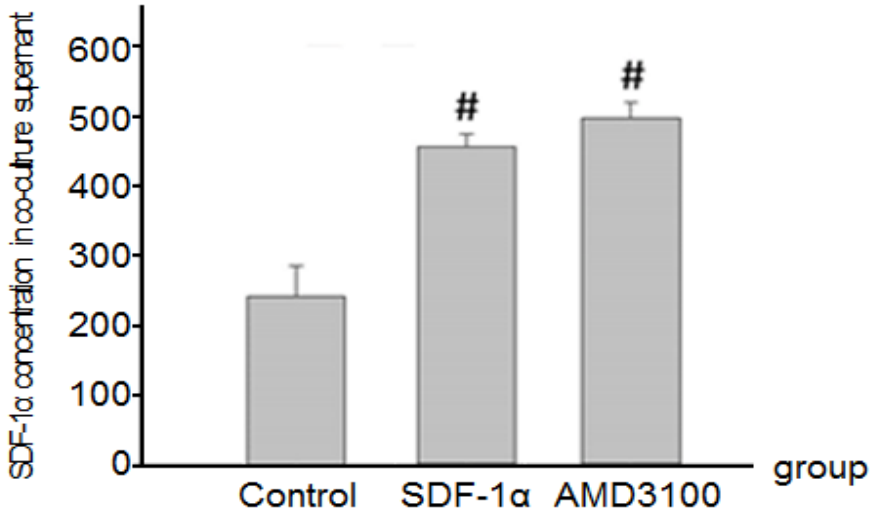


Fig. 7: The GFP expression of fibroblasts with transfected LV-SDF-1α for 4 days.



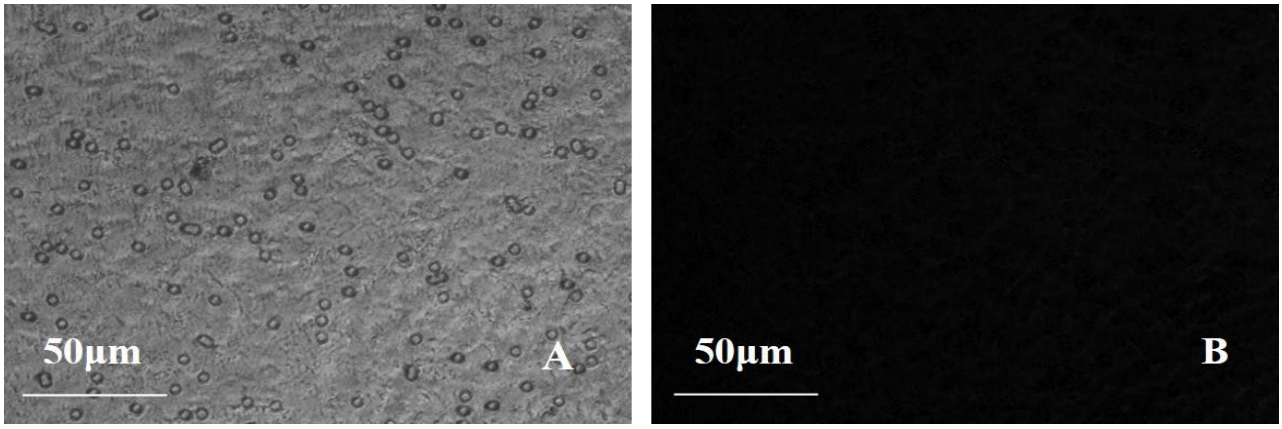
A. The migration of c-kit$^+$ cardiac stem cellsin co-cultured groups; B. Quantitative analysis of migrating cell number in each group, $^{\#}P<0.05$.

Fig. 8: Effect of SDF-1α on the migration of c-kit$^+$ cardiac stem cells.



The expression level of SDF-1α was significantly different among groups, $^{\#}P<0.01$.

Fig. 9: The expression level of SDF-1α in cell co-culture medium was quantitatively analyzed by ELISA.



A. The morphology of c-kit^{+} cardiac stem cells observed under white light channel; B. EdU-labeled c-kit^{+} cardiac stem cells were not observed under fluorescence channel.

Fig. 10: The proliferation of c-kit^{+} cardiac stem cells was detected by EdU tracing.

SDF-1α is highly expressed in damaged organs, such as pulmonary epithelial damage region and nerve injury (Fukushima and Akira 2021; Zanetti *et al*. 2019). CXCR4 is highly expressed in multiple *In Vivo* cells, which participate in damage repair, including neural stem cells, mesenchymal stem cells and endothelial progenitor cells (Deng *et al*. 2021; Liu *et al*. 2020; Wang *et al*. 2021). In addition, SDF-1α plays a significant role in many physiological processes. At present, certain research progress has been harvested with regard to paracrine-promoted repair and recovery of myocardial infarction through stem cells. Wojakowski *et al*. reported that SDF-1α can promote the homing of very small embryonic-like cells (VSELs) in peripheral blood into infarcted myocardial tissues and then differentiate into myocardial tissue, thus increasing the cardiac ejection fraction and improving cardiac functions (Wojakowski *et al*. 2011). When comparing CXCR4$^{+/-}$ myocardial infarction animal model with wild type mice, Liehn *et al*. found that the infarction area is reduced, which is related to the recruitment of inflammatory cells and the reduction of neutrophile cells (Liehn *et al*. 2011).

In addition, delayed monocyte infiltration results in a decrease in basal coronary blood flow and repair capacity, which is correlated with the decrease in vascular formation, myocardial vascular density and the number of endothelial cells. According to research findings, SDF-1α can drive the mobilization and homing of mesenchymal stem cells into ischemic tissues and accelerate the repair of ischemic cardiac diseases. The transplantation of mesenchymal stem cell patches can activate epicardial-derived cells and boost their differentiation endothelial cells, smooth muscle cells and myocardial cells and increase the density of blood vessels and lymph vessels. More c-kit^{+}cells are recruited into the infarcted myocardium to promote the myocardial repair and significantly improve cardiac functions (Song *et al*. 2019). A clinical study shows that the SDF-1α level is elevated in the plasma of patients with coronary heart diseases (Ji

et al. 2020). Therefore, the paracrine action of SDF-1α plays a significant role in the repair of myocardial infarction.

In this study, myocardial fibroblasts were transfected with LV-SDF-1α, which could secrete SDF-1α. The MOI value was 10 and the optimal time for Dot-blot detection of plasmid expression was the 4th day after transfection, which was similar to the transfection conditions described in relevant literatures. After transfection, the cultured myocardial fibroblasts grew well and showed good tolerance to this lentivirus. Furthermore, it was proved that myocardial fibroblasts would over-secrete SDF-1α due to lentiviral transfection. If the myocardial fibroblasts were co-cultured with c-kit$^+$cardiac stem cells to simulate *In Vivo* paracrine action, the migration of c-kit$^+$ cardiac progenitor cells could be effectively promoted, which could be blocked by AMD3100, indicating that SDF-1α is an important target molecular site for promoting migration.

The proliferation of c-kit$^+$ cardiac stem cells was not observed by EdU tracing, indicating that c-kit$^+$ cardiac stem cells could not be promoted by co-culture with LV-SDF-1α transfected myocardial fibroblasts *via* Transwell. We analyzed that the high-purity c-kit$^+$ cardiac stem cells existed in the upper Transwell chamber, which were separated from their microenvironment while not directly contacting other myocardial cells, such as vascular endothelial cells and cardiomyocytes and lacked the influence of other extracellular matrix or growth factors. The specific internal mechanism needs to be further investigated.

CONCLUSION

In conclusion, high purity c-kit$^+$ cardiac stem cells can be obtained by culturing cardiac tissue cells with tissue block method and sorted by flow cytometry. C-kit$^+$ cardiac stem cells can be directly differentiated into cells with myocardial cell features through *In Vitro* culture, which has the prospect of participating in the repair of cardiac injury. SDF-1α is an important target molecule for promoting migration, which lays a foundation for further experimental and clinical treatment.

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CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

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