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Cardiac Fibroblasts have the Ability to Self Differentiate into Cardiomyocytes

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Abstract

Objective: The purpose of this study is to take the biology of bone marrow-derived stem cells (BMSCs) as a comparative standard to explore whether cardiac fibroblasts (CFs) have the biological characteristics of stem cells and whether they can self-differentiate into cardiomyocytes. Simultaneously exploring the effects of aging on the growth and functionality of CFs in rats.

Methods: Rat BMSCs and CFs were isolated, purified and cultured in vitro. Immunofluorescence was performed to detect CD29, CD44, and CD45 in BMSCs and vimentin and DDR2 in CFs. Difference in cell growth was determined via MTT assay. Immunofluorescence and immunohistochemistry were performed to detect surface markers expression in BMSCs and CFs. The osteogenic, adipogenic, and cardiogenic differentiations were performed via inductive culture in special media and analyzed using alizarin red, oil red O and immunofluorescence staining of cTnT, respectively. The morphological distribution and self-differentiation potential of myocardial fibroblasts were detected by immunohistochemistry and immunofluorescence techniques.

Results: CFs and BMSCs share the same growth pattern, both expressing stem cell markers and have the ability of adipogenesis, osteogenesis, and expression cTnT in vitro. Some CFs co express vemintin or DDR2 and cTnT in myocardial tissue without any induction.

Conclusion: 1) The neonatal CFs possess the biological characteristics of BMSCs and can self differentiate into

myocardial like cells both in vitro and in vivo. 2) Aging can affect the growth rate and expression levels of various surface markers in CFs, but it does not have a significant impact on their multipotent differentiation potential.

Keywords: BMSCs; CFs; Surface Marker; Differentiative Potential; Aging

Introduction

Cardiac fibroblasts (CFs) account for approximately 70% of the cardiac volume and are the main cellular components of myocardial stroma. CFs have a strong ability to proliferate and can maintain this ability and metabolize even in hypoxic environments. These properties allow CFs to quickly repair heart injury and prevent heart rupture and heart failure. CFs are also crucial in myocardial injury repair. Fibroblasts can differentiate into tendon cells, osteocytes, chondrocytes, and adipocytes [1,2]. These research evidence demonstrate that all these cells have certain multiple differentiation potentials, that is, fibroblasts possess stem cell characteristics. Our previous studies have shown that CFs have many characteristics of stem cells in a variety of biological phenotypes [3-6]. In recent years, fruitful research has been done on CFs, but it mainly focuses on CFs subgroup classification [7], CF-iPs, Single--cell sequencing and fate map [7,8]. In terms of function, it mainly focuses on the contribution of CFs to tissue fibrosis [8-11]. There is still little literature on the stem cell characteristics of CFs and whether they can self differentiate into myocardium. However, there is no clear conclusive report on whether CFS is stem cells. The main reason is that there is no stem cell standard that can define CFS. This study uses bone marrow stromal stem cells as the comparative standard for stem cells, and attempts to prove whether CFs are a type of stem cell in terms of expression of various stem cell markers and cell differentiation potential, especially in vivo and in vitro, whether CF has the ability to differentiate into myocardial like cells. This is of great significance for further mobilizing endogenous CFs to differentiate into myocardium and accelerating the repair of damaged myocardium. Our first set of experiments revealed that neonatal CFs can express various stem cell surface markers and exhibit certain stem cell functional characteristics. With the increase in culture passages, CFs activated and transitioned into an increased number of myofibroblasts. However, whether there are differences in the growth and functionality of CFs with increasing age or months remains unreported in the current literature. This study aims to investigate potential changes in the growth and functionality of CFs with aging by analyzing their variations over different time intervals. The objective is to explore the potential impact of aging on myocardial regeneration by providing experimental evidence for basic research and clinical applications in myocardial repair.

Material and Methods

Culture of BMSCs

The cell lines of SD rat BMSCs (Guangzhou Saiye Biotechnology Co., Ltd.) were rapidly heated and melted in a water bath pot at 37 °C, resuscitated within 2 min, moved to a 15 ml centrifuge tube, suspended with complete medium, and then centrifuged for 5 min at 1200 r/min. The supernatant was discarded and added to 12 ml of a cell-specific medium (SD rat bone marrow-derived stem cells complete medium). After blowing and suspending, the cells were transferred to a 75 cm^2 culture flask and cultured in an incubator at 37 °C and 5% CO_2. After 24 h, the complete medium was changed for the first time. The medium was replaced every 2 days. When the cells converged to approximately 90%, they were digested and passaged with 0.25% trypsin (Amrescao, USA) at a ratio of 1:2.

Acquisition and Culture of CFs

Healthy SD rats (purchased from the Experimental Animal Center of Xinxiang Medical University), a total of 120, with no restriction on gender, were used in this study. Among them, neonatal rats (60) were disinfected with 75% ethanol, while 1-month-old (30) and 3-month-old (30) rats were anesthetized with ethyl ether, followed by hair removal, disinfection with 75% ethanol, and placement on a sterile dissecting board. The lower left side of the sternum xiphoid process of

the rats were located, and the thoracic cavity was rapidly opened under sterile conditions. Ophthalmic bending forceps were used to take the heart apex and placed it in a precooled DMEM. The apex was rinsed three times to remove the blood, and then the apical tissue was transferred into a 5 ml glass bottle, which was added with 2 ml precooled HBSS. Using phthalmic scissors, the apical tissue was cut it into small pieces about 1 mm^3 in size. The supernatant was then discarded. A digestive enzyme solution (containing 0.07% trypsin and 0.05% collagenase II solution) was added in the glass bottle. The bottle was placed in an incubator for 8 min and then gently shaken every 4 min. The supernatant was then collected after 8 min. A total of 1.5 ml of digestive enzyme solution were added again. The glass bottle was placed in an incubator for 8 min and then gently shaken every 4 min. These steps were repeated 11–13 times until the myocardial tissues became soft. A total of 5 ml of the complete medium were added, and the remaining tissue blocks were gently blown until they almost disappeared. The supernatant was collected and placed in complete medium. The collected supernatant was centrifuged for 5 min at 1000 rpm, and the supernatant was discarded and precipitated with 5 ml of complete medium. The suspended cells were gently blown through 200-mesh cell sieves. The cells were transferred into a 25 cm^2 culture flask for differential attachment two times for 45 min each time. The adherent cells were mainly fibroblasts, and then 5 ml of complete medium was added. The cells were cultured in an incubator with 5% CO_2 at 37 °C. When the cells converged to 90%, they were digested and passaged with 0.25% trypsin (Amrescao, USA) at a ratio of 1:2.

Identification of BMSCs and CFs via Immunofluorescence Staining

Third-generation BMSCs and CFs were digested, suspended, and inoculated into 96-well plates, with 3000–5000 cells per holes. When the cells achieved about 80% confluence, they were fixed in 4% paraformaldehyde for 30 min at room temperature, subsequently washed with PBS, and treated with 0.3% Triton X-100 in PBS for 10 min. Then, the cells were incubated in 10% goat serum for 40 min at room temperature. BMSCs were incubated overnight 4 °C with rabbit-derived primary antibodies (CD44, 1:500, abcam; CD44, 1:500, abcam; CD73, 1:500, abcam; CD45, 1:500, abcam). CFs were incubated overnight at 4 °C with rabbit-derived primary antibodies (DDR2, 1:50, Santa; Vimenten, 1:500, abcam). The cells were subsequently incubated with Cy3-conjugated secondary antibodies (1:500, Beyotime). The nuclei were counterstained with 10 μg/ml DAPI for 10 min at room temperature. Negative controls were obtained by following the same protocol but with primary antibodies. The samples were observed and photographed under a fluorescence microscope. All experiments were performed in triplicate.

Cell Proliferation via MTT Assay

Third-generation BMSCs and CFs were digested, suspended, and inoculated into 96-well plates, with 3000–5000 cells per holes. Testing was started after 24 h. From day 1 to day 6, one 96-well plate was taken every day in which 5 g/l MTT (10 μl)(Sigma, USA) per hole and 90 μl of DMEM containing (Hyclone, USA) 10% fetal bovine serum, which incubated at 37 °C for 4 h, were added. Subsequently, 150 μl of DMSO was added to each hole, and the sample was slowly shaken in a shaking bed for 10 min. The absorbance (OD) value was determined at 490 nm by using a microplate reader. The OD values of 96-well plate cells were measured for 6 days, analyzed, and compared. All experiments were performed in triplicate.

Detection of BMSC and CF Surface Marker via Immunofluorescence Staining

Third-generation BMSCs and CFs were digested, suspended, and inoculated into 96-well plates, with 5000–10,000 cells per holes. When the cells achieved about 70% confluence, they were fixed in 4% paraformaldehyde for 30 min at room temperature. Subsequently, the cells were washed with PBS and treated with 0.3% Triton X-100 in PBS for 10 min. The cells were then incubated in 10% goat serum for 40 min at room temperature. BMSCs and CFs were incubated overnight at 4 °C with rabbit-derived primary antibodies (nanog, 1:500, abcam; sox2, 1:500, abcam; oct4, 1:500, abcam; scal-1, 1:500, abcam; c-kit, 1:500, abcam). The samples were subsequently incubated with Cy3-conjugated secondary antibodies (1:500, Beyotime) for 1 h at room temperature. The nuclei were counterstained with 10 μg/ml DAPI for 10 min at room temperature. Negative controls were obtained by following the same protocol but with primary antibodies. The samples were observed and photographed under a fluorescence microscope. All experiments were performed in triplicate.

Detection of BMSC and CF Surface Marker via Immunohistochemical Staining

Third-generation BMSCs and CFs were digested, suspended, and inoculated into 96-well plates, with 5000~10,000 cells per holes. When the cells achieved about 70% confluence, they were fixed in 4% paraformaldehyde for 30 min at room temperature, washed with PBS, and treated with reagent A (3% H2O2) for 8 min at room temperature. The cells were washed three times with distilled water and added with reagent B (serum blocking liquid) at room temperature for 20 min. BMSCs and CFs were incubated overnight at 4 °C with rabbit-derived primary (nanog, 1:500, abcam; sox2, 1:500, abcam; oct4, 1:500, abcam; scal-1, 1:500, abcam; c-kit, 1:500, abcam) antibodies. The samples were subsequently added with reagent C (biotin labeled goat anti-rabbit) for 20 min at room temperature. DAB showed color, the nuclei were counterstained by hematoxylin, and then the samples underwent routine dehydration, transparency, and sealing. Negative controls were obtained by following the same protocol but with primary antibodies. The samples were observed and photographed under an inverted microscope. All experiments were performed in triplicate.

Adipogenic, Osteogenic, and Myocardial Differentiation Cultures

Third-generation BMSCs and CFs were digested, suspended, and inoculated into 96-well plates, with 5 000–10,000 cells per holes. When the cells achieved about 70% confluence, adipose, osteogenesis, and myocardial induction solutions were added. The adipogenesis- and osteogenesis-inducing media were replaced once every 2–3 days, whereas the myocardial-inducing medium was replaced by complete medium after 24 h and then changed into complete medium every 2–3 days. After 7–9 days of lipogenesis induction, the samples were fixed in 4% polyformaldehyde, stained with oil red O staining kit (Solarbio, Beijing), and photographed under a microscope. After 21 days of osteogenesis induction, the samples were fixed in 4% polyformaldehyde, stained with alizarin red staining kit (Solarbio, Beijing), and photographed under a microscope. After 21 days of myocardial induction, the samples were stained with cTnT via immunofluorescence and photographed under a fluorescence microscope. All experiments were performed in triplicate.

Immunohistochemical Staining of Myocardial Tissue

Take fresh left ventricular myocardial tissue, routine and freeze slices, with a thickness of 5um. It was fixed in 4% paraformaldehyde at room temperature for 30 minutes, and then washed with PBS. Then, incubate the tissue slices in 10% goat serum at room temperature for 40 minutes. Incubate the tissue slices with rabbit derived primary antibodies (DDR2, 1:50, Santa; Vimenten, 1:500, abcam) overnight at 4 ° C. Subsequently, the second antibody (1:500, Beyotime) was added dropwise for incubation. Cell nucleus at room temperature using 10 μ G/ml DAPI restaining for 10 minutes. A negative control was obtained by following the same protocol, but without the use of primary antibodies. Observe the results under a light microscope and take photos

Image Processing and Statistical Analysis

The immunofluorescence results of each protein were randomly selected from five to seven fields. The average optical density was defined as the ratio of total optical density to area, and semiquantitative analysis was performed using Image J version 6.0 software. The Western blotting results for the relative protein expression were determined using Image J version 6.0 software. Relative protein expression was defined as the ratio of gray value between the target protein and the internal parameters, and semiquantitative analysis was performed. Data were analyzed using SPSS version 20.0 software. Results were shown as mean ± standard deviation $(\bar{x} \pm s)$. Two different samples were compared via t-test. $P < 0.05$ was considered as a statistically significant difference.

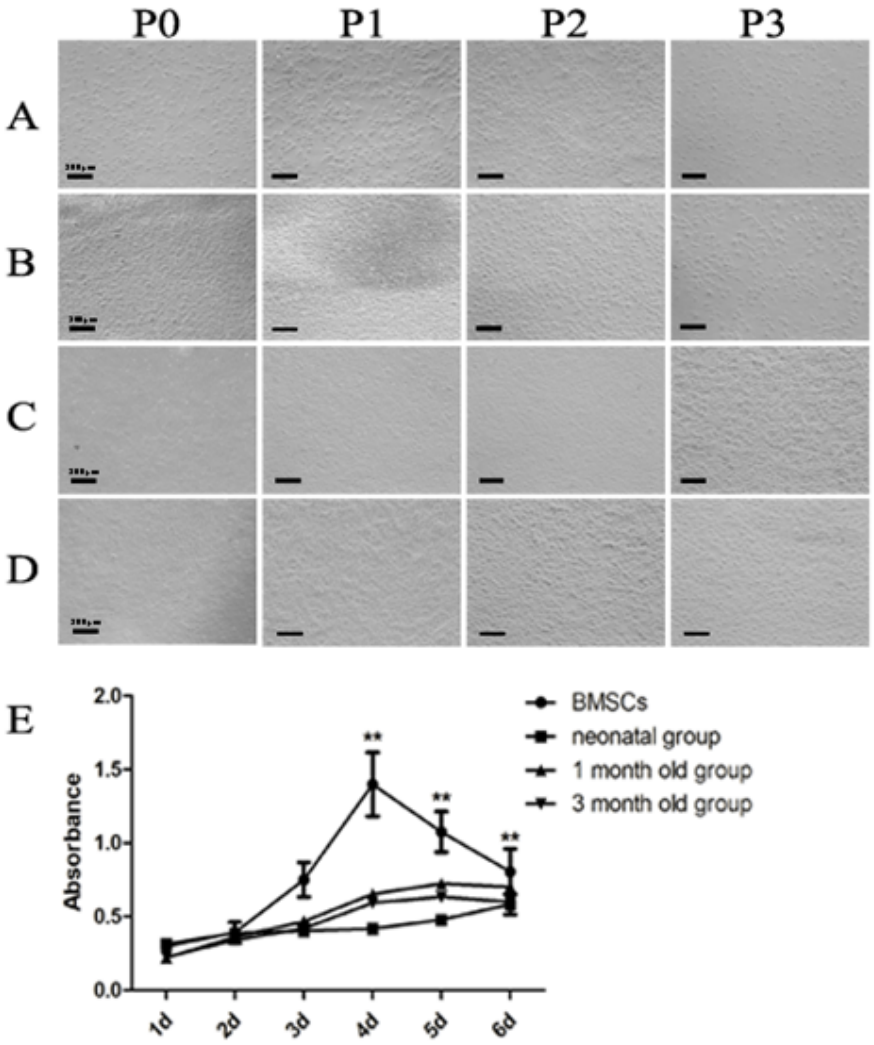

Figure 1: Morphological characteristics and cell growth curves of different generations of BMSCs and CFs

A represents different generations of BMSCs. B represents different generations of neonatal CFs. C represents different generations of 1-month-old CFs. D represents different generations of 3-month-old CFs. The scale is 200 μm. E: The abscissa indicated the incubation time, the ordinate indicated the absorbance, * : $P < 0.05$, **: $P < 0.01$.

Results

The Biological Identification of BMSCs and CFs

To demonstrate that the cells studied in this study are MBCS and CFS, rather than any other cells, the growth patterns and specific labeling proteins of bone marrow stem cells and fibroblasts were first labeled.

Morphological Characteristics

Resuscitated and cultured BMSCs exhibited adherent growth and showed elongated, circular, and triangular structures. The growth rates of P0 and P1 were slower than those of P2 and P3 (Figure 1A). Primary CFs grew fast in P0 and P1 generations but slower in P2 and P3 generations (Figure 1B).

The Growth Curves

The total growth trend of rat P3 BMSCs and P3 CFs showed that BMSCs initially increased first and then decreased, whereas that of CFs steadily increased steadily but the increase was not significant. The growth rates between BMSCs and CFs within the first 24 h after stabilization were not different (Figure 1E). The growth rate of BMSCs was significantly higher than that of CFs on days 2~3, and the OD value of BMSCs was 1.7-fold higher than that of CFs. The difference in growth rates between BMSCs and CFs reached its peak on days 3~4, and the OD value of BMSCs was about 2.3-fold higher than that of CFs. On days 4~5, the OD value of BM-

SCs began to decrease, whereas that of CFs continued to increase. The curve of CFs did not substantially change on days 5–6. The growth period of CFs was stationary. By contrast, BMSCs did not show evident growth stationary period because of their rapid growth rate (Figure 1E). The overall growth trend of P3 CFs in each group of rats has been consistently increasing steadily, but with varying degrees of ascent. Figure E shows that from 24 hours of stable adherence to 48 hours of adherence, there is no significant difference in the growth rate of CFs among groups. From 2 to 3 days, the growth rate of neonatal group CFs is significantly higher than that of the 1-month-old group and the 3-month-old group, but the difference is not statistically significant ($P>0.05$). From 3 to 4 days, the growth rate differences among groups peak, with the neonatal group higher than the 1-month-old group and the 3-month-old group, and the difference is statistically significant ($P<0.05$). There is no significant difference in growth rate between the 1-month-old group and the 3--month-old group. From 4 to 5 days, the absorbance (OD) values of CFs in each group show no significant changes compared to the previous period. The growth rate of the 1-month-old group is higher than that of the neonatal group and the 3-month-old group, while the OD values of neonatal group CFs remain higher than those of the 1-month-old group and the 3-month-old group. From 5 to 6 days, there is little change in the curve of the neonatal group CFs. This suggests that CFs of different ages enter a growth plateau phase around day 4, but there are still proliferative differences among different age groups (Figure E).

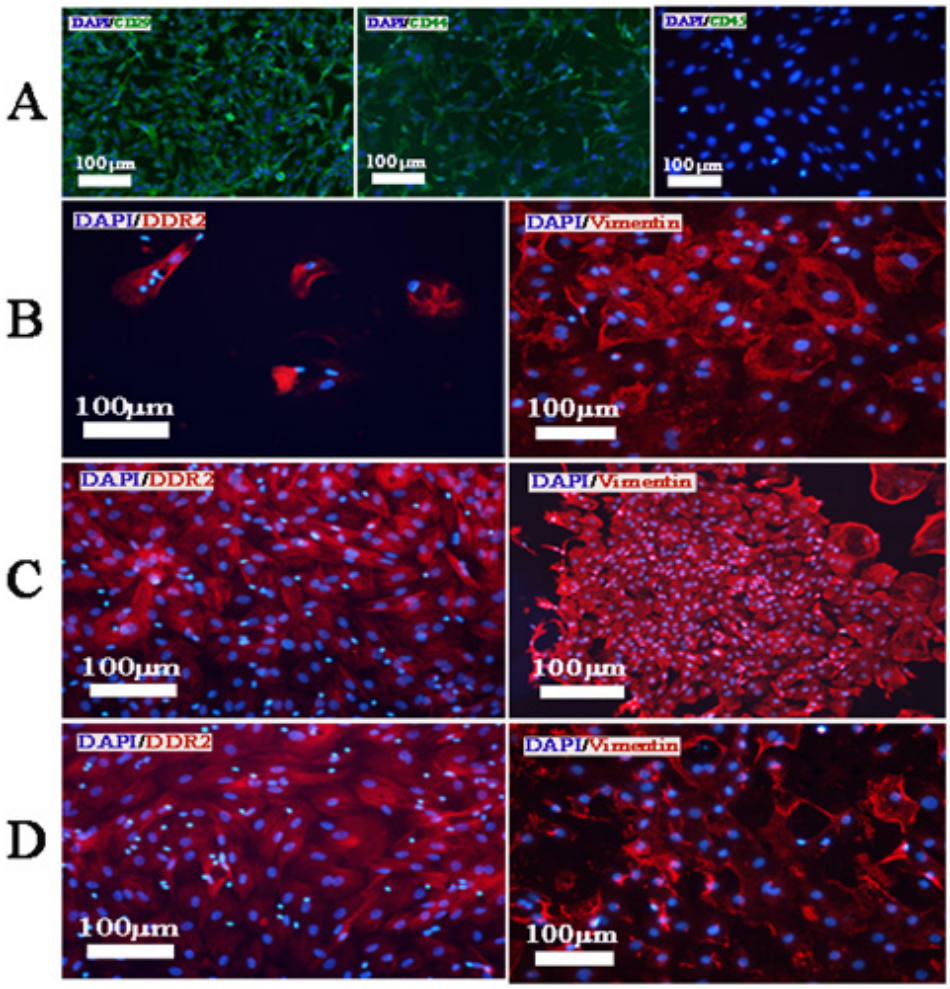

Figure 2: The surface markers expression of P3 BMSCs and P3 CFs, immunofluorescence staining, observed by inverted fluorescence microscopy

A: The blue represents the nucleus and the green FITC-labeled CD29 and CD44. Rat BMSCs express CD29 and CD44, but not CD45. The scale is 100 μm. B, C, D: Vimentin and DDR2 were labeled by Cy3 in red. Rat CFs expressed vimentin and DDR2. B represents the neonatal group, C represents the 1-month-old group, and D represents the 3-month-old group. The scale is 100 μm.

Specific Surface Marker Expression

Immunofluorescence staining showed that P3 BMSCs expressed CD29 and CD44 but not CD45. These results were in accordance with BMSC characteristics (Figure 2A). Vimentin and DDR2 expression in P3 CFs was consistent with CF characteristics (Figure 2B/2C/2D). These results meet the biomarker standards of BMSCs and CFs respectively.

Expression of five surface markers of of stem cells in rat BMSCs and CFs

In order to prove whether CFs have the characteristics of bone marrow stem cells, we took the five common biomarker of BMSCs as the standard, immunolabeled and compared the relevant information of CFs.

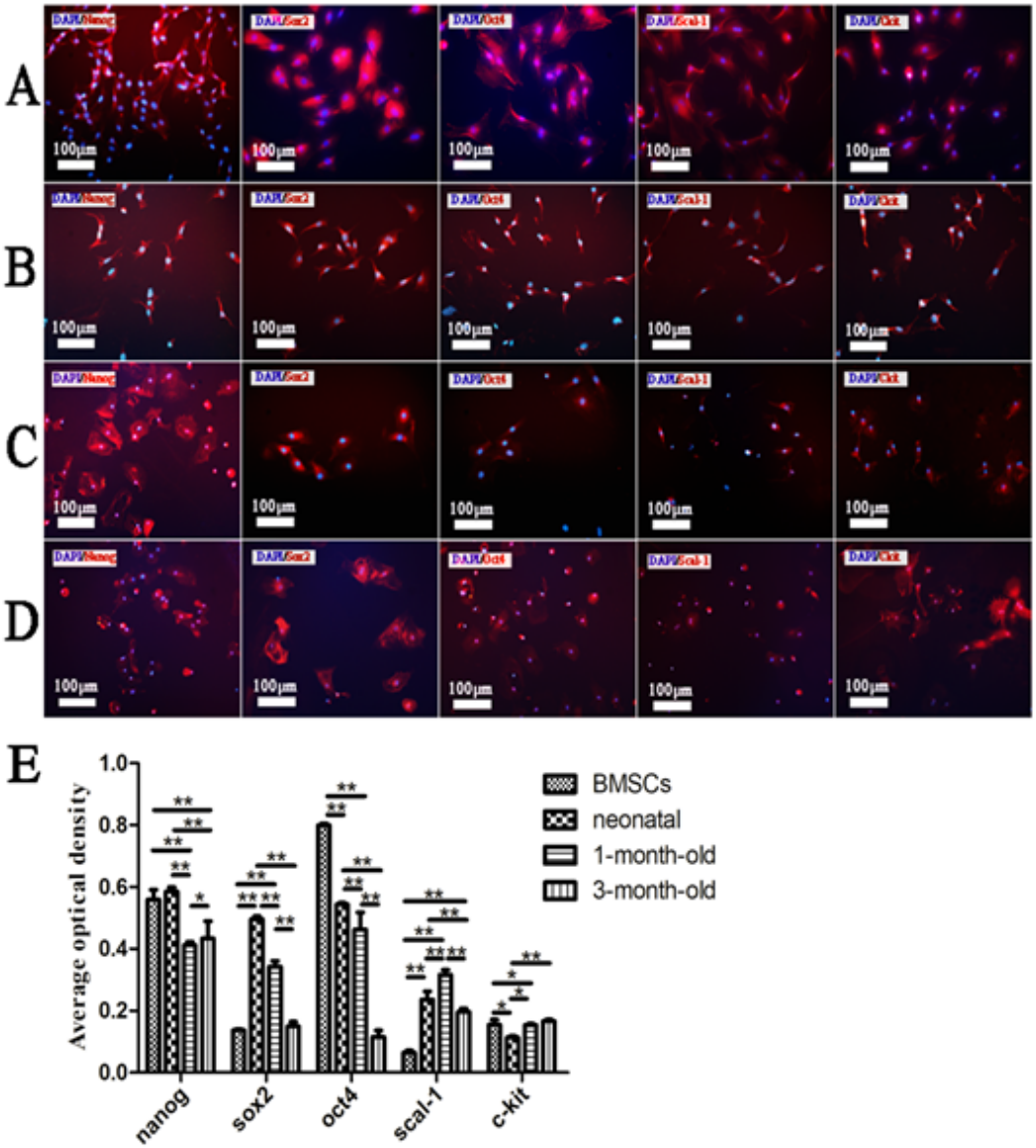

Figure 3: A Expression of five surface markers of stem cells in P3BMSCs and CFs, Immunofluorescence staining, observed by inverted fluorescence microscopy

DAPI represents the nucleus and red represents the Cy3-labeled nanog, sox 2, oct 4, scal-1, c-kit. A shows P3 BMSCs; B represents the neonatal group, C represents the 1-month-old group, and D represents the 3-month-old group. The scale is 200μm. E: Comparison of the mean optical density expressions of five surface markers between P3 BMSCs and P3 CFs

Compared with P3 BMSCs and P3 CFs, * means $P < 0.05$, ** means $P < 0.01$, the difference is statistically significant.

Immunofluorescence Results

The expression of nanog, sox2, oct4, scal-1, and c-kit surface markers in rat P3 BMSCs and P3 CFs was observed (Figure 3). Five to seven fields from each group were randomly selected, and the average optical density ratio (total optical density/area) was calculated. Results showed no significant difference in nanog expression between P3 BMSCs and P3 neonatal CFs ($P > 0.05$). However, the surface marker expression levels of P3 BMSCs and P3 CFs in the neonatal group were higher than those in the 1-month-old and 3-month-old groups ($P<0.01$). At the same time, the expression of nanog in the 1-month-old group is lower than that in the 3-month-old group ($P<0.05$). Sox2 and scal-1 expression levels in P3 BMSCs were lower than those in P3 CFs, and the difference was statistically significant ($P < 0.05$). The expression of sox2 decreases gradually in the neonatal group, 1-month-old group, and 3-month-old group, but it is higher in the neonatal and 1-month-old groups compared to BMSCs ($P<0.05$). Scal-1 expression in CFs is higher than in BMSCs ($P<0.01$), but its trend changes with age, initially increasing and then decreasing, with the highest expression in the 1-month-old group ($P<0.01$). Oct4 expression in BMSCs is higher than in CFs, but with increasing age, Oct4 expression gradually decreases ($P<0.01$).C-kit expression in BMSCs is higher than in the neonatal and 1-month-old CFs ($P<0.05$) and is comparable to the 3-month- old group. However, with increasing age, ckit expression gradually increases, with lower expression in the neonatal and 1-month-old groups compared to the 3--month-old group, while the expression levels in the neonatal and 1- month-old groups are similar ($P>0.05$). (Figure 3E).

Immunohistochemical Expression of Five Surface Markers

Immunohistochemical staining of rat P3 BMSCs and different ages of P3 CFs showed that both cells expressed nanog, sox2, oct4, sca-1, and c-kit surface markers, and the expression site was located in the cytoplasm (Figure 4).

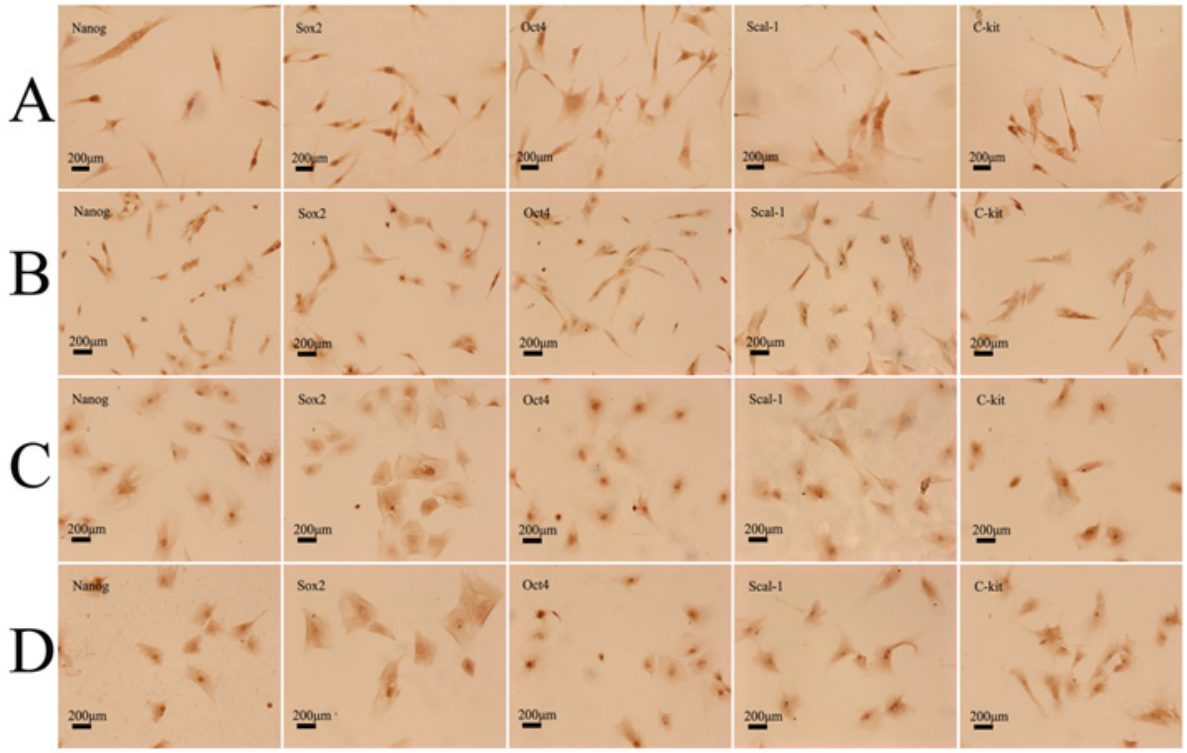

Figure 4: Immunohistochemical detection of the expression of five surface stem cell markers in P3BMSCs and P3CFs, observed by inverted microscopy

A shows P3 BMSCs; B represents the neonatal CFs group, C represents the 1-month-old CFs group, and D represents the 3-month-old CFs group. The scale is 200μm.

Comparison of adipogenesis, osteogenesis, and myocardial differentiation between BMSCs and different ages of CFs in rats

In Vitro Cell Differentiation

After the adipogenic induction of BMSCs and different ages of CFs in rats, red lipid droplets formed, indicating that both cells possess the ability of adipogenic differentiation . After osteogenesis induction, alizarin red staining showed that BMSCs and CFs had red calcium nodules, indicating that both cells possess osteogenic differentiation ability. After myocardial induction, cTnT immunofluorescence staining showed that BMSCs and different ages of CFs possess myocardial differentiation ability (Figure 5).

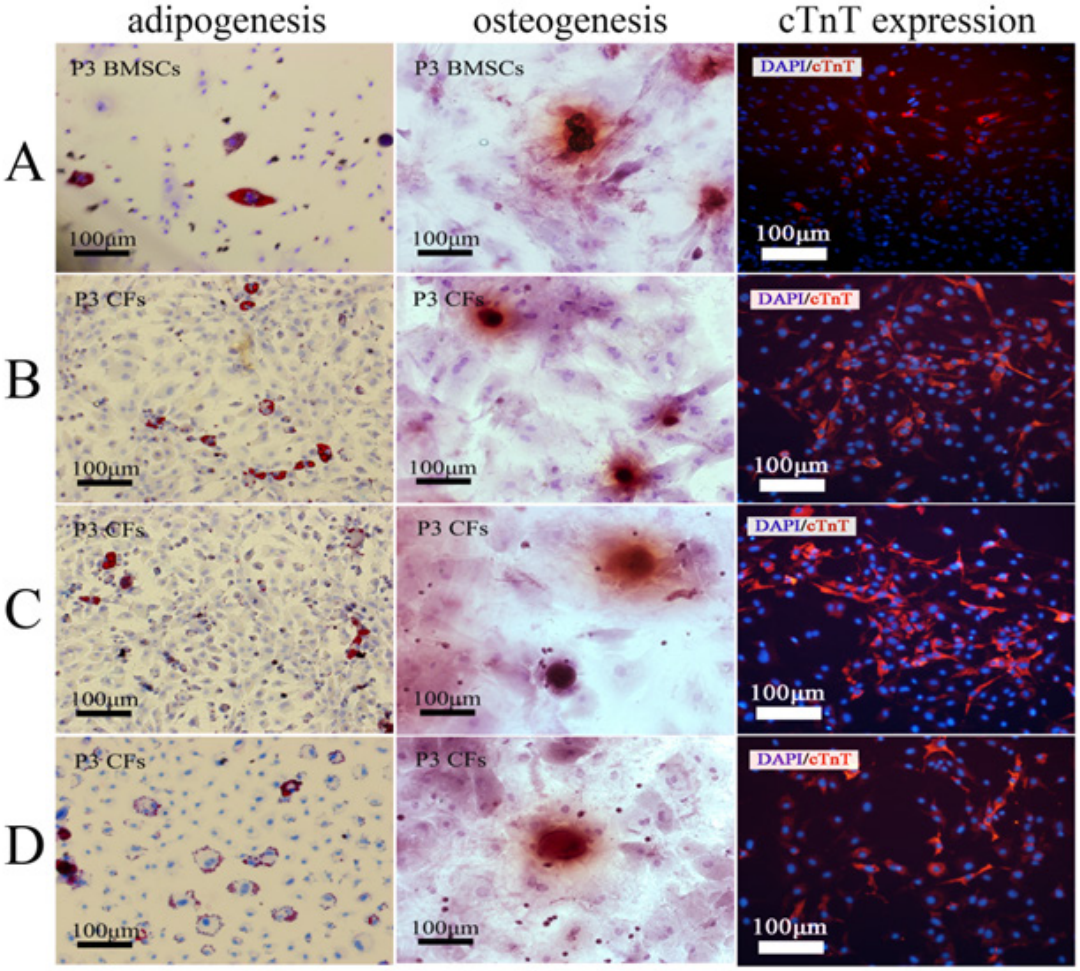

Figure 5: Comparison of adipogenesis, osteogenesis and cTnT expression in P3 BMSCs and P3 CFs, Oil red O, Alizarin red staining and myocardial induction, inverted microscope

A shows differentiative capacity of BMSCs, B shows those of neonatal CFs group, C represents the 1-month-old CFs group, and D represents the 3-month-old CFs group. Red represents lipid droplets, calcium deposition, and cTnT expression, while blue represents cell nuclei.

Differentiation of CFs in Cardiac Tissue

Normal left ventricular tissue sections show that fibroblasts in the myocardial interstitium are positive expression of fibroblast markers vemintin and DDR2. These two markers are mainly expressed in the CF cytoplasm and myocardial cell gap. Immunofluorescence results showed that some cells between myocardial cells co-expressed vemintin or DDR2 and cTnT (the two substances were yellow after merged). It is suggested that some fibroblasts can differentiate into cardiomyocytes in normal myocardium.

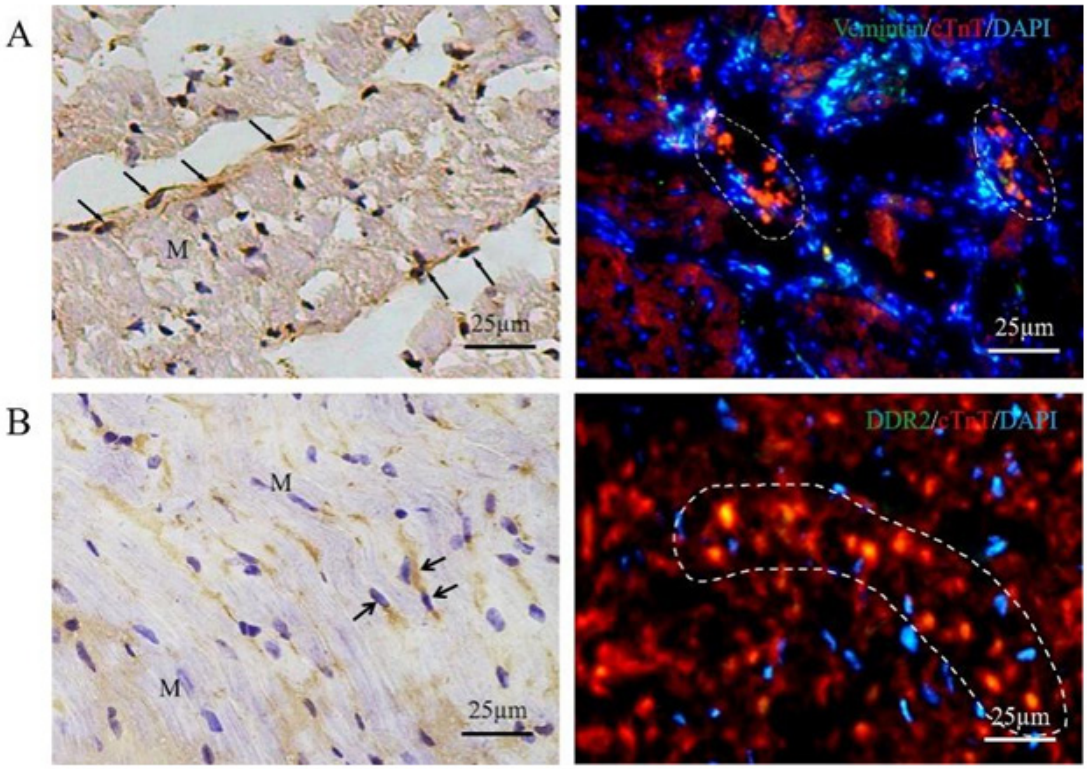

Figure 6: Evidence for myocardial differentiation of myocardial tissue fibroblasts

A: The expression of vemintin in myocardial fibroblasts (arrow), and the yellow cell groups in the dashed box on the right picture is the co-expressing cells of vemintin and cTnT. B: The expression of DDR2 in fibroblasts of myocardial tissue (arrow) and the expression of myocardial cell gap. The yellow cell population in the right picture is the co-expression cells of DDR2 and cTnT.

Discussion

BMSCs include hematopoietic stem cells and stem cells with multiple differentiation potentials. In 1991, Arnold Caplan first used MSCs to describe the stem cells isolated from bone marrow that can differentiate into cartilages, bones, and adipocytes. In 2006, the International Society for Cell Therapy defined mesenchymal stem cells according to the following criteria: they have adherent growth in monolayer cell culture and can potentially differentiate into chondrocytes and adipocytes under standard differentiation conditions in vitro. Meanwhile, mesenchymal stem cells can express CD105, CD73, and CD90, but they do not express surface markers, such as CD45, CD34, CD14, CD11b, CD37, CD19, and HLA--DR. These observations indicate that bone marrow-derived mesenchymal stem cells have clear definitions and criteria in terms of growth mode, morphological characteristics, cell surface markers, and differentiation potential [12]. Therefore, the designation of this international standard has made MBCS a scale for determining whether cells from other tissues are stem cells. This study compared the surface markers and differentiation potential of BMSCs with CFs cells, The results demonstrated that CFs, like MBSCs, express nanog, sox2, oct4, scal-1, and c-kit positively, but do not express hematopoietic stem cell markers. At the same time, it has been proven that both have the ability of adipogenesis, osteogenesis, and myocardial differentiation, which largely confirms that CFs fully possess the characteristics of stem cells. This result is basically consistent with our previous research [3,5,6].

Many studies have reported that when CFs is cultured in vitro, it can spontaneously differentiate into adipocytes, osteocytes, and chondrocytes [12,13]. CFs exhibits significant phenotypic diversity in cardiac fibroblasts [6,14]. The multidifferentiation potential of CFs suggests that [15] they possess some biological characteristics of stem cells. Fibroblasts with modified Myod can be transformed into skeletal muscles. CFs modified with *Gata4*, *Mef2c*, and *Tbx5* genes can be transformed into cardiomyocyte-like cells [16]. Given that the repair ability of myocardium after myocardial injury is poor, no method can satisfactorily restore the function of the injured myocardium. CFs are the most abundant cell types in the heart. CFs is the most abundant cell type in the heart. We assume that if CFs have the ability of myocardial differentia-

tion, they will play a positive role in the process of myocardial injury repair, and can achieve the regeneration of myocardial cells after myocardial injury to supplement the lost myocardial cells..

CTnT is a specific marker of striated muscle tissue (including myocardium and skeletal muscle), so only seeing the expression of cTnT at the single cell level cannot prove that it must be a myocardial cell (or a skeletal Muscle cell). Therefore, this study further validated the co localization of vemintin or DDR2 with cTnT at the myocardial tissue level. If only the cells that express fibroblast markers vemintin or DDR2 and cTnT in myocardial tissue express cTnT at the same time, it is reasonable to assume that the cells that express cTnT are not skeletal muscle cell, but myocardial cells. Because skeletal muscle cell cannot appear in myocardial tissue. Since these differentiated cells have not yet appeared striations and beats, we believe that they are still early immature cardiomyocytes. Once striations and pulsations occur, these cells will completely lose their ability to express vemintin and DDR2, making it difficult to distinguish between existing or newly differentiated cells at the myocardial tissue level.

There have been many studies on the differentiation of fibroblasts into cardiomyocytes, but almost all of the studies have induced CF to differentiate into cardiomyocytes by reprogramming CF [17-20]. The important finding of this study is that CF has the ability to differentiate itself into cardiomyocytes without adding any induction component, which is one of the highlights of this study. It should be mentioned that the myocardial cells differentiated by CF-IPS are easy to beat [21], while the self differentiated CF in this study is not, because reprogramming technology has increased many incentives for CF to promote differentiation.

Nanog, as a necessary gene for embryonic stem cell renewal, is the first key gene found to maintain the pluripotency of embryonic stem cells. Although sox2 and oct4 cooperate with nanog to regulate the transcription of embryonic stem cells [22], the absence of either of these factors can induce the differentiation of embryonic stem cells [23]. These three proteins are considered as the markers of embryonic stem cells [24,25]. Scal-1, as a stem cell antigen, is a marker of hematopoietic stem cells [26]. Scal-1^{+} hepatocytes have strong proliferation and differentiation abilities [27]. Scal-1 expression increases in myocardial injury, suggesting that these cells may participate in myocardial injury repair. C-kit is a proto-oncogene mainly expressed in endothelial cells and is a subgroup in the heart. This subgroup may participate in angiogenesis in myocardial injury repair [28]. In the present study, we investigated the expression of nanog, sox2, oct4, sca1-1, and c-kit proteins in BMSCs and CFs via various techniques. These stem cell markers were expressed in BMSCs and CFs, although slight differences were observed in terms of their expression levels. No difference in nanog expression level was observed between the two kinds of cells, but sox2 and sca-1 expression levels in BMSCs were lower than those in CFs. These results indicated that CFs possess strong stem cell characteristics and may be higher than BMSCs in several aspects.

In terms of differentiation potential, CFs have more unique advantages than BMSCs. CFs can self-differentiate into many kinds of cells without induction [9,10]. In the present study, we compared the differentiation potentials of BMSCs and CFs in adipogenesis, osteogenesis, and cardiomyogenesis. BMSCs exhibited higher differentiation ability than CFs in adipogenesis and cardiomyogenesis, but the difference in osteogenesis was not significant. These results suggested that BMSCs are still higher than CFs in terms of multidirectional differentiation ability. Although the differentiation intensities of the two kinds of cells is different, they are important for tissue damage repair. Understanding their differentiation advantages is necessary to utilize their biological characteristics and use them for cell regeneration. On the basis of the results of this study, we argue that CFs may be a huge seed cell that differentiates into cardiomyocytes in myocardial injury. Further research should reveal the intrinsic dynamics and environmental factors of CFs differentiation into cardiomyocytes.

We believe that CFs possess characteristics of stem cells and high levels of immaturity, serving as a reservoir of cells for tissue differentiation and repair. What sets our study apart from previous research is the demonstration of significant age differences in CFs. Our study results demonstrate that the expression of the nanog molecule is highest in the neonatal group, followed by the 3-month-old group, and lowest in the 1-month-old group. Its expression decreases with age initially, then slightly increases but remains lower than that in the neonatal group. The expression of sox2 and oct4 follows a similar pattern, with the highest expression in the neonatal group, followed by the 1-month-old group, and lowest in the 3-month-old group. These three molecules play a crucial role

in maintaining the pluripotency of embryonic stem cells, and their overall expression decreases with increasing age, possibly related to the decreased 'stemness' of fibroblasts. Scal-1 expression is highest in the 1-month-old group, followed by the neonatal group, and lowest in the 3-month-old group, while c-kit expression is higher in the 1-month-old and 3-month-old groups compared to the neonatal group, with no significant difference between the 1-month-old and 3-month-old groups. Except for Scal-1 and c-kit, the expression of other surface markers is higher in the neonatal group than in the 1--month-old and 3-month-old groups, decreasing with age. Scal-1 expression initially increases with age before decreasing, while c-kit expression increases with age and stabilizes in adulthood. These two molecules have previously been used as surface markers for cardiac stem cells, and their expression characteristics in fibroblasts may be related to cellular aging-induced upregulation of 'stemness' expression. Thus, while age affects surface markers, the changes are not stable, and there is no consistent pattern of differences. Since these five markers are considered markers of stem cells, changes in their expression can reflect changes in the differentiation ability of CFs. Our study confirms this through induction results for adipogenesis, osteogenesis, and cardiomyogenesis. Although cells in each group have the potential for multilineage differentiation, the adipogenic ability decreases with age, while the cardiomyogenic ability initially increases and then decreases with age. Comparisons of differentiation differences between groups show that although the differentiation potential of the 3-month-old group is lower than that of the neonatal and 1-month-old groups, the difference is not significant. In conclusion, aging can affect the morphological changes, growth rate, and expression levels of various surface markers in CFs, but it does not significantly affect their multilineage differentiation ability. This indicates that CFs maintain a relatively strong differentiation ability throughout the aging process, providing new insights for better research and utilization of CFs.

Finally, the comparison between BMSCs and fibroblasts proves that CFs is no longer just a cell that secretes collagen, Elastin and cytokines, but also actively participates in the differentiation of cardiomyocytes. Therefore, CFs may be a potential reserve for myocardial regeneration. Although more evidence is needed to support this conclusion, at least we see the tip of the iceberg.

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