Original Paper

Multiple Directional Differentiation Difference of Neonatal Rat Fibroblasts from Six Organs

Yuqiao Chang^{a,b} Kang Guo^c Qiong Li^b Cixia Li^b Zhikun Guo^b He Li^a

^aDepartment of Human Anatomy and Embryology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, ^bHenan Key Laboratory of Medical Tissue Regeneration, Xinxiang Medical University, Xinxiang, ^cTumor Department, Third Affiliated Hospital, Xinxiang Medical University, Xinxiang, P.R. China

Key Words

Fibroblasts • Immunofluorescence staining • Stem cell markers • Heterogeneity • Directed differentiation

Abstract

Background/Aims: Fibroblasts are abundantly distributed throughout connective tissues in the body and are very important in maintaining the structural and functional integrity. Recent reports have proved that fibroblasts and mesenchymal stem cells share much more in common than previously recognized. The aim of this study was to investigate comparative studies in fibroblasts on the differences in the expression of molecular markers and differentiation capacity from different organs. **Methods:** Combined trypsin/collagenase enzymes digestion method was used to isolate and culture the fibroblasts derived from heart, liver, spleen, lung, kidney and skin. Cell activity was determined by methyl thiazolyl tetrazolium (MTT) assay. Common molecular markers for fibroblasts such as vimentin, DDR2 and FSP1, stem cell markers nanog, c-kit and sca-1 were detected by RT-PCR, immunofluorescence and western blotting. The osteogenic, adipogenic and cardiogenic differentiations of fibroblasts were performed by inductive culture in special mediums, and analyzed by Alizarin red, Oil red O and immunofluorescence staining of cTnT respectively. **Results:** The proliferation rate of fibroblasts in lung was faster than in other five organs. Common molecular markers for fibroblasts were expressed differently in different organs. DDR2 was strongly expressed in fibroblasts in the heart, partly expressed in the heart, skin, liver and spleen. Interestingly, no expression of DDR2 was detected in liver and kidney. However, vimentin and FSP1 were consistently expressed in fibroblasts from skin, liver, kidney, spleen and lung. nanog expression in fibroblasts from lung was less than that from heart, skin, liver and spleen ($P < 0.01$). c-kit expression in fibroblasts from heart, skin and kidney was higher than that from spleen ($P < 0.05$), while the c-kit positive fibroblasts from liver was obviously higher than that from spleen ($P < 0.01$). But sca-1 expression in fibroblasts from lung was the lowest among six organs ($P < 0.01$). Directed differentiation in vitro had demonstrated that skin fibroblasts had the strongest multiple differentiation potential, and the next was cardiac fibroblasts. And fibroblasts in liver and kidney had the advantage in myocardial differentiation, while fibroblasts in spleen only had

Zhikun Guo, MD, PhD
and He Li, MD, PhD

Henan Key Laboratory of Medical Tissue Regeneration, Xinxiang Medical University, Xinxiang 453003, (P.R. China); Department of Human Anatomy and Embryology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, (P.R. China); E-Mail gzk@xxmu.edu.cn / heli@mails.tjmu.edu.cn

the advantage in osteogenic differentiation. **Conclusions:** There are obvious heterogeneity in molecular markers and multi-directional differentiation in fibroblasts from six organs.

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Published by S. Karger AG, Basel

Introduction

Fibroblasts are abundantly distributed throughout connective tissues in the body and are very important in maintaining the structural and functional integrity. Fibroblasts have traditionally been viewed as a uniform cell type with supporting and secreting function regardless of the origins of tissue. Recent studies have proved that there are many subpopulations in fibroblasts, which have significant heterogeneity in cell morphology [1, 2], growth characteristics [3], collagen synthesis [4], cytokines and surface receptors [5, 6]. More and more scientists pay close attention to their function in organism because fibroblasts and mesenchymal stem cells share much more in common than previously recognized [7-9]. Indeed, lung fibroblasts have been shown to be heterogeneous in cell surface marker expression, as well as in their levels of collagen production [10]. Moreover, periodontal fibroblasts also show heterogeneity based on morphology, glycogen pools and collagen production [10]. Additionally, fibroblasts from different anatomic sites have distinct transcriptional patterns [11].

However, there is lack of comparative studies on fibroblasts on the differences in the expression of molecular markers and differentiation capacity from different organs. Combined enzyme digestion method was used to isolate and culture fibroblasts derived from heart, liver, spleen, lung, kidney and skin in order to explore the differences of biological phenotypes. Common molecular markers for fibroblasts such as vimentin, FSP1 and DDR2, stem cell markers Nanog, c-kit and sca-1 were detected to identify their heterogeneity. Adipogenic, osteogenic, neural and cardiac induction assays in fibroblasts were also done in order to select optimal organs, to provide the reliable experimental support and optimal seed cells in situ organ repair.

Material and Methods

Animals

Neonatal Sprague-Dawley rats (1-3 day-old, 7.1 ± 0.31 g, $n = 60$) were utilized in this study. Animal treatment was performed according to the guidelines of The Ministry of Science and Technology of the People's Republic of China [(2006)398] and approved by the Xinxiang Medical University Animal Care Committee (No 030032).

Isolation and culture of fibroblasts

Neonatal rats were weighed and executed then the heart, lung, liver, spleen, kidney and skin were obtained and washed separately with PBS in aseptic conditions. The tissues from heart, lung, liver, spleen, kidney and skin were minced into small pieces of about 1 mm^3 and incubated with 0.25% trypsin and 0.125% collagenase enzymes (Sigma-Aldrich, St. Louis, MO, USA) and the digestion time was 40 min, 30 min, 25 min, 20 min, 35 min and 80 min respectively. The suspension was centrifuged at 1200 r/min for 5 min. The cellular pellet was filtered through a $40 \mu\text{m}$ cell strainer (BD Biosciences, San Jose, CA) and was re-suspended in DMEM-LG culture medium supplemented with 8% fetal calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma-Aldrich). The cells were plated in 25 cm^2 dishes and cultured in a CO_2 incubator at 37°C . After 40 min, suspensions were discarded and complete culture media were replenished. Then the culture media were changed every 48 h, when cells reached 85% confluence, and the cells were harvested for further passage following 0.25% trypsin/0.01% EDTA digestion (Amresco, Solon, Ohio, USA). The fibroblasts of passage 3 were plated in 24-well plates and cultured in a CO_2 incubator at 37°C . When cells reached 70% confluence then fixed with 4% paraformaldehyde.

Identification of fibroblasts by immunofluorescence staining

Isolated fibroblasts from six organs were fixed in 4% paraformaldehyde for 30 min at room temperature, subsequently washed with PBS and treated with 0.3% Triton X-100(Sigma) in PBS for 10 min. Then the samples were incubated with PBS containing 2% bovine serum albumin (BSA) at 37° for 10 min. Incubation with the primary antibodies (Rabbit anti-vimentin, Abcam, 1:100; Rabbit anti-FSP1, Abcam, 1:200; Goat anti-DDR2, Santa Cruz, 1:300) was performed at 4° overnight. The samples were subsequently incubated FITC- or Cy3-conjugated secondary antibodies (Beyotime, 1:500). The nuclei were counterstained with 5 mg /L DAPI (Roche). Negative controls were obtained by following all the same protocol but the primary antibodies. All experiments were performed in triplicate.

Cell growth curve from different organs by MTT assay

Primarily cultured fibroblasts from heart, lung, liver, spleen, kidney and skin were seeded into 8,000/well in a CO₂ incubator at 37°C. MTT assay were detected by 5 mg/ml MTT dissolved in PBS, 3 h before the end of the incubation, add 20 µl of solution, then incubate the plate at 37°C for 2 h and add 150 µl DMSO to each well and pipette up and down to dissolve crystals. Then transfer to plate reader and measure absorbance at wavelength 570 nm. All experiments were performed in triplicate. The cell growth curve was determined by the optical density (OD) value.

RT-PCR of molecular markers of fibroblasts from six organs

Total RNA was extracted from passage 3 fibroblasts isolated from six organs. Reverse transcription and PCR were done referring to TaKaRa RNA PCR kit. Primers for PCR amplification were synthesized by Shanghai Sangon Biological Engineering and Technology Service Co. Ltd. Sequences of the PCR primers were seen in Table 1. PCR reaction mixture was subjected to pre-denaturation at 94°C for 5 min followed by 30 amplification cycles each consisting of denaturation at 94°C for 15 s, annealing at 55°C for 40 s and extension at 72°C for 30 s, and then a final elongation cycle at 72°C for 10 min was performed.

Western blotting assay

Fibroblasts of passage 3 from heart, lung, liver, spleen, kidney and skin were lysed and protein was extracted. The number of the cell was about 1 × 10⁸. The protein concentration was measured by BCA assay and was subjected to pre-denaturation at 100°C for 5 min. 100 µg of total protein were loaded in each

Table 1. Sequences of the PCR primers

gene	Forward primer(5'→3')	Reverse primer(5'→3')
<i>nanog</i>	AGAAGATGCGGACTGTGTTC	GCTCAGGTTTCAGAATGGTAGA
<i>c-kit</i>	CTAGCCAGAGACATCAGGA	CCATAGGACCAGACATCAC
<i>sca-1</i>	GTGCTATGATGTCCTGTGT	AACAACCTTCTGCCACCTG
<i>vimentin</i>	GCTAACTACCAGGACACTATT	CAACCGTCTTAATCAGGAGT
<i>DDR2</i>	CAATAACAACCACTCCTCATC	AACCTCTCCACAGCCACATC
<i>FSP1</i>	TCAGCACTTCCTCTCTCTTGG	CACCCTCGTTGCCTGAGTAT
<i>GADPH</i>	TCCCTCAAGATTGTCAGCAA	AGATCCACAACGGATACATT

Table 2. Source and drops of grouped antibodies for immunofluorescence staining

Primary antibodies	Secondary antibodies
①Mouse anti-vimentin (1:100)	Goat anti-mouse IgM-FITC (1:500)
Rabbit anti-sca-1 (1:200)	Goat anti-rabbit IgM-Cy3 (1:500)
②Mouse anti-vimentin (1:100)	Goat anti-mouse IgM-FITC (1:500)
Rabbit anti-c-kit (1:200)	Goat anti-rabbit IgM-Cy3 (1:500)
③Mouse anti-vimentin (1:100)	Goat anti-mouse IgM-FITC (1:500)
Rabbit anti-nanog (1:300)	Goat anti-rabbit IgM-Cy3 (1:500)

well on 8-10% SDS-PAGE gels and electrophoretically blotted onto a PVDF membrane at constant voltage of 90 V for 90 min. The membrane was blocked for 1 h with 5% nonfat dry milk at room temperature. Primary antibodies were added respectively like FSP1 (Abcam, 1:400), CD73 (BD, 1:1000), sca-1 (Merk, 1:500), c-kit (Santa Cruz, 1:1000), vimentin (Abcam, 1:1000), Nanog (Santa Cruz, 1:500) overnight at 4°C, then the PVDF membrane was washed 3 times with TBST for 10 min each. Incubate the membrane with the HRP-conjugated secondary antibody diluted to 1:2000 in blocking solution at room temperature for 1 h. Wash membrane 3 times with TBST for 10 min each. Prepare ECL substrate according to the manufacture's instructions. Incubate the membrane with substrate completely for 1-5min. Exposure autoradiography film exposure under a chemiluminescence imaging system. The results were analyzed with Image-Pro Plus 6.0 software.

Immunofluorescence staining for cell surface markers

Fibroblasts from six organs grown on coverslips were fixed with 4% paraformaldehyde for 20 min, subsequently washed with PBS three times, and treated with 0.3% Triton X-100 in PBS for 10 min at room temperature. The samples were then blocked with 2% BSA for 20 min, and incubated with the mixed primary antibodies grouped as Table 2 at 4°C overnight (Table 2). The samples were subsequently incubated with FITC- or Cy3-conjugated secondary antibodies and DAPI (Table 2). Control staining was performed without primary antibody. All experiments were performed in triplicates.

Directional differentiation culture

Fibroblasts from six organs at passage 3 were plated at 1×10^5 cells/well in 24-well plates in DMEM supplemented with 8% FBS and were cultured for 4 h. For cardiomyogenic differentiation, cells were treated with complete DMEM supplemented with 10 μ M 5-azacytidine (5-Aza; Sigma, St. Louis, MO, USA) for 24 h. Next, cells were washed twice with PBS, the medium was replaced by complete medium without 5-Aza. The medium was changed every two days. Cells were fixed on day 30 and cardiomyogenic differentiation was identified by immunostaining to measure expression of myocardial cell marker cTnT. For adipogenic, osteogenic and neural differentiation induction, the culture medium was replaced with low-glucose DMEM containing 10% FBS plus adipogenic reagents (1 μ M Dexamethasone, 0.01 mg/ml insulin, 200 μ M indometacin, 0.5 mM Isobutyl- methylxanthine), 10%FBS plus osteogenic reagents (0.1 μ M Dexamethasone, 10 mM sodium β -glycerophosphate, 0.05 mM L-Ascorbic Acid) and DMEM/F-12 medium plus neural reagents(0.2 μ g/L basic fibroblast growth factor, 0.2 μ g/L epidermal growth factor, 2% B27) respectively. The adipogenic or osteogenic media was changed every 2 days. Control group was maintained using low-glucose DMEM containing 10% FBS. Adipogenic differentiation was identified by Oil Red O following 14 days of adipogenic induction, osteogenic differentiation was identified by Alizarin Red following 21 days of osteogenic induction and neural differentiation was identified by immunofluorescence staining for neuron-specific enolase (NSE). The images were captured by microscopes and 5 fields were randomly selected from each well in 24-well plates, finally 30 fields were chosen to get the differentiation rate which was $N1/N \times 100\%$, while N1 was the number of the differentiation cells and N was the total number of viable cells evaluated under the same vision.

Statistical analysis

The data were analyzed by SPSS 17.0 software. The rate of co-expression equated the total number of cells with nuclei was divided by the number of co-expression in 30 random fields. The results were shown by mean $\pm$ standard deviation ($\bar{X} \pm S$), and two different samples were compared by T-test, while multiple samples were compared with single-factor analysis of variance according to the $\alpha = 0.05$, $P < 0.05$ was considered as a statistically significant difference.

Results

The morphology of isolated fibroblasts from six organs

Neonatal rat fibroblasts from heart, lung, liver, spleen, kidney and skin were isolated and began to adhere to the wall in 30-40 min. They showed triangle, shuttle or irregular polygons and had a branched cytoplasm surrounding an elliptical, speckled nucleus having

Fig. 1. Morphology of cultured fibroblasts passage 3 from six organs under phase-contrast microscope. (A) cardiac fibroblasts on 3 d. (B) skin fibroblasts on 2 d. (C) fibroblasts in liver on 3 d. (D) fibroblasts in kidney on 4 d. (E) splenic fibroblasts on 4 d. (F) fibroblasts in lung on 2 d. Scale bar = 50 μ m.

Fig. 2. *Vimentin*, *DDR2* and *FSP1* in fibroblasts from six organs were detected by RT-PCR. *Vimentin* and *FSP1* were detected in fibroblasts from six organs, while *DDR2* expressed in heart, spleen, liver and spleen, but not in kidney and lung.

two or more nucleoli. They could not beat spontaneously and had high proliferation and metabolic activity. Isolated fibroblasts from different organs had different morphology in cell shape and volume (Fig. 1). Fibroblasts in heart showed triangle, shuttle shaped and polygons and more than 40% with double nuclei. Proliferated phases were visible obviously (Fig. 1A). Skin fibroblasts were the most diverse in morphology, except for shuttle shaped, triangle and polygons, they showed star-like or oval shapes and mutually interwove like a mesh (Fig. 1B). There were more triangle and shuttle shaped in liver (Fig. 1C), while more polygons and larger cell body in kidney (Fig. 1D). Splenic fibroblasts had smaller cell body and more shuttle shaped, and the percentage of double nuclei accounted for 24% (Fig. 1E), but most fibroblasts in lung were triangle (Fig. 1F).

Difference gene expression in molecular markers from six organs

Vimentin, discoidin domain receptor 2(DDR2) and Fibroblast-specific protein 1(FSP1) in fibroblasts from six organs were detected by RT-PCR. Vimentin and FSP1 were picked up in fibroblasts from six organs, while DDR2 was expressed in heart, spleen, liver and spleen, but not in kidney and lung (Fig. 2).

Growth characteristics in fibroblasts from different organs

There were also differences in cell growth curve and lag phase in fibroblasts from six organs. The lag phase was about 8-48 h in skin, lung and liver, 8-72 h in spleen, heart and kidney. The log phase was nearly 48-72 h in skin and lung, then to stationary phase after 72 h, while the log phase was 72-120 h in spleen and kidney, and 96 h in heart. Isolated

Fig. 3. Cell growth curve of fibroblasts from six organs. The OD value in skin, lung and liver had significantly increased after being cultured for 48 h than that for 8 h (** $P < 0.01$). There was significantly increase in kidney and heart cultured at 96 h (** $P < 0.01$). At 120 h, the OD value in spleen had significantly increased (** $P < 0.01$).

Fig. 4. Western blotting detection of DDR2, FSP1, vimentin, nanog, c-kit and sca-1 protein in fibroblasts from six organs. vimentin and FSP1 were all detected in fibroblasts from six organs and there were no significantly difference among six organs ($P > 0.05$). DDR2 was detected mainly in heart, partly in skin, liver and spleen, not in lung and kidney. sca-1 expression in fibroblast from heart, liver and spleen were more than that in fibroblasts from other three organs (** $P < 0.01$). C-kit expression in fibroblasts from heart was the most popular than other five organs (** $P < 0.01$).

fibroblasts from six organs all showed a typical S-shaped curve from lag phase, log phase and stationary phase respectively.

Though comparison with proliferation rate in fibroblasts from skin, spleen, kidney, heart and liver in different periods, there were difference in Fig. 3. The OD value in skin, lung and liver had significantly increased after being cultured for 48 h than that for 8 h ($P < 0.01$). The OD value in kidney and heart had significantly increased after being cultured for 96 h than that for 8 h ($P < 0.01$). There was significantly difference in spleen cultured at 120 h ($P < 0.01$). The proliferation rate in fibroblasts at 72 h was skin>lung>liver>kidney=spleen>heart, skin>lung>liver>kidney>heart>spleen at 96 h, and skin>lung=heart>liver>kidney>spleen at 120 h. These results showed that the proliferation rate in skin was fasted, while the lag phase of fibroblasts in heart was longest up to almost 96 h.

Fig. 5. The co-expression of vimentin and nanog protein in neonatal rats heart, liver, spleen, lung, kidney and skin by immunofluorescence staining. The nuclear stained by DAPI is shown in A1-F1; The red in A2-F2 and the green in A3-F3 were stained by vimentin marked by Cy3 and nanog protein marked by FITC; A4-F4 shown the merge result of the left three columns, the yellow indicate co-expression of vimentin and nanog protein. Scale bar = 50 μ m.

Comparison of protein expression in cultured fibroblasts from different organs

Total proteins of fibroblasts from six organs were extracted and were detected by western blotting. Consistent with RT-PCR, there were also differences of protein expression in fibroblasts from different organs. Vimentin and FSP1 were all detected in fibroblasts from six organs and there were no significantly difference among six organs ($P > 0.05$). DDR2 was detected mainly in heart, partly in skin, liver and spleen, not in lung and kidney (Fig. 4A). There were no significantly difference in nanog expression in fibroblasts from six organs ($P > 0.05$, Fig. 4A), but sca-1 expression in fibroblast from heart, liver and spleen were more than that in fibroblasts from other three organs (Fig. 4B). C-kit expression in fibroblasts from heart was the most popular than other five organs ($P < 0.01$).

Differential expression of nanog, c-kit and sca-1 in fibroblasts from six organs by immunofluorescence staining

Isolated fibroblasts *in vitro* from six organs were detected by immunofluorescence double labeling. Vimentin was the major cytoskeletal component of fibroblasts, clearly visible in the distribution within the cell (Fig. 5-7). Nanog were mainly detected within

Fig. 6. The co-expression of vimentin and c-kit protein in neonatal rats heart, liver, spleen, lung, kidney and skin by immunofluorescence staining. The nuclear stained by DAPI is shown in A1-F1; the red in A2-F2 and the green in A3-F3 were stained by c-kit marked by Cy3 and vimentin marked by FITC; A4-F4 shown the merge result of the left three columns, the yellow indicate co-expression of vimentin and c-kit protein. Scale bar = 30 μ m.

cytoplasm, partly in nuclei of fibroblasts from six organs (Fig. 5). C-kit was also detected in cytomembrane and cytoplasm, but majority of positive fibroblasts were in round and oval shape (Fig. 6). Sca-1 expressed in nuclei of fibroblasts from liver, kidney, spleen and lung, while sca-1 was detected not only in nuclei, but also in cytoplasm of fibroblasts from heart and skin (Fig. 7).

Multipotential differentiation in fibroblasts from six organs

Fibroblasts from six organs were induced by 5-aza respectively, then cells morphology had more changes in shape and proliferation comparing to the control group without 5-aza. The majority of cells showed triangle and spindle before induction. But they steadily turned

Fig. 7. The co-expression of vimentin and sca-1 protein in neonatal rats heart, liver, spleen, lung, kidney and skin by immunofluorescence staining. The nuclear stained by DAPI is shown in A1-F1; the red in A2-F2 and the green in A3-F3 were stained by sca-1 marked by Cy3 and vimentin marked by FITC; A4-F4 shown the merge result of the left three columns, the yellow indicate co-expression of vimentin and sca-1 protein. Scale bar = 30 μ m.

to round or rod and slow proliferation after induction, and the induced cells were gradually bigger with increased secretions. The cells in control group were woven through the bottom of the dish (data not shown). After 28 d induction, some cells had positive expression for cTnT (Fig. 8A-D).

Fibroblasts were induced by neuronal induction medium and their shape turned to long rod from flat shape, their cell processes were gradually elongated and slow proliferation. At the same time, nuclei became smaller, cytoplasm shrank and cell parameters significantly enhanced. After 5 d, the cells grew into secondary branches and their long protrusions connected into a network between adjacent cells, but there were still a few fibroblasts whose morphology did not change obviously. NSE protein was detected strongly in induced cells on 7th day (Fig. 8E-H), which showed that fibroblasts could be differentiated into neuron-like cells.

Fig. 8. Differentiation detection of fibroblasts cultured *in vitro*. (A-D) cardiogenic differentiation of fibroblasts after 30 d culture following exposure to 10 μ M 5-Aza for 24 hours. (A) the nuclear stained by DAPI; (B) FSP1 immunostaining. (C) cTnT immunostaining. (D) the merged image of the A-C. (E) the nuclear stained by DAPI; (F) NSE immunostaining. (G) vimentin immunostaining. (H) the merged image of the E-G. (I) Alizarin red staining, Osteogenic differentiation following exposure to osteogenic medium for 21 d. (J) Oil Red O staining, adipogenic differentiation following exposure to adipogenic medium for 10 d. Scale bar = 30 μ m.

Fibroblasts were induced by osteogenic induction medium on 3rd, and they turned to round, oval and reniform, their volume significantly increased. Partial induced cells aggregated into colony-like with particulate matter floating on 10th day (data not shown). After 21 d, there were many red calcium salt depositions by Alizarin red staining (Fig. 8I).

Induced fibroblasts began to become oval and shuttle shaped after being cultured by adipogenic induction medium on 2nd day and the number of cells reduced sharply, cellular processes shrunk and bigger cytoplasms. Then the cell bodies were full and nuclei were smaller after 7 d. At the time of 10th day, many small and round empty bubble-like structures were located in larger cytoplasms with good dioptr. After 14 d, some small and round empty bubble-like structure gradually fused into variable bigger bubbles and were detected as multilocular fat in induced fibroblasts by Oil Red O staining (Fig. 8J).

Cultured fibroblasts from six organs were induced into adipogenic, osteogenic, myocardial and neural differentiation respectively *in vitro*, which had demonstrated that fibroblasts from different organs had multipotential differentiation although there were differences in differentiation ability. Fibroblasts isolated from skin had the most advantage to differentiate into cardiomyocytes, osteocytes, adipocytes and neurons than those from other five organs (Fig. 9). Splenic fibroblasts especially had the most poor differentiation ability except for osteogenic differentiation, as there was significant difference ($P < 0.01$). Fibroblasts derived from heart, skin, liver and kidney were detected for adipogenic differentiation which accounted for 26.09-39.03%. Fibroblasts from skin, liver, kidney and spleen had better differentiation ability in neurons. Comparing with fibroblasts from kidney, fibroblasts from

Fig. 9. Multi-directional differentiation quantitative analysis of fibroblasts from six organs. Adipogenic differentiation: fibroblasts from heart, skin, liver and kidney had significant difference comparing with in spleen (** $P < 0.01$). Osteogenic differentiation: comparing with in kidney, fibroblasts in spleen had difference (* $P < 0.05$), while fibroblasts in heart, skin and lung had more significant advantages (** $P < 0.01$). Myocardial differentiation: comparing with in spleen, fibroblasts in heart and skin had difference (* $P < 0.05$), while fibroblasts in liver, lung and kidney had more significant advantages (** $P < 0.01$). Differentiation ability in neurons: comparing with in lung, fibroblasts in lung and spleen had difference (* $P < 0.05$), while fibroblasts in skin, liver and kidney had significant advantages (** $P < 0.01$).

heart, skin and lung had more significant advantages in osteogenic differentiation ($P < 0.01$), while the differentiation ability of fibroblasts from spleen was also significantly different ($P < 0.05$). The percentage of myocardial differentiation was much higher than that of other three differentiation directions. The different myocardial differentiation ability was: fibroblasts from kidney>liver>lung>heart>skin>spleen. Compared with fibroblasts from spleen, fibroblasts from kidney, liver and lung had significant advantage ($P < 0.01$), while fibroblasts from heart and skin were also different ($P < 0.05$).

Discussion

Fibroblasts synthesized the extracellular matrix (ECM) and collagen and played a critical role in development, the process of organ fibrosis, wound healing and angiogenesis, abundantly distributed throughout connective tissues in the organisms [12]. They were morphologically heterogeneous with diverse appearances depending on their location and activity, which showed spindle, triangle, polygon, irregular and flat star shape. Fibroblasts could also express various cytokines receptors, such as platelet derived growth factor (PDGF) and transforming growth factor $\beta 1$ (TGF- $\beta 1$) which would stimulate fibroblasts to proliferated rapidly and migrate into injured site once tissue damage, leading to functional impairment and scar formation [13, 14]. At present, being lack of specific markers for fibroblasts, further researches were greatly restricted.

Vimentin was a type III intermediate filament protein that was expressed in fibroblasts, endothelial cells and neurons. Given the characteristic cyto-morphological differences between these cells, anti-vimentin had been a suitable tool for reliable identification of fibroblasts. Discoidin domain receptor 2 (DDR2) was detected in cardiac fibroblasts in rodent animals [15, 16]. DDR2 expressed also in leukocytes and tumor cells, but not expressed in cardiomyocytes, endothelial cells and smooth muscle cells [17]. In addition, Fibroblast-specific protein 1 (FSP1) was also labeled with fibroblasts [18], although FSP1 was also detected in lymphocytes and tumor cells [19]. In this study, common molecular markers

for fibroblasts were expressed differently in different organs. DDR2 was strongly expressed in fibroblasts in the heart, partly expressed in the skin, liver and spleen. Interestingly, no expression of DDR2 was detected in liver and kidney. However, vimentin and FSP1 were detected and were consistent expression in fibroblasts from skin, liver, kidney, spleen and lung. So combined expression of vimentin and FSP1 could be as effective molecular markers for fibroblast in six organs.

More recently, several reports demonstrated that fibroblasts had some heterogeneity in phenotypes and transcription patterns from different organs even though cultured *in vitro* [20]. Burstei et al. reported there was different morphology although with consistent gene expression [21]. Driskell et al. persisted in fibroblasts from skin were arised from two lineages [22]. One formed the upper dermis and regulated hair growth and arrector pili muscle, and the other formed the lower dermis and synthesized the bulk of the fibrillar ECM, and the preadipocytes and adipocytes of the hypodermis. The proliferation of fibroblasts from pulmonary fibrosis was obviously higher than normal control group [23]. Fibroblasts from lung had smaller cell and faster proliferation rate compared with skin fibroblasts cultured *in vitro*, which also supported that fibroblasts from different organs had various biological characteristics [24].

It was widely believed that mesenchymal stem cells (MSCs) were multipotential fibroblast-like cells with CD73+, CD105+, CD14-, CD34- and CD45- and plastic adherent [25-27]. Recent studies had revealed that fibroblasts shared much more in common than previously recognized. Mouse embryonic fibroblasts (MEF) expressed Sca-1+, CD73+, CD105+, CD29+, CD44+, CD106+, CD11b- and CD45- [28]. Cultured fibroblasts had the positive expression in Oct-4 [29]. Dermal fibroblasts derived from rodent and human had also mesenchymal stem cell characters, such as same surface antigen and differentiation potential [30]. Our previous studies had proved that nanog was positive for cardiac fibroblasts in the infarction border zone of 1W acute myocardial infarction (AMI) model [31, 32]. However, it is not clear whether nanog expresses in normal organs, such as heart, lung, liver, spleen, kidney and skin. Moreover, there is still lack of comparative studies in fibroblasts on the differences in the expression of molecular markers and differentiation capacity from different organs. Therefore, cultured fibroblasts from heart, liver, lung, spleen, kidney and skin were detected for nanog, c-kit and sca-1 in gene and protein level in this study. Fibroblasts from six organs were further induced into cardiomyocytes, osteocytes, adipocytes and neurons by directed induction medium respectively.

Nanog is an important transcript factor which keeps embryonic stem cells (ESCs) self-renewal and their pluripotent [33]. c-kit, also named stem cell growth factor receptor (SCFR), is a molecule marker of stem cells and plays an important role in cell differentiation and proliferation [34]. Bearzi et al. described c-kit⁺ cluster in cardiac stem cell niche and confirmed endogenous cardiac stem cells (CSCs), playing an important role in myocardial regeneration and repair [35]. Stem cell antigen 1 (sca-1) is another stem cell surface marker, which plays an important role in regulation of hematopoietic stem cell self-renewal, differentiation and c-kit expression [36]. Sca-1⁺/CD31⁻ CSCs can realize effective repair in myocardial infarction model [37]. Sca-1⁺ ESCs have strong self-regeneration and differentiation ability [38].

There are heterogeneous in c-kit and sca-1 expression in fibroblasts from different organs, no matter in gene level or in protein expression, while there were no significant difference in nanog expression in fibroblasts from six organs ($P > 0.05$). Sca-1 expression in fibroblast from heart, liver and spleen were more than that in fibroblasts from other three organs. And c-kit expression in fibroblasts from heart was more popular than that in other five organs ($P < 0.01$). The results demonstrated that their heterogeneous phenotypes in different organs responded to the respective functions in microenvironment. Fibroblasts from heart and liver have also higher expression for stem cell markers except for fibroblasts from skin, which shows similarities in phenotypes of mesenchymal stem cells among three organs.

Chen et al. reported nestin⁺/vimentin⁺ dermal fibroblasts could differentiate into adipocytes, osteocytes and chondrocytes without inducer [39]. Jeney considered fibroblasts as preadipocytes due to their ability of spontaneously transferring into adipocytes [40].

Mizuno cultivated skin fibroblasts into three-dimensional collagen sponge scaffold and detected chondrocytes [41]. In this study, fibroblasts from heart, liver, spleen, lung, kidney and skin were induced into adipocytes, osteocytes, neurons and myocytes respectively, which demonstrated fibroblasts from six organs, had all multi differentiation potential in spite of their different differentiation ability. Skin fibroblasts had strong multiple differentiation potential, and the less strong one was cardiac fibroblasts. And fibroblasts in liver and kidney had advantage in myocardial differentiation, but fibroblasts in spleen only had advantage in osteogenic differentiation. And the rest had the lowest differentiation ability. Therefore, only partial fibroblasts from six organs in neonatal rats can differentiate into adipocytes, osteocytes, neurons and myocytes, which shows different populations in fibroblasts; 2induced adipocytes are proved to be multilocular adipocytes and maybe belong to brown adipose.

In conclusion, common molecular markers DDR2, vimentin and FSP1, stem cell markers nanog, c-kit and sca-1, differentiated advantage were systematically compared in cultured fibroblast from heart, liver, spleen, lung, kidney and skin in neonatal rats. These results provide the direct evidence that fibroblasts from different organs are heterogeneous in mesenchymal stem cell characters and fibroblasts in skin, liver and heart have stronger differential potential. It also put forward new issues to select the optimal seed cells for organ repair in situ and dominant population in fibroblasts, which is our further important topic to explore.

Acknowledgements

We thank all members of our lab for sharing reagents and advice. This work was supported by the National Natural Science Foundation of China (No. 81570268) and the Foundation and Advanced Research Plan of Henan province (No. 162300410106).

Disclosure Statement

The authors confirmed that there are no conflicts of interest.

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