Analysis of how obtaining melanocytes by magnetic cell separation contributes to autoepidermal transplantation technology in treating leucoderma

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Conflict of interest

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Abstract

Background. Leucoderma, a depigmentation of the skin, is a common disease in humans, and has been observed in cattle, horses and buffalo as well.

Objectives. To analyze the correlation between melanin stem cells and the differentiation and proliferation of melanocytes (MCs).

Material and methods. Magnetic cell separation was used to separate melan-A+ cells and PAX3+ cells, which were cultured in vitro. The L-DOPA staining was used to observe cell morphology; Cell Counting Kit-8 (CCK8) was used to determine the cell proliferation rate; and flow cytometry (FCM) was used to determine cell cycle changes. The relative mRNA levels of melanocyte-inducing transcription factor (MITF), dopachrome tautomerase (DCT) and melan-A in cells were determined with reverse-transcription polymerase chain reaction (RT-PCR).

Results. The number of MC dendrites increased and extended continually during in vitro culture following magnetic cell separation. The proportion of positive L-DOPA staining cells increased from a baseline 40.70% to 82.03%, and the cell proliferation rate increased from 335.0% at D3 to 1577.4% at D20. The results of FCM showed that the cell proportion at the G1 stage in the D20 group was significantly lower than the D3 group; the cell proportion at the G2/M stage also decreased significantly. The expression of MITF and melan-A increased as the culture time increased, while the expression of DCT decreased.

Conclusions. The number of MC stem cells decreased and mature MCs increased gradually, indicating that MC stem cells can gradually differentiate into mature MCs during in vitro culture following magnetic cell separation.

Key words: MSCs, MCs, magnetic cell separation, leucoderma

Cite as

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Introduction

Leucoderma, a depigmentation of the skin, is a common disease in humans, and has been observed in cattle, horses and buffalo as well. Its main characteristic is the appearance of limited or extensive discoloration of areas of the skin, with distinct boundaries. Most of the patients are teenagers. Leucoderma affects nearly 2% of the world population.¹ The causes of leucoderma are complex and its pathogenesis can be divided into neurosexual theory, immunology theory, oxidation and reduction theory, transformation theory, among others.² The mechanisms causing melanocyte (MC) destruction or dysfunction are an important part of the pathogenesis of leucoderma. Leucoderma causing large areas of depigmentation that affect patients' appearance and quality of life is especially difficult to treat. The main treatments are external hormones, calcineurin inhibitors, photosensitizers, phototherapy, and surgical transplantation.³ Surgical transplantation includes epidermal transplantation and melanoma cell transplantation. Therefore, the cultivation of MCs is important in autologous epidermal transplantation technology.

Melanocytes are phenotypically prominent but histologically inconspicuous skin cells. They are responsible for the pigmentation of skin and hair, and thereby contribute to the appearance of the skin.⁴ Mutations of the function or number of MCs can lead to a series of refractory pigmentation diseases, such as leucoderma and chloasma. The surface dependent antigens of MCs have melanoma antigen recognized by T cells-1 (melan-A), melanocyte-inducing transcription factor (MITF), tyrosinase (TYR), and tyrosinase-related protein-2 (TRP-1).⁵ Melan-A is synthesized by the melanosomes and endoplasmic reticulum of MCs, and has been identified as a marker antigen for mature MCs.⁶ Research shows that the cultivation of MCs is difficult. As compared with the other main types of skin cells, the percentage of MCs in epidermis cells is low, as is mitotic activity, which leads to the rapid growth of other cells, such as keratinocytes (KCs) and fibroblasts (FBs).⁷ Reports have shown that MC stem cells (MSCs) can differentiate into mature MCs in leucoderma patients.⁸ Most MSCs stay in the resting stage and can turn into MCs when stimulated by the related cytokines, which induces the formation of melanin. Current research on the mechanisms of MCs and the proliferation and differentiation of MSCs is mainly based on mice, while research on human MCs and MSCs is rarer.⁹ At the same time, regarding the co-culture of MCs, few reports in the literature have discussed whether MSCs participate in the differentiation of MCs. Therefore, further study of MSCs on changes in pigment and the mechanisms and treatment of related diseases has major significance. Since foreskins contain a relatively high density of MCs,¹⁰ they are the main cell type for the cultivation of MCs.

In this study, the enzyme digestion method was used to isolate and obtain skin cells in normal human foreskin

tissue. Magnetic cell sorting (MACS) was used to isolate PAX3+ MSCs and mature melan-A+ MCs from primary hybrid cells. The L-DOPA staining was used to determine the cell proliferation rate, cell cycle and related specific markers.

Material and methods

Obtaining tissue to culture

Normal skin tissues were obtained from men aged 20–50 years who underwent circumcision in the Urinary Surgery Department of Shanghai First People's Hospital (China) between February 2017 and December 2017. All the participants signed informed consent forms. Patients with a chronic skin history, type 2 diabetes, cardiovascular disease (CVD), chronic gastritis, any history of leucoderma, hyperthyroidism, systemic lupus erythematosus, or other autoimmune-related diseases were excluded.

Tissue cell culture

The prepuce tissues were washed 3 times in 30 mL of phosphate-buffered saline (PBS). Then, they were cut into approx. 2 × 2 mm squares with sterile scissors, put in 40 mL of 0.05% trypsin-EDTA (8 mL of 0.25% trypsin-EDTA + 32 mL of PBS) and refrigerated overnight at 4°C to digest. Then, 40 mL of 0.05% collagenase was added to the digested tissues and stirred with a magnetic stirrer for 2 h to digest. Afterwards, the samples were filtered and centrifuged at 1800 rpm for 5 min. Cells were resuspended in 1% human melanocyte growth supplement-2 (HMGS-2) and 0.04 µg/mL of amikacin keratinocyte serum-free medium. Following this, 100 µL of the re-suspending solution was taken out and 25 µL of trypan blue was added to the mix for cell counting. Cell concentration was adjusted and inoculated to T75 culture flasks at a density of 1 × 10⁶ per flask, and then the cells were put in an incubator with 5% CO₂ at 37°C.

After 24 h, the primary cell culture dish was taken out of the incubator and washed with 15 mL of PBS. The cells were put back in the incubator with 5% CO₂ at 37°C and the cell morphology was observed regularly under an inverted microscope. A subculture was performed when cell growth density reached 60~70%.

Magnetic beads

Twenty microliters of magnetic beads marked with biotin, 20 µL of PAX-3 antibody marked with biotin and 20 µL of melan-A antibody marked with biotin were refrigerated overnight at 4°C. The previously separated skin-cell mixes were taken out of the incubator and pancreatin was added until the cells became round under the microscope. The cell solution was transferred to a 15 mL centrifuge

tube and centrifuged at 1000 rpm for 10 min. Five hundred microliters of PBS was used to suspend cells, and biotin magnetic bead with PAX-3 and melan-A antibody was added to the cells for 90 min. Cells were obtained by centrifugation and put in the MS separation column (Miltenyi Biotec Technology & Trading; Shanghai, China). Magnetic bead-marked cells were obtained and cryopreserved at -80°C .

Hematoxylin staining

A histological evaluation was performed after 3 weeks. The hydrogels were fixed overnight in 4% paraformaldehyde at 4°C and transferred to 70% ethanol until embedded in paraffin according to standard histological techniques. Sections were stained with hematoxylin and eosin (H&E) for general cell morphology and using the von Kossa method to characterize the mineralization.

L-DOPA staining

Cells adhering to the wall were washed in PBS, and 4% triformol was added for fixation at room temperature for 10 min. After washing 3 times with PBS, 10 mL of 0.1M phosphate buffer (PB) was added and the cells soaked for 10 min. The supernatant was discarded, 0.1% L-DOPA was added and the cells were put in an electric heat blower drier to stain for 4 h. Four fields were selected under a microscope, and the number of cells in each field was calculated.

Cell proliferation

Cell growth was analyzed using a WST-8 Cell Counting Kit-8 (CCK-8; Beyotime, Nanking, China). Cells (7.5×10^3) suspended in 100 μL of RPMI 1640 medium containing 10% fetal bovine serum (FBS) were seeded in 96-well plates and incubated for 2 days. The CCK-8 solution (10 μL) was added to each well and the cultures were incubated at 37°C for 90 min. Absorbance at 450 nm was measured using an immunoreader C10066-50 (Hamamatsu Photonics; Hamamatsu, Japan). The results were plotted as means $\pm$ standard deviation (SD) of 3 separate tests, with 4 measurements per test for each of the experimental conditions.

Cell cycle

Cell cycle distribution was analyzed using flow cytometry (FCM). After the treatments outlined above, the cells were trypsinized, rinsed with PBS, fixed with 70% ethanol at 4°C overnight, and then treated with RNaseA (0.02 mg/mL) in the dark at room temperature for 30 min. The cells were resuspended in 0.05 mg/mL propidium iodide and analyzed with a flow cytometer (Becton, Dickinson, Franklin Lakes, USA). DNA histograms were analyzed using ModFit

LT v. 2.0 software (Verity Software House, Topsham, USA). For each sample, at least 104 events were recorded.

Tyrosinase activity determination

Cultured cells that had undergone magnetic cell separation were inoculated on 96-well plates with 1×10^5 cells/well and incubated with 5% CO_2 at 37°C for cultivation. After 24 h, the supernatant was discarded and the precipitate was washed twice with PBS. To each well, 500 μL of culture medium was added; after magnetic bead separation, D3 cells were taken as the control. Next, 160 μL of 10g/L Triton X-100 (pH 7.5) was added to each well, and the culture plate was put in -80°C refrigerator for 30 min. Then, the cells were split at 37°C for 4 h with 100 μL of 0.1% L-DOPA solution added to each well. After 4 h, the absorbance value in each well was determined. Tyrosinase relative activity was calculated as „(eq. 1):

$$\text{tyrosinase relative activity} = \frac{(\text{average absorbance of the experimental group} - \text{average absorbance of the blank group})}{(\text{average absorbance of the control group} - \text{average absorbance of the blank group})}$$

RNA isolation and quantitative RT-PCR

Based on the manufacturer's instructions, we extracted total mRNA from the retinal samples using TRIzol Reagent (Life Technologies, Grand Island, USA). A pipette was used mix the samples and then transfer them to 1.5 mL Eppendorf (EP) tubes, and the samples were kept standing for 5 min to separate the nucleic acid protein complex. To each EP tube, 1 mL of Trizol and 200 μL of pre-cooled chloroform was added. The tubes were shaken for 15 s and centrifuged at 4°C , 12,000 rpm, for 15 min. We drew the aqueous phase into fresh 1.5 mL EP tubes and added 0.5 mL of isopropanol. The mixture was centrifuged at 4°C , 12,000 rpm, for 15 min. After removing the supernatant, 1 mL of pre-cooled 75% ethanol was added to wash the RNA precipitate; this procedure was repeated 3 times. Then, the RNA precipitate was dried in a vacuum and its concentration was determined with a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). One microliter of Oligo dT (0.5 $\mu\text{g}/\mu\text{L}$), 2 μg of total RNA and 12 μL of diethylpyrocarbonate (DEPC) were added to polymerase chain reaction (PCR) tubes. After being mixed evenly, the tubes were placed in a 65°C water bath for 5 min, and then immediately placed on ice. Reverse transcription was performed at 55°C for 30 min, with initial activation for 15 min at 95°C ; next, 40 cycles of denaturation were conducted at 94°C for 15 s, then annealing for 30 s at 55°C and extension for 30 s at 72°C were performed. The expression level was normalized using U6 small nuclear RNA using the $2^{-\Delta\text{Ct}}$ method. The ΔCt values were normalized to GAPDH level.

Statistical analysis

The statistical analyses were performed using SPSS software v. 20.0 (SPSS Inc., Chicago, USA). All the data was expressed as means $\pm$ SD. Student's t-test or a one-way analysis of variance (ANOVA) test was performed to determine significant differences. $P < 0.05$ was considered statistically significant.

Results

Cell morphology

Cell morphology was observed before and after magnetic cell separation; the results are shown in Fig. 1. As can be

seen, after digested separated skin cells were inoculated for 24 h, some cells stuck to the wall; the cells were round, triangular or oval; and a lot of floating cells could be seen. After 3 days, the cells gradually formed into small colonies (Fig. 1A). After magnetic bead separation, melan-A+ and PAX3+ cells comprise 10.26% of the total. After separation for 24 h, some cells adhered to the wall, and most of them grew in bipolar or multi-stage dendrites. Most of the MC cells had 2 or 3 dendrites and no obvious KCs were observed (Fig. 1B). Before the 1st transmission, multiple dendritic cells could be seen but they grew slowly. Melanocytes also grew slowly. However, multi-stage dendritic processes and slender axons were obvious and there were no obvious KCs. After a few generations, MCs grew fast and multi-stage dendritic growth appeared with 3 to 4 dendrites, and no obvious KC growth was seen (Fig. 1C). After the 2nd transmission, the lens is covered with dendritic cells, the slender axis is prominent and no obvious KC growth was seen (Fig. 1D). In conclusion, the number of MCs increased as the incubation time increased, reaching a maximum on the 20th day of culture.

Absolute cell count and morphology after magnetic bead separation

The number of mixed cells in the primary skin was approx. 2.0×10^7 . After separation, the number of PAX3+ and melan-A+ cell was about 1.9×10^6 . Cell passage cultivation was carried out on the 3rd day after magnetic bead separation and the number of cells was 2.5×10^6 (Fig. 2A). The cells were inoculated into T75 culture flasks at the density of $1 \times 10^6/\text{T75} \times 2$ bottles in vitro and cell morphology was observed under a microscope. As can be seen, the MC cells were lightly dyed, had a bipolar or tripolar form, and part of the extension was dendritic (Fig. 2B). Cell passage cultivation was carried out on the 10th day after magnetic bead separation and the number of cells was 5.8×10^6 . A large number of L-DOPA-stained positive cells were observed under a microscope. The cell bodies were larger than before, the dendrites were obvious and the length varied (Fig. 2C). Cell passage cultivation was carried out on the 20th day

Fig. 1. Cell growth situation before and after magnetic cell separation

A. Digested isolated skin cell growth after adherence to the wall of the culture flask for 3 days. Melan-A+ and PAX3+ cell growth after magnetic cell separation for (B) 1 day, (C) 10 days and (D) 20 days

Fig. 2. The counts and morphology of melan-A+ and PAX3+ cells after magnetic cell separation

A – absolute cell counts at different times after magnetic cell separation. L-DOPA staining of melan-A+ and PAX3+ cells observed under a microscope after magnetic cell separation for (B) 1 day, (C) 10 days and (D) 20 days

separation, the optical density (OD) value was 0.316 ± 0.002 ; it was 0.469 ± 0.012 on the 3rd day; 0.723 ± 0.008 on the 5th day; and 1.222 ± 0.009 on the 7th day. This indicated that the proliferative capacity of the cells increased with time after magnetic bead separation.

Flow cytometry was used to determine the proportion of cells in the cell cycle stages at different times, and results are shown in Fig. 3B. On day 3, the ratio of cells at the G1 stage was 71.30 ± 0.27 ; the ratio at the S stage was 12.81 ± 0.40 ; and at the G2/M stage the ratio was 15.9 ± 0.13 . On day 10, the ratio of cells at the G1 stage was 68.19 ± 0.80 ; the ratio at the S stage was 20.88 ± 0.75 ; and at the G2/M stage it was 10.93 ± 0.11 . On day 20, the ratio of cells at the G1 stage was 63.83 ± 0.20 ; the ratio S stage was 29.83 ± 0.41 ; and the ratio at the G2/M stage was 6.27 ± 0.30 . Compared with the control (day 3), the G2/M phase of cells was significantly decreased after magnetic bead separation, the percentage of cells at the S stage increased and the percentage of cells at the G1 stage decreased slightly.

Changes in tyrosinase activity

Tyrosinase activity in cells was determined at different times after magnetic cell separation, and the results are shown in Fig. 4. As shown, the OD value after separation for 3 days was 0.295 ± 0.008 ; on the 10th day the value was 0.486 ± 0.007 ; and on the 20th day it was 1.038 ± 0.002 . The ratio of tyrosinase activity (D10) to tyrosinase activity (D3) was 2.0, and the ratio of tyrosinase activity (D20) to tyrosinase activity (D3) was 4.91.

after magnetic bead separation and the number of cells was 1.83×10^7 . Under a microscope, a large number of heavily dyed MCs were observed; the dendrites were obvious and fully extended (Fig. 2D).

Changes in cell proliferation and the cell cycle

A CCK-8 assay was used to determine the cell proliferation rate at different times; the results are shown in Fig. 3A. As can be seen, after 1 day of magnetic bead

MITF, DCT and melan-A expression

The MITF is the main regulator of melanogenesis and a key regulatory enzyme in the biosynthesis of melanin, while DCT can be regarded as a specific MSC surface antigen, but it can also be expressed in MCs. Melan-A is a major protein involved in the formation and maturation of melanosomes, and is a specific antigen on the surface of MCs. We determined the mRNA levels of MITF, DCT and melan-A at different times after magnetic cell separation using reverse-transcription PCR (RT-PCR); the results are shown

Fig. 3. Changes in melan-A+/PAX3+ cell proliferation and cell cycle after magnetic cell separation

A. Cell proliferation at different times after magnetic cell separation using CCK-8; B. Cell cycle variations at different times after magnetic cell separation using FCM; * $p < 0.05$; ** $p < 0.01$.

Fig. 4. Tyrosinase activity in cells at different times after magnetic cell separation by L-DOPA staining method

* $p < 0.05$; ** $p < 0.01$.

in Fig. 5. As can be seen, the relative level of MITF and melan-A increased significantly over time, while the relative level of DCT decreased significantly ($p < 0.05$). This indicated that the expression of MITF and melan-A is positively correlated with the number of days, while the expression of DCT is negatively correlated with the number of days.

Fig. 5. The relative mRNA levels of (A) MITF, (B) DCT and (C) melan-A in cells cultured in vitro and magnetic cell separation for 3, 10 and 20 days

* $p < 0.05$; ** $p < 0.01$.

Discussion

At present, in vitro culture of MCs has been widely used in the experimental and clinical research of various tryptophan diseases, such as leucoderma and chloasma. Melanocytes, the main group of cells in the skin and hair coloring of vertebrates, are present in the epidermal basal layer and a small part of the hair follicle. The mammalian follicle contains a variety of cell lines and has great potential for development. Active melanin stem cells differentiate into hair follicles melanocyte (hf-mc) precursors, which

are then generated by mature hf-mcs, whose main function is to synthesize melanin. The technique of culturing hf-mcs in vitro has opened up new research pathways and solutions for the treatment of pigmentary diseases. Melanocytes cultured in vitro can also be used to study the etiology and pathology of malignant melanoma, and to examine the efficacy and toxicity of drugs that remove pigmentation. The regulation of pigmentation involves the development, heterogeneity, regeneration, and aging of MCs and their precursors. Some studies have reported that immature MCs and MSCs could be activated to differentiate into mature MCs in leucoderma patients.¹¹ Therefore, we believe that MSCs are involved in the differentiation and proliferation of MCs, and have therapeutic value for pigmentation disorders, trauma and melanoma.

Melanocyte surface antigens include melan-A, MITF, TYR, DCT, and others.^{12,13} Melan-A is one of the most important MC markers and can be expressed in melanosome and the endoplasmic reticulum of MCs. It also participates in the formation and maturation of melanosome, which as a lysosomal organelle can synthesize, store and transport melanin. Melan-A can combine with the major structural protein Pmel17 of melanosome to form a complex to participate in the transport and processing of melanosome.¹⁴ Melan-A can be expressed in mature MCs, but not on the surface of MSCs. Therefore, in this study, melan-A was selected as a marker antigen of mature MCs for MC screening in mixed cells. The MITF is a key transcription factor that encodes MC development. It plays an important role in the various processes of MC development, including neural crest differentiation, melanoblast growth and terminal MC differentiation.¹⁵ The *DCT*, also named *TRP-2*, is a mouse gene. The carboxyl terminal and membrane domain of DCT play a key role in the formation of melanosome. The DCT can form into a tyrosinase complex with TYR and TRP-1. DCT and TRP-1 mutations can induce pigment changes rather than depigmentation. Therefore, DCT and TRP-1 play a modifying role in melanogenesis. The DCT can be expressed not only in embryonic MCs, but also in mature MCs. The surface of MSCs lacks MC surface-related antigens such as TYR, MITF, melan-a, SOX10, KIT, and others, and only expresses PAX3 and DCT.^{16,17} PAX3 is an important transcription factor in the neuroectoderm of embryo development and is first expressed in anterior neural crest cells during the embryonic period.¹⁸ The pairing domain and homologous domain of PAX3 can promote the binding of PAX3 to DNA by recognizing the common sequences of DNA, which activates the transcription of target genes.¹⁹ In the embryonic stage, PAX3 can inhibit cell apoptosis by targeting p53 to make sure the cells are fully developed.²⁰ It can promote the migration of MCs from neural crest cells and promote the migration of MSCs from hair follicles to dermal papillae.²¹ Therefore, in this study, PAX3 was used as a marker to analyze and detect MSCs.

In this study, melan-A+ and PAX3+ MSCs were isolated from mixed cells digested from skin and cultured in vitro

to establish MC and MSC in vitro culture models. The key to culturing MCs is the selection of culture conditions and preparation of the culture medium, since the growth of MCs is negligible under standard cell culture conditions. In this experiment, we used a medium containing HMGS-2 and endothelin (ET), without 12-O-tetradecanoylphorbol-13-acetate (TPA) or cholera toxin (CT). HMGS-2 can reduce the possibility of cell mutagenesis, and is usually used to culture neonatal MCs or MSCs,²² increasing the availability of cultured cells for clinical use. At different stages of the culture process, we observed that dendrites of separated MCs constantly increased over time and no obvious KCs or other cell growth was observed. This means that magnetic cell separation can separate MCs from other cells in mixed skin cells and maintain MC characteristics during the culture process. However, during the culture stage, we found that some cells with large amounts of cytoplasm had no obvious dendrites. To verify whether these cells are PAX3+ MSC cells and whether they participate in the differentiation and maturation of MCs, we performed L-DOPA staining on the cells at different stages of the culture, counted the L-DOPA-positive cells and calculated their proportion in the cultured cells. The L-DOPA staining is a classic method for identifying active MCs and positive staining indicates the existence of active tyrosinase in melanosomes.²³ Both MSCs and differentiated MCs lack functional tyrosinase and cannot produce melanin; only mature MCs produce melanin.²⁴ Therefore, as primitive cells unable to synthesize melanin particles, L-DOPA staining of MSCs should be negative, while the L-DOPA staining of MCs with active tyrosinase is positive. Our results showed that the number of positive L-DOPA stained PAX3+ and melan-A+ cells increased, and most cells gradually differentiated into mature MCs. This provides a cellular biological model for further study of the process and mechanism of human MC differentiation and maturation.

In order to further study cell growth after magnetic cell separation, a proliferation curve was plotted using CCK-8 and the cell cycle was studied with FCM. The results of CCK-8 showed that the number of cells increased as the duration of the culture increased. The results of FCM showed that after magnetic cell separation, the cells in G1 phase decreased with the extension of the culture time, indicating that cells in the static phase decreased and MSCs develop into mature MCs. Cells in S phase increased and cells in the G2/M phase decreased, which indicates that the DNA replication period is prolonged and MC proliferation is active. Therefore, we inferred that cell proliferation capacity increased; the cells in the stationary phase gradually reduced; and cells in the inactive phase gradually increased after in vitro culturing and magnetic cell separation.

In order to study the biological function of cells after separation, we examined the tyrosinase activity and gene expression of the cells. Tyrosinase, as a key enzyme in melanin metabolism, mainly exists in mature MCs and cannot be detected in MSCs.²⁵ Our study showed that tyrosinase

activity increased with the duration of the culture, which indicated that the ability to secrete melanin was significantly improved and the content of melanin in MCs increased. Both MSCs and differentiated MCs lack functional tyrosinase and could not produce melanin; only mature MCs can produce melanin. The MITF is the main regulator of melanogenesis and the key regulator of melanin biosynthesis. The DCT can be used as a specific surface antigen of MSCs and can also be expressed in MCs. Melan-A is a major protein involved in the formation and maturation of melanosomes, and a specific surface antigen of MCs. Therefore, we mainly studied the relative mRNA levels of MITF, DCT and melan-A in cells cultured in vitro after magnetic cell separation. Our study showed that the relative expression levels of MITF and melan-A in cells increased with culture time, while the relative mRNA levels of DCT decreased over time. Therefore, we believe that after magnetic bead separation, the cells have the characteristics of MSCs and MCs, and that MSCs constantly differentiate into mature MCs.

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