Original Article

Melanocyte spheroids are formed by repetitive long-term trypsinization

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Abstract

Background: Autologous melanocyte transplantation plays an important role in the treatment of vitiligo.
Objective: Previous studies have indicated that, compared with melanocytes growing in monolayers, melanocyte spheroids have a better survival in growth factor- and serum-deprived conditions.
Methods: Melanocyte spheroids were obtained from human epidermis by repetitive long-term trypsinization and maintained an aggregated morphology for a short period in certain conditions.
Results: Melanocyte spheroids were capable of growing into normal dendritic melanocytes in monolayer when they were harvested and reinoculated in 24-well plates. Immunohistochemical analysis of the melanocyte spheroids revealed that they were positive for HMB45, a melanosome-specific marker. No melanomas occurred when melanocyte spheroids were transplanted into mice.
Conclusion: Our study provides a promising approach for melanocyte transplantation to treat vitiligo.

Key words: Melanocytes, spheroid, trypsinization

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Introduction

Vitiligo is an acquired dermatological disorder characterized by circumscribed depigmented macules due to the loss of functional melanocytes in the epidermis.[1,2] Therapeutic options for vitiligo include the application of topical steroids, psoralen plus ultraviolet A (PUVA), narrowband ultraviolet B, biologics, immunosuppressants, low energy laser irradiation and surgical therapy.[3,4] In intractable cases of vitiligo, autologous melanocyte transplantation may be used to repigment the skin, wherethe vitality and purification of the transplanted melanocytes are crucial for effective results.

In the process of cell transplantation, providing a ''survival advantage''to cells is very important.It has been found that melanocytes spontaneously grow into three-dimensional spheroids on a chitosan-coated surface in growth factor- and serum-deprived conditions, and preculturing melanocytes into spheroids can provide them a survival advantage.[5] Lin *et al*. reported that, depending on the seeding density and culture time, melanocytes can assume a monolayer or a spheroid morphology.[6]

Melanocytes in human skin are roughly divided into two distinct populations: mature differentiated cells and melanocyte stem cells (MSCs). Melanocyte stem cells and/or their immediate progenies are slow-cycling and self-renewing, and generally stress-tolerant. In 2013, Shigemoto proposed a novel approach to collect satellite cells from skeletal muscles based on their stress tolerance. They suggested that long-term trypsinization is a safe and inexpensive method to efficiently collect tissue stem cells.[7]

The aim of this study was to observe the changes of primary melanocytes in culture treated by repetitive long-term

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trypsinization and to investigate the biological functions of melanocyte spheroids.

Methods

All procedures and human experiments were approved by the Ethics Committee of the First People's Hospital of Changzhou (Changzhou, China) [permit number (2013) number 4].

Preparation of adult human epidermal melanocytes

Primary cultured cells were harvested from adult foreskin by digesting it with dispase and trypsin sequentially. These cells were cultured in M254 Medium supplemented with human melanocyte growth supplement, which is suitable for melanocyte growth but not for other types of cells.[18-20]

Skin specimens were obtained from adult circumcisions and were immediately immersed in iodophor solution for 5 min, then washed with phosphate-buffered saline supplemented with 400 U/mL penicillin and 400 μg/mL streptomycin. Subcutaneous adipose tissues were removed manually using eye scissors. The remaining tissues, including the epidermis and dermis, were washed again with phosphate-buffered saline and cut into small pieces (approximately 0.5 cm × 1 cm). The epidermis was separated from the dermis after treatment with 0.25% dispase (cat. no. 4942078001, Roche, Basel, SUI) for 2 h. The epidermal sheets obtained were treated with 0.25% trypsin- ethylenediaminetetraacetic acid (cat. no. 25200-056, Gibco, Grand Island, NY, USA) for 15 min to produce a cellular suspension which was filtered through a 200-μm filter and then centrifuged at 1000 rpm for 3 min.[8] The cells obtained were resuspended and maintained in a growth medium consisting of 254 Medium (cat.no. M-254-500, Gibco, NY, USA) containing human melanocyte growth supplement-2 (cat.no.S-016-5, Gibco, NY, USA), in 5% CO_2 at 37°C.Culture media were changed every 3 days.[9,10]

Repeated long-term trypsinization

When melanocytes in primary culture reached 70–80% confluence, the medium was discarded, adherent melanocytes were gently rinsed with 0.01 M phosphate-buffered saline and then incubated with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution for 2 h. Subsequently, the cells were washed with 0.01 M phosphate-buffered saline and suspended in a 5 mL Falcon tube which was vortexed for 1 min at 1,800 rpm and then centrifuged at 400 rpm for 10 min. Finally, the supernatant containing any dead cells was removed. The surviving cells were resuspended in 254 Medium and were then reseeded in 24-well plates. Approximately 1 week later, when cells in the second passage were 70–80% confluent, the above processes of trypsinization and reseeding were performed, but with the incubation time being 4 h. A similar sequence was again carried out when the third passage cells became 80–90% confluent, with an incubation time of 6 h.[11]

Morphological observations and immunofluorescence staining

For morphological observations, cells and spheroids adhering to the walls of the dishes were photographed using an inverted phase contrast microscope. Melanocyte spheroids were picked up using the blunt point of a transfer pipette. Actually, with a smooth surface they could be detached intact by gently pipetting the culture medium. They were then reseeded in another 24-well culture plate. When these spheroids attached to the bottom of the plate and grew outwards, staining could be carried out. The cells were fixed in 4% paraformaldehyde for 15 min at room temperature.Then they were incubated in 0.3% Triton X-100 in phosphate-buffered saline for 10 min. After two successive washes with phosphate-buffered saline, the cells were incubated with a blocking solution containing 5% bovine serum albumin (cat.no. 10099141, Gibco) at room temperature for 60 min. They were subsequently stained with HMB45 antibody (1:50; cat.no.M0634, Dako), which was diluted in 1% bovine serum albuminin phosphate-buffered saline and then incubated overnight at 4°C. After being washed three times in phosphate-buffered saline, these cells were incubated with a secondary antibody (1:400; cat.no.ab150105, Abcam) in 1% bovine serum albumin in phosphate-buffered saline for 2 h at room temperature in the dark. An isotype-matched donkey anti-mouse IgG (1:400;cat.no.ab150105, Abcam) was used as a negative control.[12] The results were observed using a fluorescence microscope (Olympus, Tokyo, Japan).[13]

Electron microscopy

Melanocyte spheroids were collected and fixed in 2.5% glutaraldehyde (buffered to pH 7.2 with sodium cacodylate) for 4 h. Then they were post-fixed in 1% (wt/vol) osmium tetroxide for 1 h. The specimens were dehydrated sequentially using ethanol at concentrations of 30, 50, 70, 80, 90 and 100%, each phase for 10 min. The specimens were then embedded in Epon-Araldite and cut into ultrathin sections (70 nm thick) using a Leica Ultracut UCT ultramicrotome (EM UC6, Leica, Wetzlar, Germany), mounted on copper grids and stained with lead and uranyl solutions.[14,15] Finally, these sections were observed using a transmission electron microscope (JEM-2100, JEOL, Tokyo, Japan).

Transplantation

To check if the cells after repeated trypsinization might become melanoma cells, we inoculated them subcutaneously into nude mice.[16] Subcutaneous transplantation is easy to perform and allows simple monitoring of tumor development. Prior to inoculation, melanocyte spheroids were dissociated into single-cell suspensions to enable the transplantation of approximately 1.5×10^5 cells. To increase sensitivity, the transplanted cells were mixed with Matrigel. We monitored the transplanted animals for the development of tumors for a period of 4 months.

Immunohistochemical examination of tissues

After 4 months, the skin tissue of mice at each transplanted site was removed, fixed, embedded and cut into

sections for immunohistochemical examination.[17]After deparaffinization and hydration, these sections were subjected to blocking of endogenousperoxidase activity, and were then treated with 10 mMEDTA at pH 6.0, at 100°C in a steamer to allow antigen retrieval. Immunohistochemical investigations were performed using the HMB45 antibody (1:50; cat.no. M0634, Dako) diluted in 1% bovine serum albuminin phosphate-buffered saline at pH 7.6. The slides were incubated with the primary antibody described above for 2 h at 37°C. Antigen-antibody binding was detected by means of the EnVision peroxidase system (code K1491; Dako). Staining was achieved using 3,3′-diaminobenzidine (Dako) diluted in 3% H_2O_2 in phosphate-buffered saline at pH 7.6. The same specimens were used as negative controls by omitting the primary antibody and replacing it with 1% bovine serum albumin.

Results

Primary cultures

When reaching 70–80% confluence, the cultured cells comprised a majority of melanocytes, a small number of keratinocytes and a few fibroblasts [Figure 1].The melanocytes had small bodies with a good diopter, and multiple dendrites were obvious; however, they did not show clonal growth behavior. In contrast, the keratinocytes showed colony-like growth and were arranged densely with a clear boundary.

Enrichment of melanocytes after the first long-term trypsinization

In preliminary experiments, we subjected primary cultured cells to long-term trypsinization for 0.5, 1, 2, 3, 4, 5, 6 and 8 h, to determine the best conditions for high survival ratio of melanocytes and the elimination of keratinocytes and fibroblasts. The results indicated that long-term trypsinization

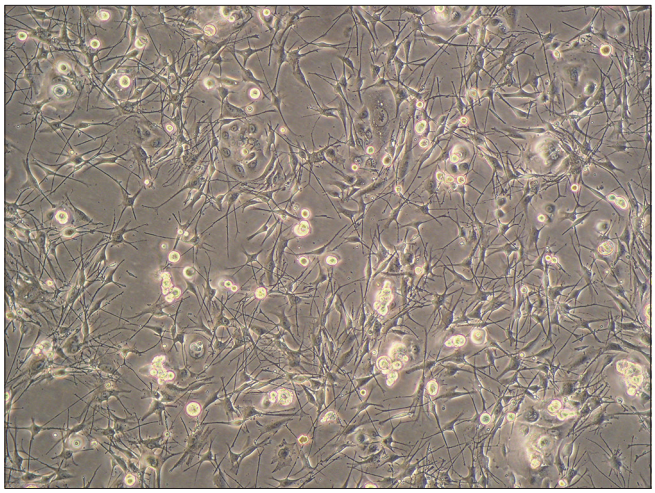

Figure 1: Primary culture. The cultured cells comprised a majority of melanocytes, a small number of keratinocytes and a few fibroblasts (inverted phase contrast microscopy, ×100)

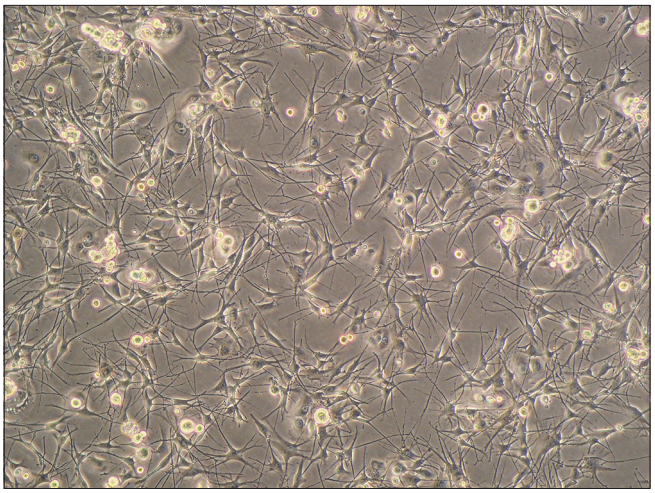

Figure 2a: These cells displayed the classic form of melanocytes, each cell having multiple dendrites, each of which was very long (inverted phase contrast microscopy, ×100)

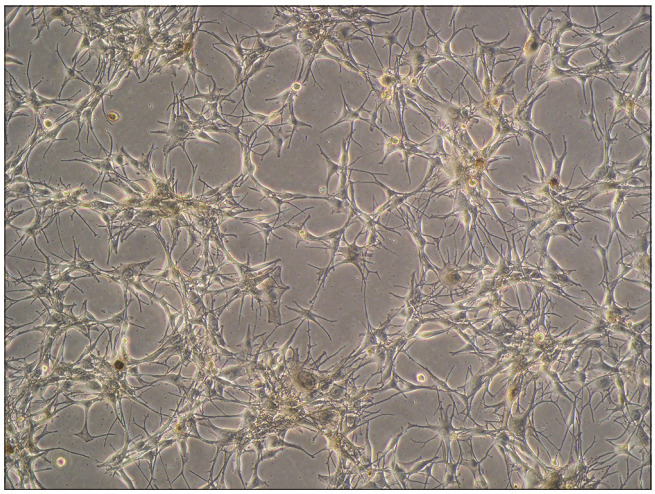

Figure 2b: Long-term trypsinization of 4 h was performed on these cells and they had a tendency to form clusters (inverted phase contrast microscopy, ×100)

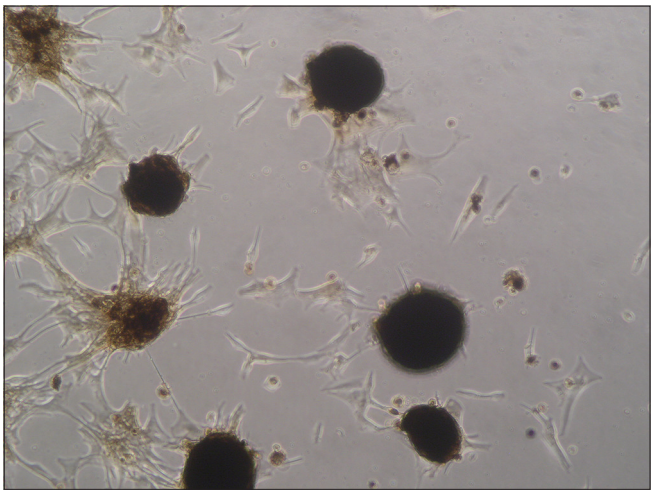

Figure 2c: Larger compact cellular spheroids were observed on the third day after another long-term trypsinization of 6 h (inverted phase contrast microscopy, ×100)

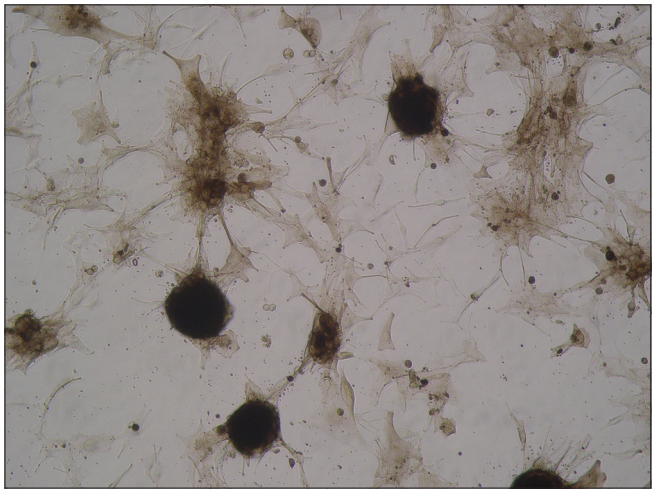

Figure 2d: Larger compact cellular spheroids were observed on the third day after the further long-term trypsinization of 6 h (inverted phase contrast microscopy, ×100). Between the melanocyte spheroids, dendritic melanocytes were still present

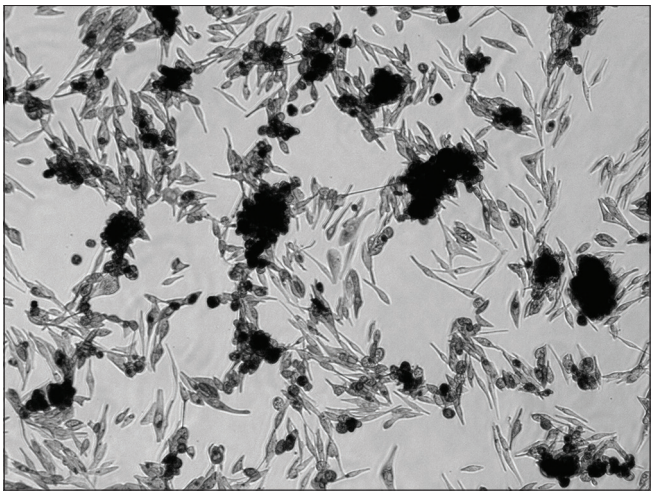

Figure 3a: Larger compact cellular spheroids before they were picked up (inverted phase contrast microscopy, ×100)

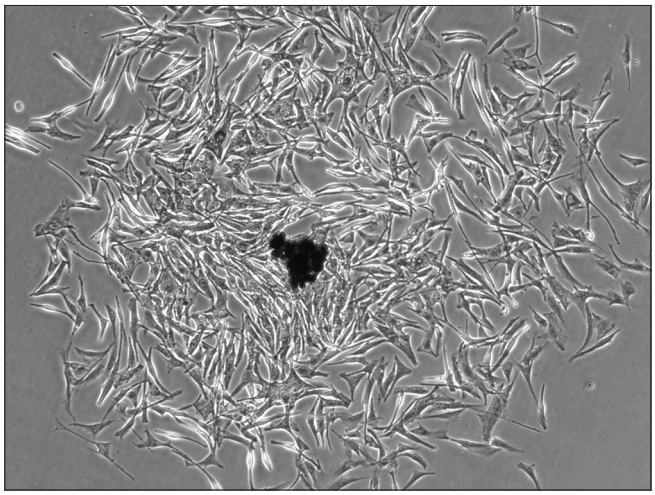

Figure 3b: Spheroids picked up by the blunt point of a pipette..The spheroids were then capable of growing into monolayer dendritic melanocytes when reseeded onto a culture plate (inverted phase contrast microscopy, ×100). Over time, these melanocytes spheroids gradually disintegrated

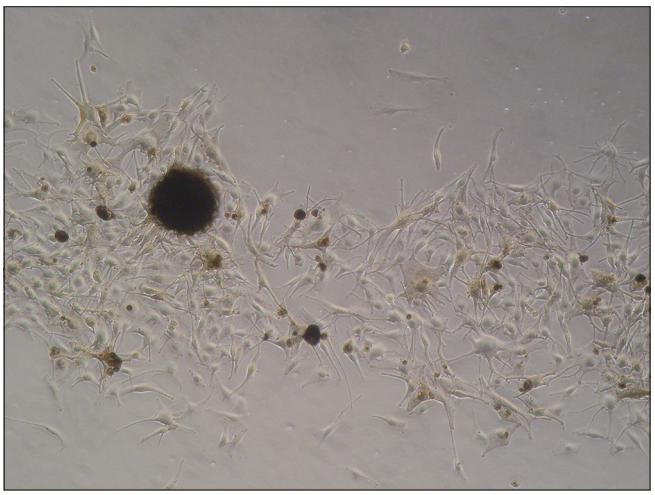

Figure 3c: Spheroids picked up by the blunt point of a pipette. The spheroids were then capable of growing into monolayer dendritic melanocytes when reseeded onto a culture plate (inverted phase contrast microscopy, ×100)

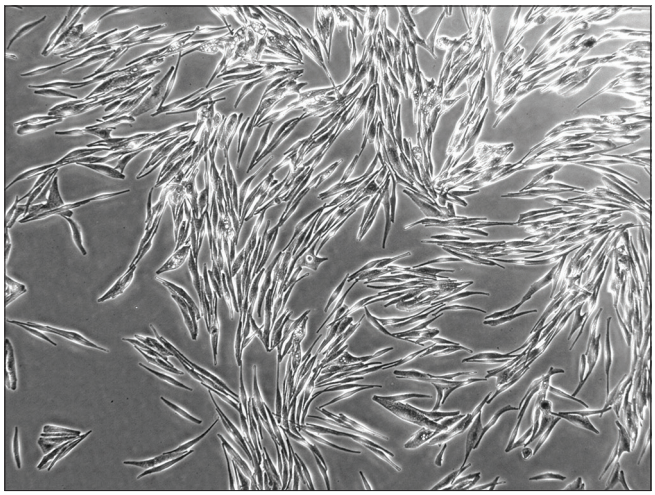

Figure 3d: Over time, these spheroids gradually disintegrated and were replaced by a monolayer cells that grew vigorously (inverted phase contrast microscopy, ×100)

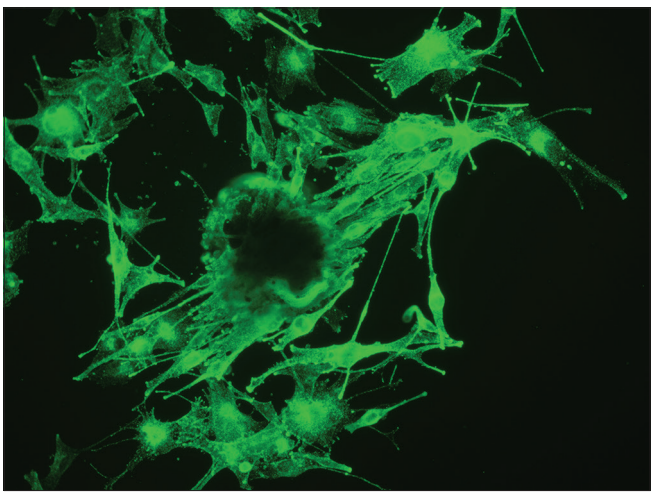

Figure 3e: Immunofluorescence staining with HMB-45 showed that the spheroids exhibited an intense green fluorescence (×200)

for 2 h was the most suitable condition for the primary culture. In that case, the proportion of melanocyte stem cells increased, and keratinocytes and fibroblasts almost disappeared, although we do not know the underlying mechanisms.

The surviving cells grew quickly and achieved 70–80% confluence on the seventh day after long-term trypsinization. At this time, the cells displayed the classic form of melanocytes, with each cell having multiple very long dendrites [Figure 2a]. A long-term trypsinization of 4 h was performed on these cells. Four days later, the cells achieved 70–80% confluence and tended to form clusters [Figure 2b]. Larger compact cellular melanocyte spheroids were observed on the third day after yet another long-term trypsinization of 6 h [Figure 2c and d]. Between the melanocyte spheroids, dendritic melanocytes were still present.

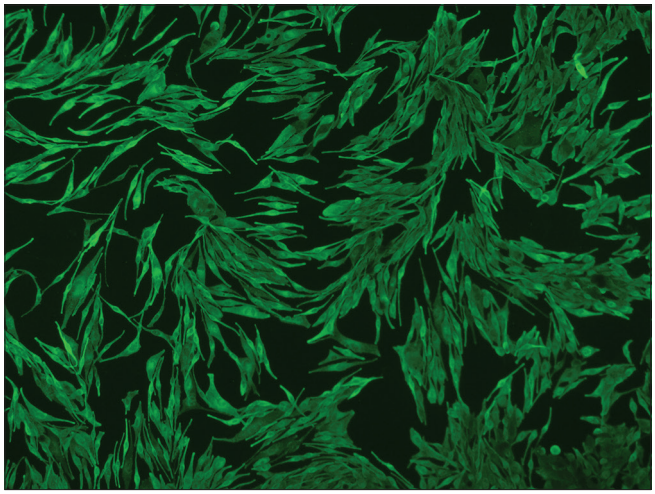

Figure 3f: The second-generation cells showed a short spindle-shape, with good diopters and bipolar protrusions. Immunofluorescence staining with HMB-45 showed that most cells exhibited an intense green fluorescence (×200)

Culture of cellular spheroids

The diameters of melanocyte spheroids ranged from 50–250 μm and they were capable of growing into monolayer dendritic melanocytes when reseeded in culture

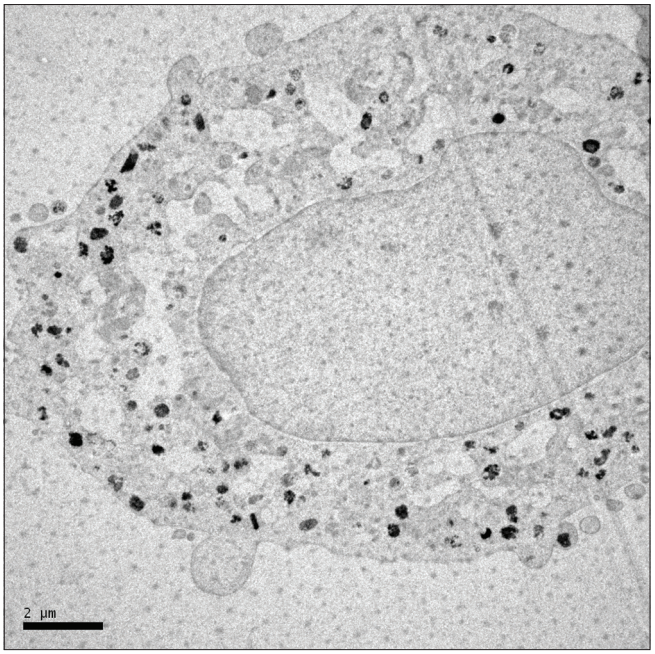

Figure 4a: The nucleus/cytoplasm ratio of these cells was high and chromatin aggregation in the nuclei appeared poor (transmission electron microscopy, ×8000)

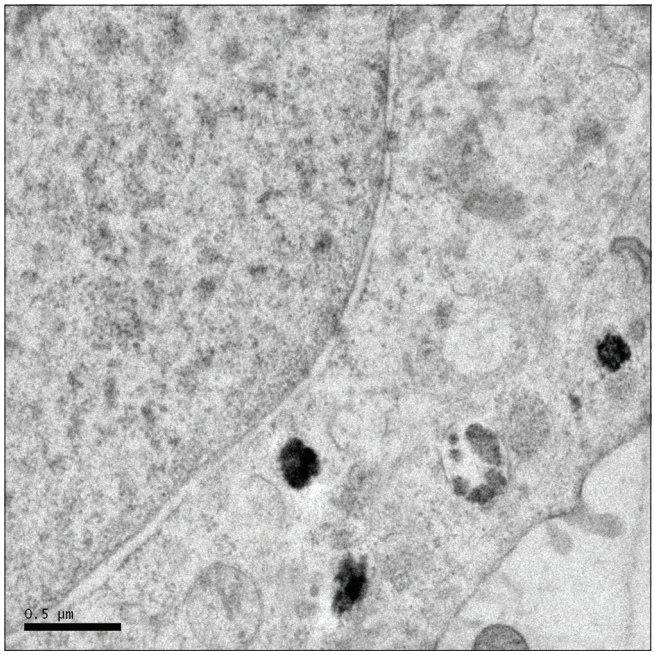

Figure 4b: The dense melanin granules appear as uneven masses (transmission electron microscopy, ×40000)

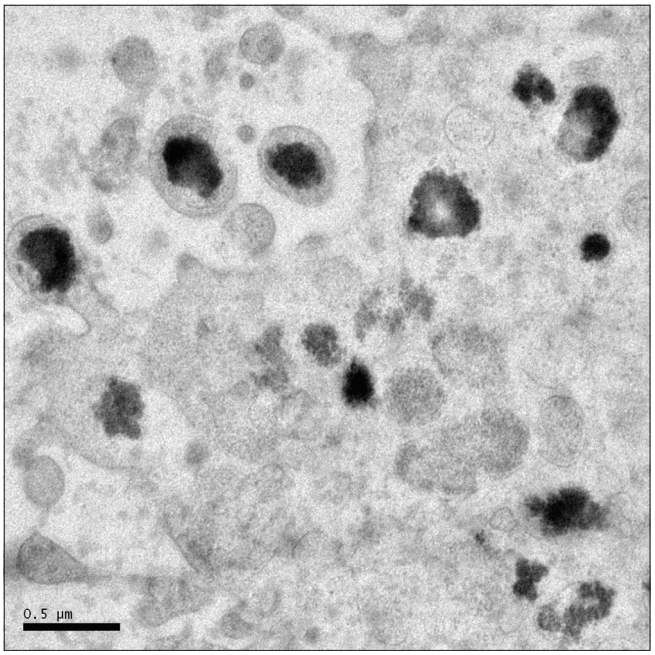

Figure 4c: Double-membrane-walled structures containing electron-dense material were found, typical of melanin-containing autophagosomes (transmission electron microscopy, ×40000)

plates [Figure 3a-c]. Over time, these melanocyte spheroids gradually disintegrated and were replaced by a monolayer of cells that grew vigorously. After passage, few melanocyte spheroids could be seen [Figure 3d]. The second-generation cells showed a short spindle-shape, with good diopters and bipolar protrusions, characteristic of immature melanocytes.

On immunofluorescence staining with HMB45, most cells exhibited an intense green fluorescence [Figure 3e and f], indicating the presence of moderate to high levels of HMB45 antigen, which is a specific marker for melanosomes.[21] Fluorescence was stronger at the tips of the cellular dendrites and at the periphery of the cell bodies, demonstrating that those regions were rich in melanosomes. The staining pattern confirmed that these cells were indeed melanocytes, and further demonstrated that the melanocyte spheroids were viable.

Electron microscopy

Transmission electron microscopy revealed that the nucleus/cytoplasm ratio of these cells was high and chromatin aggregation in the nuclei was poor [Figure 4a].The dense melanin granules appeared as uneven masses [Figure 4b]. Double-membrane-walled structures containing electron-dense material were found, typical for melanin-containing autophagosomes [Figure 4c].[22] A few melanin-containing autophagosomes had exocytosed into the intercellular spaces [Figure 4d]. These findings indicated that autophagy may have occurred in melanocytes under conditions of stress.

Transplantation and immunohistochemical examination of tissues

30 µl of melanocyte suspension (approximately 1.5 × 10^5cells) was slowly injected into the subcutaneous tissue of the right rear leg of each mouse. We observed the skin for changes on a daily basis, and found a black spot at the injection site on the sixth day. Four months later, punch biopsies were taken. Hematoxylin and eosin staining of the excised tissue was normal. Immunohistochemical staining revealed many HMB45-positive cells located in the deep dermis and subcutaneous tissue [Figure 5], but there was no evidence of melanoma tissue.

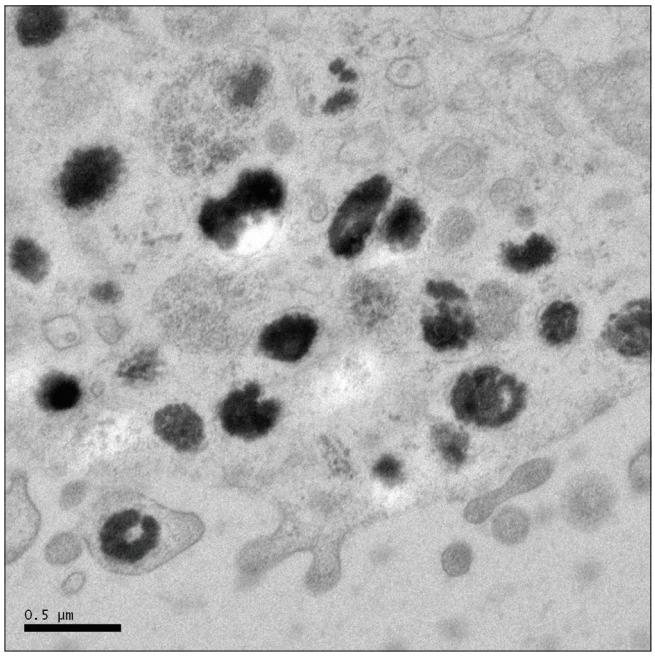

Figure 4d: Some of the melanin-containing autophagosomes were exocytosed into the intercellular spaces (transmission electron microscopy, ×40000)

Discussion

Vitiligo is an acquired depigmenting disorder with great cosmetic importance. Various treatment modalities have been tried to repigment vitiliginous skin.[23] Melanocyte transplantationis a cellular grafting procedure that aims to replenish the lost melanocytes inthe vitiliginous epidermis. In general, epidermal melanocytes are mature cells showing multiple dendrites and pigmentation. Melanocyte stem cells and/or their immediate descendants have a potential to provide a sufficient supply of melanocytes for cell-based treatments due to their high proliferative capacity, but the separation and purification of these cells is still a challenging problem.

Flow cytometry is widely used to collect stem cells from tissues by detecting specific surface markers, but it cannot be directly used for the collection of some tissue stem cells with no known specific markers, such as melanocyte stem cells. Some authors have developed a very efficient and inexpensive method to collect satellite cells.[24] They reported that a long-term trypsinization of 6 h was the most effective method to enrich mouse satellite cells and 3 h for human cells, due to species-specific differences.We tried long-term trypsinization for varying periods, and found that lengthening the time did less damage to cell activity. Shigemoto *et al*. also described that the ratio of stem cells among primary cultured cells was substantially increased by long-term trypsinization. This was also verified in our experiments, considering the time required to reach cell confluence.It took about 7–8 days to reach 70–

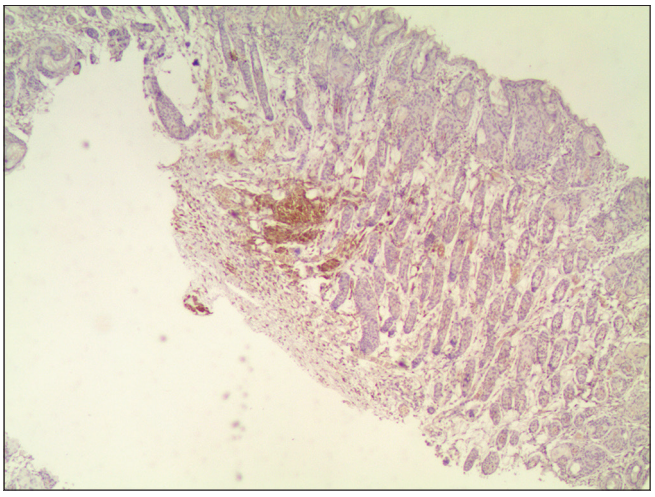

Figure 5: Immunohistochemical staining revealed many HMB45-positive cells located in the deep dermis and subcutaneous tissue (light microscopy, ×40)

80% cell fusion after the first long-term trypsinization, but the second and third periods were approximately 3–4 days. As the proportion of melanocyte stem cells gradually increased, the total number of cells increased rapidly. In other words, the repeated trypsinization greatly increased the proportion of melanocyte stem cells.

In previous experiments, trypsinization took only 3 min at 37°C, in the third generation of melanocytes mixed with a large number of fibroblasts. In our experiments, we subjected primary cultured cells to long-term trypsinization for 2, 4 and 6 h. The activity and purity of obtained melanocytes by our method were higher. Furthermore, it requires no expensive devices and can be easily performed in any laboratory. In the transplantation experiment, we confirmed that although these cells underwent repeated long-term trypsinization, they did not become melanoma cells. Though these findings suggest that it does not significantly affect the properties of the original cells, they are insufficient to recommend using these melanocytes immediately in the clinic, and genetic testing may be needed.

It is known that neuron precursor cells can only maintain their long-term proliferative potential and stem cell characteristics when they are cultured into neurospheres.[25,26] The mechanism for the superior survival of spheroids is unknown. The increased cell density may enhance the survival of neurons in stringent conditions via the autocrine and/or paracrine secretion of growth factors. Lin *et al*. reported that the development of melanocytes in monolayers or in spheroids on a chitosan-coated surface is dependent on seeding density, and it is assumed that melanocytes at high density form spheroids via migration after close intercellular contact. They concluded that melanocyte spheroid formation needs two signals: one is chitosan as the substrate, and the other is the intercellular communication by way of contact. They also proposed that when cell–cell interactions exceed cell–substrate interactions, cells may aggregate into multicellular spheroids. In our study, cellular spheres were successfully obtained without any biomaterial as a substrate, indicating that the substrate is not a necessary condition. Melanocytes in the spheroids proliferated continuously, resulting in a gradual enlargement of the spheres, and they were capable of growing into monolayered dendritic melanocytes when they were reinoculated in culture plates.

Many recent studies have suggested that the pathways of autophagy, apoptosis and necroptosis are connected to one another as mechanisms of cell death.[27] At first, nutrient starvation, including long-term trypsinization, causes autophagy. When the starvation is prolonged, autophagy-dependent cell death occurs, and eventually apoptosis and/or necroptosis occurs. We observed some melanosome-containing autophagosomes in melanocytes derived from spheroids using transmission electron microscopy, suggesting the involvement of autophagy.

Our findings show that melanocytes have two interchangeable morphologies. Repetitive long-term trypsinizationcan induce the formation of cellular spheroids, in which the melanocytes retain the capacity of proliferation and differentiation. Melanocytes from the spheroids had a morphology similar to that of melanocyte stem cells and proliferated quickly. Hence, the formation of melanocyte spheroids by repetitive long-term trypsinization seems to be a promising method to enhance the activity and purification of melanocytes, and thus facilitate their transplantation for the treatment of vitiligo.[28] Further studies are needed to determine the clinical applications of our findings.

Acknowledgments

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Conflicts of interest

There are no conflicts of interest.

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