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Biological characteristics of mouse skin melanocytes

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ABSTRACT

The objective of this research was to evaluate the optimal passage number according to the biological characteristics of mouse skin melanocytes from different passages. Skin punch biopsies harvested from the dorsal region of 2-day old mice were used to establish melanocyte cultures. The cells from passage 4, 7, 10 and 13 were collected and evaluated for their melanogenic activity. Histochemical staining for tyrosinase (TYR) activity and immunostaining for the melanocyte specific markers including S-100 antigen, TYR, tyrosinase related protein 1 (TYRP1), tyrosinase related protein 2 (TYRP2) and micropthalmia associated transcription factor (MITF) confirmed purity and melanogenic capacity of melanocytes from different passages, with better melanogenic activity of passage 10 and 13 cells being observed. Treatment of passage 13 melanocytes with α-melanocyte stimulating hormone (α-MSH) showed increased expression of MITF, TYR and TYRP2 mRNA. However, considering the TYR mRNA dramatically high expression which is the characteristics of melanoma cells, melanocytes from passage 10 was the optimal passage number for the further research. Our results demonstrate culture of pure populations of mouse melanocytes to at least 10 passages and illustrate the potential utility of passage 10 cells for studies of intrinsic and extrinsic regulation of genes controlling pigmentation and coat color in mouse.

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1. Introduction

The phenotype of coat and skin color of animal is attributed to two types of melanin, black to brown eumelanin and yellow to reddish brown pheomelanin (Ito et al., 2000; Ito and Wakamatsu, 2008). The quality and ratio of eumelanin to pheomelanin dictate the color of skin, fleece and eyes (Ito et al., 2000; Wakamatsu et al., 2006). The synthesis of melanin occurs in melanocytes located in the basal layer of the epidermis where melanin is packaged into melanosomes (Barsh and Cotsarelis, 2007; Lin et al., 2007). The production of melanin, melanogenesis, is hormonally and genetically regulated (Yamaguchi and Hearing, 2009). The coat color in fleece producing species (such as alpaca and sheep) and other animals has received considerable attention. However, the molecular and cellular mechanisms that regulate coat color are not completely understood. Melanocytes from skin of several species including alpaca (Bai et al., 2010), human (Tobin et al., 1995; Tsuji and Karasek, 1983) and mouse (Bennett et al., 1989) have been isolated and cultured, and used in studies to investigate factors that regulate genes controlling pigment production and coat color. Cultured mouse melanocytes are often used as a model to study regulation of pigmentation/coat color in fiber producing species. To date, suitable passages of mouse melanocytes with high melanogenic activity have not been reported. The objective of the present study was to characterize the expression of coat color genes and the response to α-MSH treatment in mouse melanocytes of different passages in order to determine which passages of cultured melanocytes were more active.

2. Materials and methods

2.1. Animal care and sample collection

Housing and care of animals used for skin sample collection were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals (http://www.cioms.ch/publications/guidelines/1985_texts_of_guidelines.htm), and were approved by the Animal Experimentation Ethics Committee of Shanxi Agricultural University. Punch skin biopsies (8 mm) were obtained from three C57BL/6C male mice (2-day

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old) under local anesthesia. Skin tissues were placed in DMEM-12 basal medium (Gibco BRL Co. Ltd., USA) containing 25 mM N-2-hydroxyethyl-piperazine-Nc-2-ethanesulfonic acid (HEPES), 400 U/ml penicillin and 400 μg/ml streptomycin, and transported to the laboratory on ice.

2.2. Cell dispersion and culture

Upon return to the laboratory, tissues were immersed in 70% ethanol for 1 min, then rinsed three to five times with Dulbecco's phosphate buffered saline (DPBS; Ca^{2+} and Mg^{2+} free) supplemented with 400 U/ml penicillin and 400 μg/ml streptomycin. After removing the underlying connective tissue, the skin tissue from each mouse was minced into 0.2 cm × 0.5 cm pieces, and digested in 0.2% dispase II solution in DPBS at 4 °C for 20 h. After digestion, epidermal tissue was separated from underlying dermal tissue using fine forceps and washed three to five times in DPBS. Single cell suspensions were prepared from epidermal tissue by digestion in 0.25% trypsin, 0.02% EDTA for 8 min at 37 °C, followed by vigorous pipetting in 5× volume of DMEM-12 basal medium supplemented with 10% newborn calf serum and filtration through a 200-pore steel sifter (Jinke Net Ltd Co., Shijiazhuang, China) with a pore diameter of 76 μm. The dissociated cell suspensions were then centrifuged at 1,000 × *g* for 10 min, and total cell number and yield of viable cells were determined.

Cultures were observed daily with an inverted phase contrast microscope and culture media replaced every 48 h. When the cells became confluent (~90%), they were detached using a 0.25% trypsin, 0.02% EDTA solution, centrifuged, counted, diluted 1:3, and plated for subculture or frozen. Cells obtained following dispase II treatment were incubated for specified number of days or passages prior to initiation of experiments. Melanocytes from three animals were pooled and the pooled cells were used in the subsequent experiments.

2.3. Validation of purity of melanocyte cultures

Purity of cells at different time points after initiation of culture was determined by visual observation of cell morphology and histochemical and immunocytochemical analyses. Cells were classified morphologically as melanocytes based on the characteristic dendritic morphology with multiple long processes and variable pigmentation. The epithelial cells of epidermal origin were identified based on their characteristic round nuclei. At 24 h of culture, the number of epithelial cells and melanocytes were counted separately in 3 different low magnification fields (×200) per culture dish, and the average number of melanocytes relative to contaminating epithelial cells was calculated.

TYR activity, a specific histochemical marker of melanocytes (Clarkson et al., 2001), was used to confirm melanocyte purity and phenotypic characteristics. TYR activity in cultured cells was assayed by L-DOPA reaction as described previously (Iijima and Watanabe, 1956). At passage 4, 7, 10 and 13, cells were trypsinized, plated on coverslips and cultured for ~3 days before detection of TYR activity. For L-DOPA reaction, culture media was removed and cells rinsed twice in PBS, fixed for 20 min in fixative solution (ethanol:chloroform:acetic acid = 6:3:1), washed three times with PBS and then incubated at 37 °C for 18 h in dark with 10 mM L-DOPA (Sigma, St. Louis, MO, USA). Negative control cells were treated similarly, but incubated in the absence of L-DOPA. After incubation, the cells were rinsed with distilled water, dehydrated, and mounted. Cells positive for TYR activity was observed using light microscopy.

Immunocytochemical localization of melanocyte markers including TYR (Clarkson et al., 2001), TYRP1, TYRP2, S-100 and MITF (King et al., 1999) was performed on melanocytes at passage 4, 7, 10 and 13. Cells were grown on coverslips for ~3 days in culture media with bovine pituitary extract omitted 48 h prior to staining to reduce potential background. Immediately prior to immunocytochemical staining, cells were rinsed briefly in PBS for 5 min and then fixed in fixative solution for 10 min at 20 °C and subsequently re-hydrated in PBS for 5 min. Cells were then incubated with the primary antibodies for 60 min at 37 °C. The primary antibodies included polyclonal mouse anti-rabbit TYR (Abcam, Hong Kong, Ltd, China) at 1:100 dilution, polyclonal mouse anti-goat MITF (Santa Cruz Biotechnology, Santa Cruz, CA USA) at 1:100 dilution, polyclonal mouse anti-rabbit TYRP1 (Abcam, Hong Kong, Ltd, China) at 1:100 dilution, polyclonal mouse anti rabbit TYRP2 (Abcam, Hong Kong, Ltd, China) at 1:100 dilution and polyclonal rabbit anti S-100 antigen (Zhongshan Biotechnological Ltd Co, Guangzhou, China) at 1:50 dilution. After incubation with appropriate secondary antibodies (rabbit anti-mouse IgG for TYR, TYRP1, TYRP2 and MITF. Goat anti-rabbit IgG for S-100), immunocytochemical staining was performed using the streptavidin/peroxidase method with a SP kit according to the manufacturer's instructions (Zhongshan Biotechnological Ltd Co, Guangzhou, China).

2.4. Proliferation of melanocytes from different passages

The proliferation of melanocytes from passages 4, 7, 10 and 13 was determined using RTCA iCELLigence Analyzer (ACEA Biosciences Inc., San Diego, CA USA). Approximately 5,000 melanocytes in culture medium were placed in each well (4 wells/passage) of an electronic plate (E-Plate L8) placed inside a cell culture incubator. Following 30 min incubation at room temperature, cell proliferation was recorded every 5 min for a period of 144 h.

2.5. Quantitative real time PCR analysis of gene expression

To compare the expression of TYR, TYRP1 and MITF mRNA in melanocytes from different passages, cells were plated into 25 cm^2 culture flasks at passage 4, 7, 10 and 13, allowed to grow to ~80% confluence, and then harvested for RNA isolation.

To study the effect of α-MSH treatment on mRNA abundance of TYR, TYRP2 and MITF in passage 13 melanocytes, cells number of 1×10^6 were cultured in the presence of 16.7 ng/ml α-MSH for 72 h. Cells were then harvested and subjected to RNA isolation.

Total RNA was isolated from cultured melanocytes using Trizol reagent (Invitrogen, Carlsbad, CA USA). Approximately 1 μg of total RNA per sample was converted to cDNA using previously published procedures (Li et al., 2004). Quantitative real-time PCR analysis of mRNA abundance for TYR, TYRP1, TYRP2 and MITF was conducted using the SYBR Green-based detection system (TaKaRa, Dalian, China) and the comparative threshold cycle (Ct) method (Livak and Schmittgen, 2001). The 25 μl PCR reaction included 12.5 μl of Platinum SYBR Green qPCR SuperMix-UDG, 0.5 μl of forward primer (10 pM), 0.5 μl of reverse primer (10 pM), 0.5 μl of ROX reference dye (Invitrogen, Carlsbad, CA USA), 2 μl of template and 9 μl of water. The reactions for a particular gene were performed in a single 96-well plate at 95 °C for 2 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 30 s. Quantitative real time PCR was performed in triplicate for each cDNA sample on a Stratagene Mx3005P Real-Time qPCR system. Abundance of TYR, TYRP1, TYRP2 and MITF mRNA was normalized relative to abundance of β-actin mRNA. All primer sequences are listed in Table 1. Data were analyzed using analysis of variance and Fisher's protected least significant difference test. Data are reported as mean ± SE. All experiments were replicated three times ($n = 3$).

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