Isolation and culture of melanocytes from the arctic fox (Alopex lagopus)

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Abstract

Coat colour is a phenotypic marker of fur animal species, which was determined by the pigment generated from melanocytes. In this study, we developed and validated a method for isolation, purification and passage culture of melanocytes from the arctic fox (Alopex lagopus). Skin biopsies were harvested from the dorsal region of adult foxes and enzyme digestion by Dispase II. The primary culture of melanocytes from arctic fox skin was obtained by using keratinocyte serum-free medium supplemented with epidermal growth factor and bovine pituitary extract with/without phorbol-12-myristate-13-acetate, and by carrying out a medium change strategy. After serial passages, it yielded pure population of melanocytes, which become efficient tools for investigating the function of colour genes and unraveling the process of melanin synthesis.

Introduction

Artic fox (Alopex lagopus) belongs to the Canidae family that includes domestic dogs, wolves and coyotes. The arctic fox includes two different types upon the coat colour, one is white arctic fox, which is dark gray to bluish brown in the summer and turns into white or creamy white in the winter. Another is blue arctic fox, also known as blue fox, which is blue all the year (Grzegorczyk et al., 2000; Vage et al., 2005; Stasiak and Janicki, 2014). The blue foxes were important fur-bearing animals for fur industry. Pelts were graded and sold at the auction house according to character traits: size, colour, and so on. Their colour types had some effects about the price of pelts (Wierzbički, 2005).

Colour variation is determined by melanin (eumelanin and pheomelanin), which is synthesized in melanocytes and aggregated into pigment granules in special subcellular organelles, namely melanosomes (Aspangren et al., 2009). Melanocytes differentiate from undifferentiated precursors, called melanoblasts, which originates from embryonic cells named neural crest cells (NCC) (Li, 2014). The melanocytes are normally found in the bottom layer of epidermis, also known as the stratum basal of the skins epidermis, can also be found in the middle layer of the eye, which is known as the uvea, the inner ear and the meninges as well as the bones and the heart (Jule et al., 2003; Fang et al., 2006).

Variation in colour and pigment patterning can be made as a powerful model for many classic genetics studies of naturally phenotypic variation. The genetic mechanisms that had been studied on determine phenotype formation created an understanding of the developmental mechanisms responsible for the phenotypic effects. Moreover, pigmentation has played an important role in the intersection of evolution, genetics, and developmental biology (Hoekstra, 2006; Ng et al., 2008; Hines et al., 2012).

Thus, it is vital to set up an appropriate method to obtain the pure melanocytes in vitro for carrying out melanocytic associated studies. Thereby, we established the model system to obtain the melanocytes from the skin of foxes successfully, and to provide a tool to investigate the biology of melanocytes, the process of melanin synthesis and the mechanisms of coat colour formation.

Materials and methods

Sample collection

We obtained the skin samples from the blue foxes (1-2 yrs old) from Zuojia Experimental Animal Base (Jilin, China), in accordance with the International Guiding Principles in Biomedical Research Involving Animals. After taking an intravenous anesthesia, the authors shaved the hair, disinfected the local skin and cut the skin biopsies (5x5mm) from the dorsal region, immediately rinsed in the phosphate-buffered saline (PBS) containing 100U/penicillin and 400 g/mL streptomycin against microbial contamination and took the samples into Dulbecco’s Modified Eagle Medium (DMEM) back to the laboratory on ice.

Isolation and primary culture

In the lab, we cut the dissected tissues into small stripes and used the 1.07U/mL Dispase II solution in Hanks Balanced Salt Solution (HBSS) to digest the skin biopsies at 4°C overnight. Then, we separated the epidermis from the dermal tissue with forceps, isolated the melanocytes from the epidermis layer with blowing repeatedly, and obtained the single cell suspensions with cell sieves (200 meshes). The cell suspensions were centrifuged at 1200g for 5min, and seeded into 25cm2 flasks at a density of 5x106 cells. In the beginning, the culture medium was keratinocyte serum-free medium (K-SFM) supplemented with EGF, bovine pituitary extract (BPE) without phorbol-12-myristate-13-acetate (PMA) for three days. After that, we changed the medium into the K-SFM with PMA for several days (about 7-10 days). All the media were added 10% FBS and 1% antibiotics of Penicillin-Streptomycin (Cat. No. 15140-148) against microbial contamination. The primary cultures were incubated at 37°C in a humidified atmosphere of 5% CO2 in air.

Purity culture of melanocytes

The primary cultures contained the melanocyte, keratinocyte and fibroblast. In order to obtain pure melanocytes, we carried...
out a medium replacement strategy that was Dulbecco’s Modified Eagle Medium (DMEM) for half an hour, K-SFM supplemented with EGF, BPE and PMA for two or three days. All the media were added 1% antibiotics and 10% FBS. After second or third passage under that treatment, the primary culture would yield purity melanocytes.

**Dopa-staining**

Melanocytes were fixed with 5% formalin in PBS (pH7.0) at 4°C, for 20 min, rinsed with cold PBS twice, and incubated with 0.1% L-Dopa (3,4-dihydroxyphenylalanine; Sigma Aldrich, St. Louis, MO, USA) at 37°C for 4 h, with one change of Dopa solution. Then they were fixed with 10% formalin in PBS (pH7.0) for 30 min, and counterstained with nuclear fast red.

**Transmission electron microscopy**

Melanocytes in culture were scraped, and then were washed in PBS and prepared for electron microscopy. Cell pellets were fixed in 2.5% glutaraldehyde in PBS (pH7.2) at 4°C; they were conventionally embedded and sectioned. Slices were viewed in a JEM-1200EXII transmission electron microscope.

**Results**

**The primary cell cultures**

The primary cells were isolated from the dissected tissues of the arctic fox. After three days culturing, they demonstrated single, paired or small colonies nearly without fibroblasts contamination, and presented several melanocytic features with fibroblasts as the control cells, such as dendrites and halo in the cytoplasm (Figure 1). When they were transferred to the medium of K-SFM with PMA, the melanocytes proliferated and showed pigment granules in the cytoplasm under the electron microscope (200×) as the arrows pointed (Figure 2). We obtained the primary culture of melanocytes.

**Purity culture of melanocytes**

After second or third pure passage, we can obtain relative pure melanocytes (Figure 3A). Melanocytes had attached to the culture plate and had many typical morphological characteristics as the arrows showed, such as two or three, even more dendrites (Figure 3B), pigment granules (Figure 3C) and melanin shedding from the dendrites (Figure 3D). Melanosomes transferred along the dendrites of melanocytes, reaching to neighboring keratinocytes in vivo. But by *in vitro* co-culture systems, melanosomes were packaged into...
sheddings. These behaviours confirmed these cells to be melanocytes in turn.

Dopa-staining

The melanocytes were stained with L-Dopa, and counterstained with nuclear fast red with the control melanocytes; the dendrites of the cells stained were grayish or black under light microscopy. The cytoplasm of melanocytes appeared reddish (Figure 4), which is explained by tyrosinase reaction of melanocytes.

Transmission electron microscopy

Mature melanosomes among various stages of melanosomes specially can be observed by electron microscope (Figure 5). As the arrows showed, melanosomes were large organelles, their diameter was about 500nm, which contained dark melanin, and were easily observed by bright field microscopy. Thus, these cells were confirmed to be melanocytes by transmission electron microscope.

Discussion

In this study, we established a method for isolation and culture melanocytes from the skin of blue foxes, which were provided an excellent carrier to investigate pigment synthesis and colour genes function. However, pure melanocytes in vitro culture have an obstacle to establish without fibroblasts contamination (Navarro et al., 2001). The epidermis was separated from the underlying dermis is a crucial step to reduce fibroblasts. Unfortunately, even if spending many attentions on the separation, it is hard to avoid fibroblasts. In the process of melanocytes subculture, a small amount of fibroblasts may also lead to the failure of melanocytes culture. Even the most experienced cell breeder is also difficult to ensure that never mixed the fibroblasts. So, it is important to inhibit the proliferation of fibroblasts in the primary and passage cultivation. When fibroblasts mixed happened, most researchers used to add G418, which is one of the mitotic cell inhibitors, to remove fibroblasts (Zhu et al., 2004). Moreover, some researchers have reduced the calcium ion concentration, which can induce fibroblasts growth (Guo et al., 2014) and adopted the mechanical scrape method (Carey and Prunieras, 1984; Nielsen and Don, 1984; Abdel-Naser, 2003). Although most of the methods can inhibit fibroblasts grow, they also affect the growth of the melanocytes at the same time.

In this study, we established the method for isolation of the pure melanocytes from fox skin without fibroblasts mixed. Melanocytes can survive in the K-SFM medium for the short time and have a good growth status. However, the fibroblasts cannot proliferate in the medium. By this way, we can eliminate the mixed fibroblasts. After three days’ culture in K-SFM, we changed the medium to the K-SFM medium supplemented with PMA – was a diester of phorbol and a potent tumor promoter to activate the signal transduction enzyme protein kinase C, which can promote the melanocytes and inhibit the keratinocytes proliferation (Deveci et al., 2001). After in vitro culture for seven to ten days, we could obtain primary culture melanocytes. Yet, the primary culture

![Figure 4. Melanocytes were stained by L-Dopa staining: A) melanocytes before L-Dopa staining; B) melanocytes after L-Dopa staining.](image1)

![Figure 5. Mature melanosomes can be viewed by transmission electron microscope (arrows).](image2)
remained some keratinocytes. Thus, in the subculture, we also used DMEM and K-SFM medium exchange strategies to minus keratinocytes. Melanocytes and keratinocytes have different adherent abilities in the medium (Jule et al., 2003; Zhu et al., 2004). In the DMEM medium, the melanocytes adhered to the surface of flasks faster than keratinocytes do. Firstly, we passed the primary culture in the DMEM medium for half an hour. Most of melanocytes had attached to the culture flask, whereas keratinocytes had a round morphology and were more poorly attached. Then, replacing the DMEM medium to the K-SFM with PMA, we obtained the normal highly purity melanocytes. We can observe melanin granules and melanosomes by the electron microscope. These demonstrated that using Dispal II digestion and mediums transfer strategies is a simple and efficient method for isolation and culture the pure melanocytes from arctic fox skin.

Melanin plays a crucial role in many biological processes, such as camouflage, and especially protection against UV radiation. In keeping with these multiple roles, melanin synthesis was controlled by a complex regulation system, which is activated since early embryogenesis. Melanin is produced by melanocytes under highly structured mechanisms. The significance of melanin synthesis extends beyond the mere assignment of a coat colour trait (Slominski et al., 2004). The disorders of melanin synthesis and metabolism are thought to be associated with disease, such as the skin pigmentation sickness, melanoma and Alzheimer’s disease (Ando et al., 1995; Rees, 2003; Qin et al., 2004; Heidenreich et al., 2014). Thus, it is very significant for investigating the melanin synthesis and metabolism in normal and diseased tissues. Melanocytes were an efficient carrier for investigation of coat colour gene function in vitro, melanosomes formation from subcellular organelle, colour variation pattern and molecular mechanisms of pigment genes associated diseases and so on (Vage et al., 1997; Nagai et al., 2006; Baxter et al., 2009).

**Conclusions**

This study established an efficient procedure for isolating and culturing melanocytes from the skin of the arctic fox. Unlike other assays reported for melanocyte isolation, we can obtain melanocytes with scarcely fibroblasts and epithelial cells and it was not necessary to use the geneticin. Our melanocytic cultures were identified by Dopa staining and TEM, showing they had good biological characteristics. Therefore, we have successfully established a method for isolating and culturing melanocytes from the skin of arctic fox. We hoped the method described here could be useful for investigating the function of pigment genes and unraveling the process of melanin synthesis.

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