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Validation of Methods for Isolation and Culture of Alpaca Melanocytes: A Novel Tool for *In vitro* Studies of Mechanisms Controlling Coat Color*

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ABSTRACT : The objective of the present studies was to develop and validate a system for isolation, purification and extended culture of pigment-producing cells in alpaca skin (melanocytes) responsible for coat color and to determine the effect of alpha melanocyte stimulating hormone treatment on mRNA expression for the melanocortin 1 receptor, a key gene involved in coat color regulation in other species. Skin punch biopsies were harvested from the dorsal region of 1-3 yr old alpacas and three different enzyme digestion methods were evaluated for effects on yield of viable cells and attachment *in vitro*. Greatest cell yields and attachment were obtained following dispersion with dispase II relative to trypsin and trypsin-EDTA treatment. Culture of cells in medium supplemented with basic fibroblast growth factor, bovine pituitary extract, hydrocortisone, insulin, 12-O-tetradecanolphorbol-13-acetate and cholera toxin yielded highly pure populations of melanocytes by passage 3 as confirmed by detection of tyrosinase activity and immunocytochemical localization of melanocyte markers including tyrosinase, S-100 and micropthalmia-associated transcription factor. Abundance of mRNA for tyrosinase, a key enzyme in melanocyte pigment production, was maintained through 10 passages showing preservation of melanocyte phenotypic characteristics with extended culture. To determine hormonal responsiveness of cultured melanocytes and investigate regulation of melanocortin 1 receptor expression, cultured melanocytes were treated with increasing concentrations of α-melanocyte stimulating hormone. Treatment with α-melanocyte stimulating hormone increased melanocortin receptor 1 mRNA in a dose dependent fashion. The results demonstrated culture of pure populations of alpaca melanocytes to 10 passages and illustrate the potential utility of such cells for studies of intrinsic and extrinsic regulation of genes controlling pigmentation and coat color in fiber-producing species. (**Key Words :** Alpaca, Alpha Melanocyte Stimulating Hormone, Coat Color, Melanocortin 1 Receptor, Melanocyte, Pigmentation)

INTRODUCTION

Popularity of alpacas as agricultural and companion animals is increasing with populations of over 3 million animals worldwide used for fiber and meat production and packing (Lupton et al., 2006). Alpaca fiber consists of over 22 natural colors of varying economic value (Gregor, 2006). Despite numerous phenotypic descriptions (Sponenberg et al., 1988; Renieri, 1995), regulation of coat color variation in the alpaca has been very poorly explored at the molecular, cellular and genetic levels.

Coat and skin color is attributed to the pigments known as melanin. Synthesis of melanin occurs in melanocytes located in the basal layer of the epidermis where melanin is packaged into melanosomes (Lin et al., 2007). Skin and coat color are dictated by both number of melanocytes and their phenotypic properties including extent and composition of melanin produced (Wakamatsu et al., 2006). Evidence indicates that melanogenesis is hormonally and genetically regulated in other species (Yamaguchi and Hearing, 2009). For example, intrinsic factors such as alpha melanocyte stimulating hormone (α-MSH) regulate melanin production (Tsatmali et al., 2002) and mutations in the melanocortin 1 receptor (MC1R) influence coat color phenotype (Andersson, 2003). Understanding of the intrinsic and extrinsic factors that influence coat color is far from complete, particularly in fiber producing domestic species.

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In vitro culture of melanocytes from alpaca skin represents a potentially useful tool to investigate factors that regulate genes controlling pigment production and coat color. To our knowledge, *in vitro* models for study of regulation of pigmentation/coat color in fiber producing domestic species have not been previously established. The objectives of the present studies were to develop and validate a system for isolation, purification and extended culture of alpaca skin melanocytes responsible for coat color and to determine responsiveness of cells to α-MSH treatment.

MATERIAL AND METHODS

Animal care and sample collection

Housing and care of animals and collection of skin samples for use in described experiments were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals http://www.cioms.ch/frame 1985 texts of guidelines.htm. Punch skin biopsies (4×8 mm) were obtained from three alpacas (1-3 yrs old) under local anesthesia, immediately immersed in melanocyte basal medium (Promocell GMBH, Heidelberg Germany) 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.

Cell dispersion and culture

Upon return to the laboratory, tissue was immersed in 70% ethanol for 1 min, then rinsed 3-5 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 and underlying connective tissue removed. Tissue from each alpaca was minced into 0.2 cm ×0.5 cm pieces and evenly allocated for dispersion using either i) digestion in 0.25% trypsin solution (Sigma, Beijing, China) at 4°C for 20 h, followed by incubation at 37°C for 2 h, ii) digestion in 0.05% trypsin, 0.02% EDTA solution (Sigma) at 4°C for 20 h, followed by 37°C incubation for 2 h or iii) digestion in 0.2% dispase II solution in DPBS at 4°C for 20 h, followed by 37°C incubation for 2 h. After incubation, epidermal tissue was separated from underlying dermal tissue using fine forceps and washed 3-5 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 X volume of Melanocyte 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 numbers and yield of viable cells obtained with each dispersion method determined. Cells were then seeded into 6-well plates at a density of 1×10^5 cells per well in Melanocyte Basal Medium supplemented with 0.2 μg/ml cholera toxin (Sigma), 0.05 mg/ml gentamicin, 2.5 μg/ml fungizone, 50 μg/ml bovine pituitary extract (BPE), 0.5 μg/ml hydrocortisone, 1 ng/ml bFGF, 5 μg/ml insulin and 10 ng/ml TPA.

For above described comparison of cell dispersion methods, at 24 h after initiation of cultures, numbers of unattached cells present in culture medium in each well were counted. Plating efficiency (number of cells attached) was calculated as number of total cells plated-number of unattached cells. For remaining experiments, cultures were observed daily with an inverted phase contrast microscope and culture media replaced every 48 h. When the cells became confluent (80-90%) 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 as described below.

Validation of purity of melanocyte cultures

Purity of cells at different timepoints after initiation of culture was performed by visual observation of cell morphology and by histochemical and immunolocalization studies (passage 3). Cells were classified morphologically as melanocytes based on a characteristic dendritic morphology with multiple long processes and variable pigmentation with remaining epithelial cells of epidermal origin possessing 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 average number of melanocytes relative to contaminating epithelial cells calculated.

Visualization of tyrosinase activity, a specific histochemical marker of melanocytes (Clarkson et al., 2001), was also used to confirm melanocyte purity and phenotypic characteristics. Tyrosinase activity in cultured cells was assayed by the DOPA reaction as described previously (Iijima and Watanabe, 1956). At passage 3, cells were trypsinized, plated on coverslips and cultured for ~3 days before detection of tyrosinase activity. For DOPA reaction, culture media was removed and cells rinsed twice in PBS, fixed for 20 min in 4% formaldehyde solution in PBS, washed three times with PBS and then incubated at 37°C for 18 h in the dark with 10 mM L-DOPA (Sigma). Negative control cells were treated similarly, but incubated in the absence of L-DOPA. After incubation, the cells were rinsed with distilled water, dehydrated, mounted and number of cells positive for tyrosinase activity observed

using light microscopy.

Immunocytochemical localization for the melanocyte markers S-100 (Clarkson et al., 2001), tyrosinase (Clarkson et al., 2001) and micropthalmia associated transcription factor (MITF) (King et al., 1999) was also performed on melanocytes at passage 3 grown on coverslips for ~3 days, with BPE omitted from media 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 were fixed in ice cold 75% ethanol for 10 min at 20°C and subsequently re-hydrated in PBS for 5 min. Cells were then incubated in presence of following primary antibodies for 60 min at 37°C at dilutions indicated: i) polyclonal rabbit anti S-100 antigen IgG (Zhongshan Biotechnological Ltd Co, Guangzhou, China) at 1:400 dilution; ii) monoclonal mouse anti human tyrosinase (Santa Cruz Biotechnology, Santa Cruz, CA USA) at 1:75 dilution and iii) mouse monoclonal anti human MITF (Neomarkers, Fremont, CA, USA) at 1:75 dilution. After incubation in presence of appropriate secondary antibodies, immunocytochemical staining was performed using the streptavidin/peroxidase method (SP kit) according to manufacturer's instructions (Zhongshan Biotechnological Ltd.).

Effect of passage number on tyrosinase mRNA abundance

For studies of changes in tyrosinase mRNA associated with passage number, melanocytes were plated into 25 cm^2 culture flasks at passage 3, 5 and 10. Cells were allowed to grow to ~80% confluence, and then harvested for RNA isolation as described below.

Regulation of alpaca melanocyte MC1R mRNA by α-MSH

For studies of effects of α-MSH treatment on melanocyte mRNA abundance for MCIR, cells were plated into 25 cm^2 flasks at passage 5. At 80% confluence, cells were cultured as described above in the presence of 0, 1.6, 16.7 and 166.5 ng/ml α-MSH for 72 h. Cells were then harvested and subjected to RNA isolation as described below.

RNA isolation, cDNA synthesis and real time PCR analysis

Total RNA was isolated from cultured melanocytes using the Trizol reagent (Invitrogen, Carlsbad, CA USA). Approximately 1 μg 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 tyrosinase and MC1R was conducted using the SYBR Green-based detection system (TaKaRa, Dlian, China) and the comparative threshold cycle (CT) method (Livak and Schmittgen, 2001). The 25 μl PCR reactions included 12.5 μl Platinum SYBR Green qPCR SuperMix-UDG, 0.5 μl forward primer (10 pM), 0.5 μl reverse primer (10 pM), 0.5 μl ROX reference dye (Invitrogen), 2 μl template and 9 μl water. The reactions were incubated in a 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. All reactions were performed in triplicate on the Stratagene Mx3005P Real-Time QPCR system. Abundance of tyrosinase mRNA was normalized relative to abundance of 18S rRNA and abundance of MC1R mRNA normalized relative to abundance of β-actin mRNA. All primers sequences are found in Table 1.

Statistical analysis

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.

RESULTS

Comparison of alpaca skin digestion procedures

Average yield of cells and plating efficiency (cells attached) following dispersion with the three isolation methods is depicted in Table 2. The greatest yields of total cells and plating efficiency were obtained using dispase II

Table 1. Sequence information for real time PCR primers used in described studies

Gene	Primers[1] (5′→3′)	Primer locations	Product (bp)	Gene bank accession
TYR	F:GGAAGATNGGATCNTTGGCAGA R:TTACTGGGATAGCNGATGCC	5-112	108	EU293064
18SrRNA	F:GAAGGGCACCACCAGGAGT R:CAGACAAATCACTCCACCAA	250-408	158	EF426477
MC1R	F:TCTATGCACTGCGCTACCAC R:GACATATAGCACCGCCATGA	273-458	185	FJ517582
β-actin	F:AGCCATGTACGTAGCCATCC R:ACCCTCATAGATGGGCACAG	471-585	148	NM_031144.2

[1] F (forward primer), R (reverse primer).

Table 2. Influence of alpaca skin digestion methods on total cell yields and plating efficiency (cells attached)

	Trypsin	Trypsin-EDTA	Dispase II
Cell yields*	11.5±0.5[a]	7.8±0.3[b]	17.8±0.3[c]
Cells attached*	9.1±0.3[a]	6.0±0.4[b]	13.1±0.2[c]

* Mean +/- SE $\times 10^4$; within row. [a, b, c] $p<0.001$.

treatment relative to the two procedures using trypsin ($p< 0.001$), whereas the lowest yield of total cells and attached cells was obtained using trypsin:EDTA combination ($p< 0.001$). Based on morphological examination, most cells (obtained using dispase II method) attached to wells were skin epithelial cells (keratinocytes), and accounted for 92.5±0.2% of the total attached cells, with melanocytes accounting for. 7.5±0.2% of total attached cells.

Phenotypic characteristics of cultured cells

The morphological characteristics of cells at various timepoints relative to initiation of cultures are depicted in Figure 1. Immediately after plating (1 h), melanocytes appeared as irregularly shaped ovoid cells, whereas epithelial cells (keratinocytes) had a round morphology (Figure 1A). Within 12 h, melanocytes had attached to the culture plate and initiated a dendritic appearance, whereas keratinocytes had a round morphology and were more poorly attached (Figure 1B). By 24 h post plating, appearance of melanocytes with multiple dendritic processes was evident and remaining keratinocytes showed an aggregated appearance and were poorly adhered to the culture dish (Figure 1C). On day 7 after plating (data not shown) cells were >80% confluent and >80% of the cells displayed typical dendritic processes characteristic of melanocytes. Purity of cells was further increased following passage and typical morphology of cells at passage three is illustrated in Figure 1D. Cells appear to be >99% melanocytes based on morphological characteristics. Further evidence of phenotypic properties and purity of cultured melanocytes was revealed by detection of tyrosinase activity using L-DOPA reaction. As depicted in Figure 1E, cells demonstrated positive staining for tyrosinase activity (black or gray) in cytoplasm of virtually all cells and long dendritic appearing processes were evident. No staining was detected in negative control cells incubated in absence of L-DOPA (Figure 1F). Furthermore, alpaca skin melanocytes cryopreserved at various passages were routinely recovered after thawing and maintained characteristic melanocyte morphology through 22 passages (data not shown).

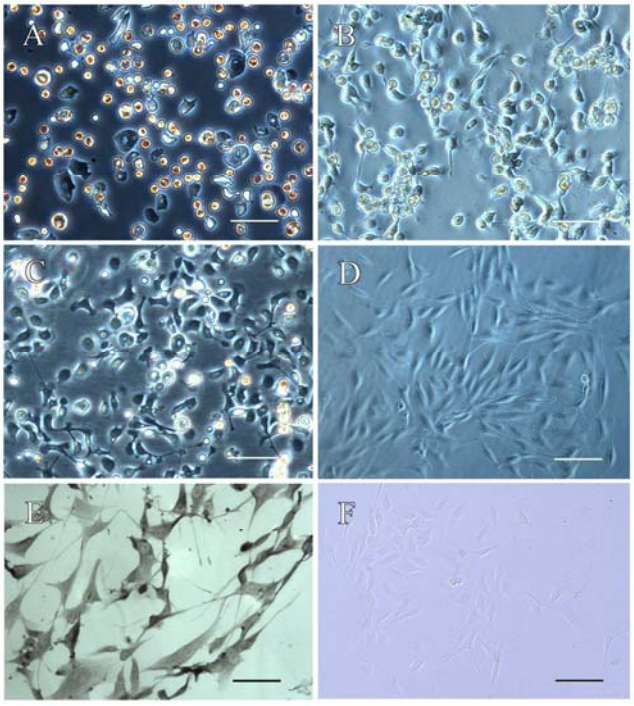

Figure 1. Morphology and tyrosinase activity of cultured alpaca skin melanocytes. Phase contrast micrographs illustrating morphology of cultured alpaca skin cells at (A) 1 h of culture, (B) 12 h of culture, (C) 24 h of culture, (D) passage 3 (21 d of culture). (E) Detection of tyrosinase activity in cultured alpaca melanocytes (passage 3, 21 d of culture) using the DOPA reaction. (F) Negative control for DOPA staining. Grey arrows denote melanocytes and black arrows denote epithelial cells (keratinocytes). 200× magnification. Bar = 50 μM.

Cultured melanocytes also showed positive immunoreactivity for the melanocyte specific markers S-100, tyrosinase and MITF (Figure 2A, C and E). Immunoreactivity was observed in all cells with characteristic melanocyte morphology, and immunonegative cells were rare. For S-100, tyrosinase and MITF, immunoreactivity was not detected when staining was performed in the absence of respective primary antibodies (Figure 2B, D and F).

To characterize changes in potential melanogenic activity of cells with extended culture, abundance of tyrosinase mRNA was measured in cells at passage 3, 5 and 10. As indicated in Figure 3, melanogenic capacity of cells (as indicated by tyrosinase mRNA abundance) did not decrease with increasing passage number. Rather, tyrosinase mRNA abundance was increased through passage 5 ($p< 0.01$) and maintained through passage 10 ($p<0.01$), suggesting that loss of melanogenic capacity does not likely occur with extended culture.

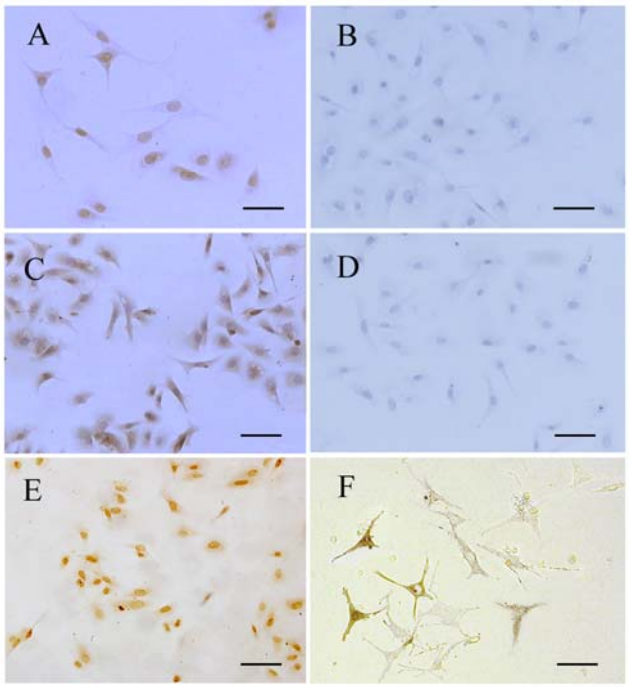

Figure 2. Detection of characteristic melanocyte cell markers in cultured alpaca skin melanocytes. (A) Immunocytochemical localization of S-100 antigen in primary cultures of alpaca skin melanocytes (passage 3, d 21 of culture). (B) Negative control for S-100 immunolocalization. (C) Immunocytochemical localization of tyrosinase in primary cultures of alpaca skin melanocytes (passage 3, d 21 of culture). (D) Negative control for tyrosinase immunolocalization. (E) Immunocytochemical localization of micropthalmia associated transcription factor (MITF) in primary cultures of alpaca skin melanocytes (passage 3, d 21 of culture). (F) Negative control for MITF immunolocalization. 200× magnification. Bar = 50 μM.

Hormonal responsiveness of cultured melanocytes to α-MSH

To establish that cultured alpaca melanocytes are hormonally responsive and to begin to examine regulation of key genes linked to coat color control, effects of 72 h treatment with increasing concentrations of α-MSH on abundance of MC1R mRNA were determined. As depicted in Figure 4, treatment with 1.7 ng ($p<0.05$) and 16.7 ng ($p<0.01$) α-MSH increased abundance of MC1R mRNA in cultured alpaca melanocytes. No increase in MC1R mRNA was observed in response to remaining dose (167.5 ng/ml) of α-MSH tested.

DISCUSSION

The mechanisms influencing coat color in fiber producing domestic species, such as the alpaca, are not well understood. Given the fact that alpacas exhibit over 22 different natural fleece colors of varying economic value (Gregor, 2006) a greater understanding of the factors that

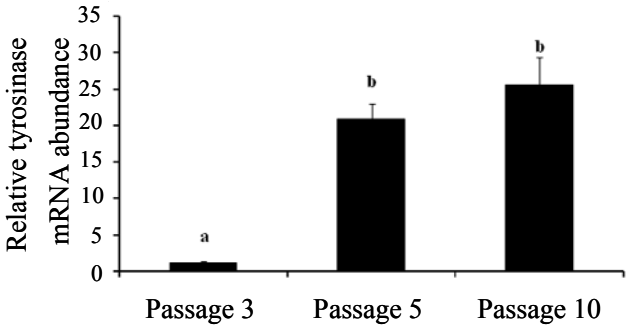

Figure 3. Effect of passage number on abundance of tyrosinase mRNA in cultured alpaca skin melanocytes. Real time PCR analysis of relative abundance of tyrosinase mRNA in alpaca melanocytes harvested at passage 3, 5 and 10. Abundance of tyrosinase mRNA was normalized relative to abundance of 18S rRNA. Bars in each panel represent the mean±SE. [a,b] $p<0.01$.

regulate melanocyte pigment production *in vitro* may lead to novel pharmacological and genetic approaches to predict coat color and or regulate pigmentation and enhance value of this agricultural commodity. Our goal in the present studies was to develop an *in vitro* model system appropriate for studies of the influence of intrinsic and extrinsic factors on key genes linked to coat color regulation in other species. Results of the present studies established a protocol for effective isolation and culture of melanocytes from alpaca skin for 10 passages. Results also demonstratee that cultured cells exhibit key phenotypic features of normal melanocytes including expression of the melanocyte markers S-100, tyrosinase and MITF (King et al., 1999; Clarkson et al., 2001), tyrosinase activity, and responsiveness to stimulation with α-MSH, a key local regulator of pigmentation (Tsatmali et al., 2002). To our knowledge, such model systems have not been established

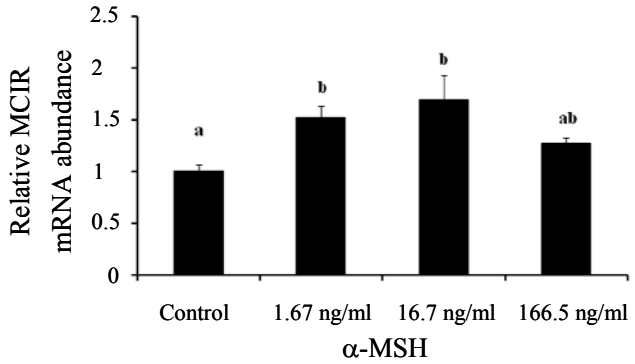

Figure 4. Hormonal responsiveness of cultured alpaca melanocytes. Effect of treatment of cultured alpaca melanocytes (passage 5) with increasing concentrations of α-MSH (0, 1.67, 16.7 and 166.5 ng/ml) on relative abundance of mRNA for the melanocortin 1 receptor (MC1R). Abundance of MC1R mRNA was normalized relative to abundance of β-actin mRNA. Bars in each panel represent the mean±SE. [a,b] $p<0.05$.

previously in fiber producing domestic species.

Culture of melanocytes from humans (Eisinger and Marko, 1982; Vancoillie et al., 1999) mice (Bennett et al., 1989) and pigs (Jule et al., 2003) for physiological and pathological studies has been reported previously. The main obstacle to establishment of pure melanocyte cultures *in vitro* is the elimination of contaminating fibroblasts. Separation of epidermis from dermis and underlying connective tissue is crucial to help eliminate contamination of fibroblasts. In our study, minimal connective tissue attached following skin punch biopsy was carefully removed before digestion and epidermal tissue was isolated from dermis by a two-step enzyme treatment using dispase II. Many previous studies used trypsin digestion to obtain normal melanocytes (Eisinger and Marko, 1982), but we found dispase II treatment to be more effective in terms of cell yield and attachment. Furthermore, media used for melanocyte culture in the present studies contained TPA and CT which have been shown in previous studies to promote melanocyte growth and inhibit growth of fibroblasts (Eisinger and Marko, 1982). Results of histochemical staining for tyrosinase activity and immunostaining for S-100 antigen, tyrosinase and MITF confirmed purity of melanocyte cultures in the present studies. Furthermore, marker gene expression and presumably melanogenic capacity of cultured melanocytes was maintained across multiple passage in the current study as tyrosinase mRNA expression increased through passage 5 and was maintained through passage 10. Collectively, results demonstrate that cells possess functional characteristics of normal melanocytes across multiple passages.

Phenotypic characteristics of melanocytes were confirmed using immunostaining for three distinct antigens in the current study. S-100 is a highly sensitive marker for melanocytes and other cells of neural crest origin, but is not 100% specific unless used in conjunction with other melanocyte markers such as tyrosinase (Clarkson et al., 2001). In contrast, tyrosinase and MITF are more specific as they are directly involved in melanogenesis (Yamaguchi and Hearing, 2009). Tyrosinase catalyzes the rate limiting step in melanogenesis and is functionally linked to expression of a pigmented phenotype (Yamaguchi and Hearing, 2009). The transcription factor MITF functions as a key regulator of melanocyte development, function and survival and regulates genes involved in cell cycle and melanocyte differentia (Hemesath et al., 1998; Levy et al., 2006). MITF acts as a master regulator of pigment production and mediates effects of extracellular signals triggered by α-MSH, agouti signaling protein and Kit ligand (Aberdam et al., 1998; Hemesath et al., 1998). Furthermore, MITF mutations cause pigmentation defects (Steingrimsson et al., 1994; Shibahara et al., 2000). Understanding the factors that regulate MITF and tyrosinase expression in cultured alpaca melanocytes will be of considerable interest in future studies.

We also demonstrated hormonal responsiveness of cultured alpaca melanocytes in the current studies. Treatment of cells with α-MSH resulted in a dose dependent increase in MC1R mRNA expression. The functional role of MC1R in regulation of hair and coat color is well established. Mutations in MC1R have been linked to differences in hair color in humans (Rees, 2000; Kadekaro et al., 2003) and coat color in multiple other species including farm animals (Andersson, 2003). Intracellular signaling via MC1R is stimulated by its native agonists including α-MSH and by its native antagonist, Agouti signaling protein (Suzuki et al., 1997). Agonist induced activation of the MC1R stimulates melanogenesis (Tsatmali et al., 2002). Upregulation of MC1R gene expression by α-MSH in cultured murine melanocytes has been reported previously (Rouzaud et al., 2003) and may therefore enhance sensitivity of cells to melanogenic stimuli. Demonstrated hormonal responsiveness of cultured alpaca melanocytes further supports the utility of our model for studies of regulation of key genes and components of the melanogenic machinery linked to coat color.

In conclusion, methods for isolation and culture of pure populations of melanocytes from alpaca skin across multiple passages have been demonstrated in the current studies. Results illustrate the potential utility of our model for studies of the intrinsic and extrinsic regulation of key genes controlling melanocyte pigment production and hence the cellular and genetic mechanisms responsible for the significant variation in pigmentation (over 22 natural coat colors) observed in this fiber producing domestic species.

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