Primary Culture and Subculture Methods for Preadipocytes from Cashmere Goat Lambs and Adult Sheep Postprint

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Abstract

This study primarily explored the culture and passage methods of preadipocytes from Cashmere goat lambs and adult goats to provide a cellular model for investigating fat metabolism mechanisms in Cashmere goats. Using perirenal adipose tissue from 3-month-old Albas White Cashmere goat lambs as experimental material, preadipocytes were directly isolated via the collagenase method and identified through cell morphology observation, growth curve analysis, and Oil Red O staining. Using perirenal adipose tissue from adult Cashmere goats as experimental material, mature adipocytes were first obtained through the collagenase method and the "ceiling" method, from which preadipocytes were subsequently derived via dedifferentiation; their differentiation into mature adipocytes was assessed by Oil Red O staining following induction. The optimal isolation conditions for preadipocytes from perirenal adipose tissue of Cashmere goat lambs were 0.1% Type I collagenase digestion at 37 °C for 1 h, followed by centrifugation at 250g for 10 min; during subculture, cells were dissociated using 0.25% trypsin for 60 s. The cells exhibited spindle-shaped morphology, an S-shaped growth curve, and positive Oil Red O staining. For adult goats, after obtaining mature adipocytes using the same collagenase method as employed for lambs, preadipocytes were acquired through an improved "ceiling" method, yielding positive Oil Red O staining results after induced differentiation. In conclusion, the Type I collagenase digestion method enables direct isolation and culture of preadipocytes from 3-month-old Cashmere goat lambs, while the combination of collagenase method and improved "ceiling" method is feasible for isolating and culturing preadipocytes from adult Cashmere goats.

Full Text

Primary Culture and Passage Method of Preadipocytes from Cashmere Goat Lambs and Adult Goats

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Abstract

This study aimed to establish methods for the culture and passage of preadipocytes derived from cashmere goat lambs and adult goats, thereby providing a cellular model for investigating the mechanisms of lipid metabolism in cashmere goats. Perirenal adipose tissue from 3-month-old Albas cashmere goat lambs was used to isolate preadipocytes directly via collagenase digestion. The isolated cells were characterized through morphological observation, growth curve analysis, and oil red O staining. For adult goats, perirenal adipose tissue was processed using collagenase digestion and the "ceiling" culture method to obtain mature adipocytes, which were then dedifferentiated to generate preadipocytes. Following induction of differentiation, oil red O staining confirmed their capacity to differentiate into mature adipocytes. The optimal isolation conditions for preadipocytes from lamb perirenal adipose tissue were identified as digestion with 0.1% type I collagenase at 37 °C for 1 h, followed by centrifugation at 250×g for 10 min. For cell passage, 0.25% trypsin digestion for 60 s was employed. The preadipocytes exhibited a spindle-shaped morphology, displayed an S-shaped growth curve, and tested positive for oil red O staining. Mature adipocytes from adult goats were obtained using the same collagenase method as for lambs, and preadipocytes were subsequently derived using a modified "ceiling" culture method. After induction of differentiation, these cells also tested positive for oil red O staining. In conclusion, type I collagenase digestion is a feasible approach for directly isolating and culturing preadipocytes from 3-month-old Albas cashmere goat lambs, while the combination of collagenase digestion and the modified "ceiling" culture method is effective for isolating and culturing preadipocytes from adult cashmere goats.

Keywords: cashmere adult goats; cashmere lambs; preadipocytes; cell culture

Introduction

Fatty acid metabolism within adipocytes is closely associated with meat flavor. Since adipocytes differentiate from preadipocytes through a process involving a series of transcription factors that determine adipocyte formation, preadipocytes

represent an ideal model for studying animal lipid metabolism mechanisms. In vitro culture of preadipocytes can recapitulate the entire process of adipocyte development, proliferation, and differentiation, facilitating observation of various factors affecting these cells. This approach enables investigation of adipogenesis mechanisms and the study of effective regulatory methods for fat formation.

Currently, the collagenase method has been used domestically to establish preadipocyte culture models for various animals including cattle [1], sheep [2], pigs [3], and cashmere goats [4], though these models have predominantly been derived from young animals. The number of adipocytes is largely determined during early life [5], and as animals age, preadipocytes progressively differentiate into adipocytes. Consequently, direct isolation of animal preadipocytes via collagenase digestion is only applicable to embryonic and young animals, limiting mechanistic studies of lipid metabolism by age. Therefore, developing methods for isolating and culturing preadipocytes from adult animals is essential. Currently, preadipocytes from adult animals are primarily obtained through dedifferentiation of mature adipocytes using the "ceiling" culture method. Dedifferentiation of mature adipocytes is a lipid metabolic process dominated by lipolysis accompanied by a certain level of lipogenesis [6]. During the accumulation of mature adipocytes, fatty acid metabolism and lipid droplet metabolism are regulated by genes, ultimately leading to lipid droplet disappearance [7]. Through autocrine and paracrine pathways, adipocyte decomposition is stimulated, particularly by reducing lipoprotein lipase production and activity, thereby preventing preadipocyte differentiation and inducing reverse differentiation of mature adipocytes to facilitate dedifferentiation [8]. However, research on the "ceiling" culture method has primarily focused on humans [8], mice [9], pigs [10], cattle [11], and rabbits [12], with few reports on isolating and culturing preadipocytes from adult cashmere goats using this approach. Moreover, the traditional "ceiling" culture method utilizes culture flasks, requiring large volumes of culture medium and high initial cell seeding densities. Although some studies have improved the traditional method by combining culture dishes with coverslips, circular coverslips are difficult to source and contamination is hard to avoid during cutting, which hinders research progress [13].

In view of these limitations, the present study aimed to explore culture and passage methods for preadipocytes from 3-month-old lambs and adult goats of the Albas cashmere breed, using perirenal adipose tissue as experimental material. Additionally, we sought to improve the "ceiling" culture method by using culture dishes instead of flasks, providing a foundation for in-depth investigation of cashmere goat lipid metabolism through preadipocyte models, which holds important theoretical and practical significance for regulating lipid metabolism and improving meat quality in cashmere goats.

Materials and Methods

1.1 Experimental Animals and Main Reagents and Instruments

Experimental animals: Three-month-old Albas cashmere goat lambs and adult Albas cashmere goat does.

Main reagents: DMEM/F12 medium, 0.25% trypsin, penicillin-streptomycin dual antibiotics, and insulin-transferrin-selenium (ITS) were purchased from Gibco (USA). Type I collagenase, dexamethasone (prepared as 1 mg/mL working solution), 3-isobutyl-1-methylxanthine (IBMX, prepared as 100 mg/mL working solution), dimethyl sulfoxide (DMSO), amphotericin B (prepared as 2.5 mg/mL working solution), and oil red O were purchased from Sigma (USA). Phosphate-buffered saline (PBS) and fetal bovine serum (FBS) were purchased from Hyclone (USA). All other reagents were of analytical grade.

Main instruments: Centrifuge, inverted microscope, laminar flow hood, 37 °C CO_2 incubator, 37 °C constant temperature water bath, 37 °C constant temperature shaker, 25 cm^2 Corning cell culture flasks, and 35 mm Corning cell culture dishes.

1.2.1 Primary and Passage Culture of Lamb Preadipocytes

Isolation and primary culture of lamb preadipocytes: Preadipocytes were isolated using the type I collagenase method. Perirenal adipose tissue was collected from Albas cashmere goat lambs, immersed in 75% ethanol for 30 s, and immediately transferred to PBS containing dual antibiotics. Within a laminar flow hood, the tissue was washed several times with PBS containing dual antibiotics, visible blood vessels and connective tissue were removed, and the tissue was cut into small pieces approximately 0.5–1.0 mm in size. The tissue fragments were transferred to a sterile wide-mouth glass bottle, and 0.1% type I collagenase was added (volume ratio of adipose tissue to collagenase = 1:3). Digestion was carried out in a 37 °C constant temperature shaker for 1 h. The digest was filtered through a 200-mesh cell strainer, and the filtrate was centrifuged at 250×g for 10 min. The supernatant was discarded, and the pellet was resuspended in basal medium (containing 15.6 g/L DMEM/F12 medium and 1.2 g/L sodium bicarbonate) to prepare a cell suspension, which was centrifuged again at 250×g for 10 min. After discarding the supernatant and repeating the centrifugation step, the pellet was resuspended in 5 mL complete medium (containing 25% FBS, 2.5% dual antibiotics, and 0.25% amphotericin B working solution) and seeded into a 25 cm^2 culture flask. Cells were cultured at 37 °C with 5% CO_2, with medium changes every 2 days.

Passage culture of lamb preadipocytes: When primary cells reached 80–90% confluence (approximately day 5), the culture medium was removed, cells were washed twice with PBS, and 2 mL of 0.25% trypsin solution was added to each flask. Upon microscopic observation of most cells retracting into round dots and beginning to detach, 3 mL of complete medium was added to terminate

digestion. The cell suspension was collected and centrifuged at 250×g for 10 min. After discarding the supernatant and washing with PBS, cells were resuspended in 5 mL complete medium and subcultured at a 1:3 ratio (first passage, F1). Cells were incubated at 37 °C with 5% CO_2, with medium changes every 2 days until they reached confluence and covered the entire flask (approximately day 7), at which point they were passaged again to generate the second passage (F2).

1.2.2 Primary and Passage Culture of Adult Goat Preadipocytes

Isolation and primary culture of adult goat preadipocytes: Mature adipocytes were first isolated and cultured using collagenase digestion and a modified "ceiling" culture method, then preadipocytes were obtained through dedifferentiation. Perirenal adipose tissue was collected from adult Albas cashmere goats, immersed in 75% ethanol for 30 s, and immediately transferred to PBS containing dual antibiotics. The tissue was washed, minced, digested, filtered, and centrifuged using the same method as for lamb adipose tissue. After centrifugation, the supernatant (containing adipocytes floating in the supernatant) was mixed with an equal volume of basal medium, and 1 mL of this mixture was seeded into a 35 mm sterile culture dish. Complete medium was added until the dish was nearly overflowing. The original culture method was improved by inverting the matching dish lid and sliding it over the dish to cover it without creating air bubbles. This utilized surface tension to firmly adhere the lid to the dish, ensuring a sealed environment. The dish was inverted, and mature adipocytes were cultured at 37 °C with 5% CO_2. Due to buoyancy, the mature adipocytes floated to the "ceiling" surface to grow. Medium was changed every 2 days thereafter. The mature adipocytes re-entered the cell cycle and gradually expelled intracellular lipid droplets. By day 7, they had essentially dedifferentiated into spindle-shaped cells capable of adherent growth. The culture dish was then returned to the upright position for normal culture until lipid droplets completely disappeared and cells dedifferentiated into preadipocytes with fibroblast-like morphology.

Passage culture of adult goat preadipocytes: The passage method was identical to that described for lamb preadipocytes.

1.2.3 Growth Curve of Lamb Preadipocytes

Following the method of Situ Zhenqiang et al. [14], lamb preadipocytes were counted using a hemocytometer. Primary lamb preadipocytes were seeded into 35 mm culture dishes at 80% confluence. Cells were randomly divided into 8 groups with 3 replicates per group. Cells in each dish were counted every 2 days, with averages calculated until day 14. The doubling time of preadipocytes was calculated based on the cell counts, where DT represents cell doubling time, t represents culture time, N_0 represents the initial cell count, and Nt represents the cell count after t time.

1.2.4 Identification of Lamb and Adult Goat Preadipocytes

Second-passage (F2) preadipocytes were induced to differentiate into adipocytes and subjected to oil red O staining to confirm that the cultured cells were indeed preadipocytes.

Induction of differentiation for lamb and adult goat preadipocytes: When F2 cells reached 80–90% confluence, the medium was replaced with induction medium (containing 10% FBS, 0.04% dexamethasone working solution, 0.011% IBMX working solution, 0.5% ITS, 2% dual antibiotics, and 0.2% amphotericin B working solution) for 2 days. The medium was then changed to insulin medium (containing 10% FBS, 0.5% ITS, 4% dual antibiotics, and 0.4% amphotericin B working solution) for another 2 days. Finally, cells were cultured in complete medium with medium changes every 2 days until lipid droplets appeared in 80% of the cells.

Identification of lamb and adult goat preadipocytes: Oil red O staining was used to identify differentiated adipocytes. After induction of differentiation, the complete medium was removed from lamb preadipocytes and adult goat dedifferentiated preadipocytes, and cells were rinsed twice with PBS. Cells were fixed with 4% paraformaldehyde for 30 min, rinsed twice with PBS, and stained with oil red O (1% oil red O in isopropanol, diluted 1.67-fold with distilled water) for 1 h. After removing the staining solution and rinsing several times with distilled water, cells were observed under an inverted microscope.

Results

2.1 Morphological Observation of Primary Cultured Lamb Preadipocytes

After 2 days of culture, most preadipocytes were short spindle-shaped or irregular triangular cells with obvious extension [Figure 1: see original paper]A. With extended culture time, cells on days 3 and 4 exhibited larger volume, greater extension, and more prominent fibroblast-like morphology [Figure 1: see original paper]B, [Figure 1: see original paper]C. Subsequently, cells began to proliferate rapidly with significantly increased density, even showing overlapping growth. Cells underwent contact inhibition and density-dependent growth cessation. After extensive confluence, some cells showed spontaneous differentiation, with differentiated cells beginning to deposit fat granules and numerous small lipid droplets appearing within the cytoplasm [Figure 1: see original paper]D.

A. Cells on day 2 of primary culture ($\$\times 200$); $B.Cellsonday3ofprimaryculture(\times 200); C.Cellsonday4ofprima$

Fig. 1 Morphological observation of primary cultured preadipocytes of lambs

2.2 Morphological Observation of Passaged Lamb Preadipocytes

First-passage (F1) and second-passage (F2) cells were morphologically similar to primary cells, displaying a fibroblast-like appearance. The main differences

were that F1 and F2 cells were more homogeneous, exhibited more uniform morphology, grew more vigorously, showed stronger resistance to contamination, and demonstrated reduced spontaneous differentiation [Figure 2: see original paper]A, [Figure 2: see original paper]B.

A. Cells of F1 on day 2 of culture ($\times$100); $B.Cells of F2 at 4h of culture$($\times$\$100)

Fig. 2 Morphological observation of passaged cultured preadipocytes of lambs

2.3 Growth Curve of Lamb Preadipocytes

As shown in [Figure 3: see original paper], the growth curve of lamb preadipocytes was S-shaped. Cells remained in a lag phase with slow growth during the first 4 days after seeding. After 4 days, cell growth accelerated and entered the logarithmic growth phase. After 12 days, adipocyte growth entered the plateau phase. The calculated doubling time of preadipocytes was 6.9 days.

Fig. 3 Growth curve of preadipocytes of lambs

2.4 Induction, Differentiation, and Identification of Lamb Preadipocytes

Passaged F2 cells were induced to differentiate, and microscopic observation revealed they sequentially progressed through differentiation preparation, initiation, rapid differentiation, and maturation phases, ultimately differentiating into adipocytes.

Differentiation preparation phase: F2 cells at 90% confluence were cultured for an additional 2 days (designated as day 2 of differentiation) to exit the growth cycle and prepare for subsequent differentiation [Figure 4: see original paper]A.

Differentiation initiation phase: Cells were cultured in induction medium for 2 days (designated as day 4 of differentiation). Under the influence of inducers, cells entered the differentiation initiation phase with more prominent fibroblast-like morphology [Figure 4: see original paper]B.

Rapid differentiation phase: Cells were then cultured in insulin differentiation medium for 2 days (designated as day 6 of differentiation). Cells rapidly entered the differentiation state, with small lipid droplets beginning to appear in the cytoplasm, though in small numbers.

Differentiation maturation phase: Cells were cultured in complete medium for an additional 2 days (designated as day 8 of differentiation). Intracellular lipid droplets became more prominent, and oil red O staining was positive with characteristic "ring" structures visible [Figure 4: see original paper]C. By day 10 of differentiation, lipid droplets increased in number and size, fused together, and occupied most of the cell volume. Some lipid droplets were secreted into the culture medium, making it viscous. Some cells detached and floated in the medium. After oil red O staining, lipid droplets were stained red [Figure 4: see original paper]D, confirming these cells as adipocytes and the pre-differentiation

cells as preadipocytes. Observation indicated that approximately 10 days were required for preadipocytes to develop into mature adipocytes.

A. Cells of F2 on day 2 of differentiation ($\times$200); $B.Cells of F2 on day 4 of differentiation$($\times$200); $C.Cells of F2 o$

Fig. 4 Morphological observation of inducement, differentiation and identification of preadipocytes of lambs

2.5 Morphological Observation of Trypsin Digestion of Lamb Preadipocytes

Different tissues or cells exhibit varying responses to trypsin treatment, and longer digestion times cause greater cell damage. When lamb preadipocytes were digested with trypsin for 60 s, microscopic observation revealed most cells became round dots [Figure 5: see original paper]A, indicating cell detachment. After 90 s of digestion, cells were floating [Figure 5: see original paper]B, suggesting excessive digestion time that may have caused cell damage. Therefore, the optimal digestion time was determined to be 60 s.

A. Preadipocytes trypsinized for 60 s ($\times$100); $B.Preadipocytes trypsinized for 90 s$($\times$100)

Fig. 5 Morphological observation of trypsin digestion of preadipocytes of lambs

2.6 Isolation, Culture, and Dedifferentiation of Adult Goat Mature Adipocytes

Adipocytes obtained from adult goat perirenal adipose tissue using the collagenase method were cultured for 2 days. Microscopic observation revealed that mature adipocytes floated to the "ceiling" layer of the inverted culture dish, with adherent cells showing single-chamber lipid droplet morphology typical of mature adipocytes, and dividing adipocytes were observed [Figure 6: see original paper]A. On day 5 of culture, cells began to assume a spindle shape [Figure 6: see original paper]B. With increasing culture time, spindle-shaped cells gradually increased, and cells were observed "expelling" lipid droplets [Figure 6: see original paper]C. By day 14, lipid droplets had completely disappeared from cells, which had dedifferentiated into preadipocytes with fibroblast-like morphology [Figure 6: see original paper]D.

A. Mature adipocytes on day 2 of culture ($\times$100); $B.Mature adipocytes on day 5 of culture$($\times$100); $C.Mature adi$

Fig. 6 Morphological observation of proliferation and dedifferentiation of mature adipocytes of adult goats

2.7 Induced Differentiation and Identification of Adult Goat Preadipocytes

As F2 preadipocytes differentiated, the number of oil red O-stained cells gradually increased [Figure 7: see original paper]A. By day 12 of differentiation, over 90% of cells were positive for oil red O staining [Figure 7: see original paper]B,

confirming that the differentiated cells were adipocytes and that the cultured cells were preadipocytes.

A. Preadipocytes on day 6 of inducement, stained with oil red O ($\times$200); $B.Matureadipocytesonday12ofcultur$

Fig. 7 Morphological observation of induced differentiation and identification of preadipocytes of adult goats

Discussion

The collagenase digestion method is a fundamental technique for cell isolation. Collagenase, extracted from bacteria, effectively digests collagen and interstitial matrix with minimal impact on cells themselves, allowing cells to detach from collagen components without damage. Preadipocytes can be derived from various adipose tissues, and the collagenase digestion method has been used to establish preadipocyte culture models for multiple animals both domestically and internationally, though these have predominantly originated from young animals. For example, Cai et al. [2] obtained preadipocytes from perirenal adipose tissue of 1-day-old sheep. Preadipocytes have also been isolated from intramuscular adipose tissue of 1-day-old cashmere goats [4], subcutaneous adipose tissue of 60-day-old pigs [3], subcutaneous adipose tissue of 1-day-old pigs [15], inguinal and epididymal adipose tissue of 20-day-old rats [16], inguinal and perirenal adipose tissue of newborn cattle [1], and cervical and dorsal subcutaneous adipose tissue of 7-day-old pigs [17]. Oil red O staining can stain lipid droplets red, with transparent "ring" structures appearing at lipid droplet boundaries. This method provides a simple and rapid means to determine the conversion rate of cultured preadipocytes in vitro. Ramirez-Zacarias et al. [18] reported that its sensitivity and accuracy are similar to glycerol phosphate dehydrogenase, a marker enzyme in preadipocyte differentiation, making it commonly used for in vitro preadipocyte identification. Therefore, this study employed this method to identify preadipocyte differentiation into adipocytes. The results demonstrated that preadipocytes obtained from perirenal adipose tissue of 3-month-old lambs were spindle-shaped with an S-shaped growth curve similar to fibroblasts. After passage (F2 generation) and induction of differentiation, characteristic rings appeared on day 8 of differentiation, and by day 10, lipid droplets had enlarged and fused with positive oil red O staining results. These findings meet the identification criteria for preadipocytes proposed by Ng et al. [19] and Van et al. [20], confirming the feasibility of obtaining preadipocytes from cashmere goat lambs using this method and providing an important cellular model for studying cashmere goat lipid metabolism at the cell biology level. Additionally, the induction differentiation agents used in this study constitute a classic hormone cocktail composed of insulin, dexamethasone, and IBMX, which has been successfully used to induce preadipocyte differentiation into adipocytes in numerous domestic and international reports [21-22]. However, some studies have indicated that mesenchymal stem cells from adipose tissue can also differentiate into adipocytes with functions similar to preadipocytes [23]. Therefore, further research is needed regarding the purification of preadipocytes and their

differentiation from mesenchymal stem cells.

The number of adipocytes is largely determined during early life [5], and as animals age, preadipocytes progressively differentiate into adipocytes. Therefore, direct isolation of animal preadipocytes using the collagenase method is only applicable to embryonic and young animals, restricting mechanistic studies of lipid metabolism in adult animals by age. Consequently, obtaining preadipocytes from mature adipocytes of adult animals through dedifferentiation is essential, with the "ceiling" culture method being the most commonly used approach. Mature adipocytes automatically undergo dedifferentiation to form preadipocytes after accumulation [6], and can stimulate adipocyte decomposition through autocrine and paracrine pathways, prevent preadipocyte differentiation, and induce reverse differentiation of mature adipocytes to promote dedifferentiation [8]. Chen et al. [8] used collagenase to isolate mature adipocytes from abdominal fat of 30-35-year-old women and dedifferentiated them into preadipocytes using the "ceiling" culture method. Additionally, preadipocytes have been obtained through dedifferentiation of mature adipocytes from human orbital fat with an average age of 48.5 years [24], subcutaneous fat of 12-week-old mice [16], and inguinal fat of 4-month-old rabbits [19]. In this study, perirenal adipose tissue from adult cashmere goats was used as experimental material. Mature adipocytes were isolated and cultured using the same collagenase digestion method combined with the "ceiling" culture method as for lambs, and preadipocytes were obtained through dedifferentiation. These preadipocytes were then induced to differentiate into adipocytes. The study found that primary adipocytes began to adhere to the "ceiling" layer after 2 days of culture, started to show spindle-shaped morphology by day 5, and completely dedifferentiated into preadipocytes by day 14. Based on this, passaged and induced F2 cells showed over 90% positive oil red O staining by day 12 of differentiation, indicating that mature adipocytes had been successfully induced to differentiate into preadipocytes. Therefore, the "ceiling" culture method is feasible for obtaining preadipocytes from adult cashmere goats. Compared with direct isolation and culture of preadipocytes using the collagenase method, this approach yields preadipocytes with higher purity and stronger differentiation capacity, and is not limited by animal age, despite having relatively higher costs and longer culture periods. This provides a new model for studying adipocyte differentiation and metabolism. Furthermore, the traditional "ceiling" culture method requires culture flasks, consumes large volumes of culture medium, and demands high initial cell seeding densities. Wei et al. [11] used nested culture dishes for culture, which still consumed considerable medium. Therefore, based on existing adipocyte "ceiling" culture methods, this study improved the technique by inverting the culture dish lid instead of using flasks, successfully obtaining preadipocytes. This modification not only saved culture medium and reduced costs but also was simple to perform while ensuring a sealed culture environment.

Conclusions

1. Using perirenal adipose tissue from 3-month-old Albas cashmere goat lambs as experimental material, preadipocytes can be directly obtained using type I collagenase digestion.
2. Using perirenal adipose tissue from adult Albas cashmere goat does as experimental material, preadipocytes can be obtained through collagenase digestion and the "ceiling" culture method to isolate mature adipocytes, followed by dedifferentiation. This approach is feasible and provides a cellular model for investigating lipid metabolism mechanisms in cashmere goats.

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Note: Figure translations are in progress. See original paper for figures.