Establishment of a Goat Hair Follicle Outer Root Sheath Cell Line (Postprint)

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Abstract

This study aimed to establish a homogeneous and stable goat hair follicle outer root sheath cell line. Back skin was collected in vivo from Jining Grey goat kids, and outer root sheath cells were isolated using a combined mechanical separation and enzymatic digestion method, then cultured in serum-free keratinocyte medium containing epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I), basic fibroblast growth factor (bFGF), and hydrocortisone, with primary culture initiated in a 37 °C incubator with 5% CO2 concentration. When the primary cells formed a confluent monolayer, subculture was performed; when cells were subcultured to passages 8-10, the medium was changed to DMEM/F12 containing EGF, IGF-I, bFGF, hydrocortisone, and FBS for long-term culture. Outer root sheath cells at passage 40 were selected for growth characteristic studies and chromosome analysis. The results showed that the doubling time of cells cultured in vitro was 51.9 h, and the chromosome number of cultured cells remained predominantly 2n=60, but exhibited aneuploid chromosomal characteristics; immunocytochemical identification results showed that the cell line was positive for cytokeratin 19 expression. These results indicate that the cells isolated and cultured in this study were indeed outer root sheath cells differentiated from goat hair follicle stem cells, and the goat hair follicle outer root sheath cell line cultured in vitro was successfully established.

Full Text

Establishment of a Goat Outer Root Sheath Cell Line

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Abstract

This study aimed to establish a pure and stable outer root sheath cell line from goat hair follicles. Dorsal skin tissue was collected from Jining grey goat lambs under sterile conditions. Outer root sheath cells were isolated using a combination of mechanical separation and enzymatic digestion, then cultured in keratinocyte serum-free medium supplemented with epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I), basic fibroblast growth factor (bFGF), and hydrocortisone at 37°C with 5% CO_2 for primary culture. Once primary cells formed a confluent monolayer, subculture was initiated. Cells at passages 8–10 were transferred to DMEM/F12 medium containing EGF, IGF-I, bFGF, hydrocortisone, and fetal bovine serum (FBS) for long-term culture. Passage 40 cells were selected for growth characteristic studies and chromosome analysis. Results showed that the population doubling time of cultured cells was 51.9 h, with the majority maintaining the diploid chromosome number of 2n=60, though aneuploid characteristics were observed. Immunocytochemical identification confirmed positive expression of cytokeratin 19. These results demonstrate that the isolated and cultured cells were indeed outer root sheath cells differentiated from goat hair follicle stem cells, and a goat hair follicle outer root sheath cell line was successfully established in vitro.

Keywords: outer root sheath cell; cell line; Jining grey goat; growth characteristic; chromosome number

Introduction

The hair follicle is a skin appendage formed by epidermal invagination into the dermis that controls hair growth, possessing a unique structure, the ability to synthesize various specific keratin proteins, and the capacity for cyclical regeneration. Hair follicle formation and differentiation involve at least 20 distinct cell populations, primarily composed of dermal papilla cells, hair matrix cells, inner root sheath cells, and outer root sheath cells [1-2].

Among these, outer root sheath cells (ORSCs), as important hair follicle epithelial cells, have attracted increasing attention due to their high proliferative potential and crucial roles in hair regeneration, wound healing, and differentiation of primary and secondary hair follicles.

Since Wetering et al. [3] successfully induced migration of human hair follicle outer root sheath cells from free hair follicles using cobalt-irradiated bovine lens capsule as a support, numerous scholars have improved the in vitro culture systems and techniques for outer root sheath cells. Oshima et al. [4] directly inoculated isolated hair follicles onto plastic culture dishes and, under controlled pH conditions not exceeding 7.2, observed outer root sheath cells migrating from the adherent hair follicles. However, this method yielded low cell numbers and poor regenerative capacity, making it unsuitable for further research and

large-scale applications. To date, various cell isolation techniques and culture methods have been applied to outer root sheath cell culture in vitro, with two commonly used approaches: (1) treating isolated hair follicles with trypsin digestion, preparing a cell suspension in DMEM/F12 medium containing 10% fetal bovine serum (FBS), and inoculating onto a mitomycin-treated 3T3 feeder layer for primary culture at 37°C and 5% CO_2 [5-8]; and (2) using a collagen embedding method, where hair follicles with outer root sheath portions are digested with trypsin at 37°C, then transferred to plastic culture dishes coated with Type I and Type IV collagen, cultured in DMEM medium containing 10% FBS, and switched to serum-free DMEM medium after firm attachment [9-10]. Although both methods can obtain hair follicle outer root sheath cells, they still contain small amounts of non-outer root sheath cells, and cells undergo apoptosis after 8–10 passages, preventing continued subculture.

The above outer root sheath cell cultures primarily used human hair follicles as the tissue source. For other mammals, methods were mainly adapted from human hair follicle cell culture protocols. Studies have shown that isolating mouse vibrissa follicles and inoculating them into RPMI1640 medium containing epidermal growth factor (EGF) and 10% FBS, with culture plates pre-coated with mitomycin C-treated epidermal cell feeder layers, successfully yielded pure outer root sheath cells that grew stably to passages 8–10 [11]. Other researchers have modified these methods to successfully isolate and culture outer root sheath cells and other hair follicle cells from rats, cattle, alpacas, pigs, and rabbits [12-13]. Despite extensive research on animal hair follicle cell culture, reports on in vitro culture of goat hair follicle cells, particularly regarding establishment of outer root sheath cell lines, remain relatively scarce, and few cell strains and lines are available for animal hair follicle research.

Based on isolation, purification, and inoculation methods from domestic and international researchers, this study aimed to improve protease treatment of skin tissue and secondary follicle isolation methods to increase outer root sheath cell yield. Growth characteristics and chromosome analysis were performed at passage 40 to establish cell strains and lines for animal hair follicle research.

Therefore, establishing an in vitro cultured hair follicle outer root sheath cell line and improving isolation and culture methods can lay the foundation for further research on animal skin hair follicle growth and development patterns and their influencing factors. It also provides an in vitro cell model for studying hair follicle cell growth, development, and differentiation. Research on goat hair follicle outer root sheath cells to explore hair follicle growth regulatory mechanisms and cashmere growth-degeneration patterns will ultimately open new avenues for improving the yield and quality of wool, cashmere, and leather products. Additionally, using the goat outer root sheath cell line as a model to study interactions between outer root sheath cells and dermal papilla cells, inner root sheath cells, and skin fibroblasts can provide important insights for medical applications in hair follicle regeneration and alopecia treatment.

Methods

1.1 Reagents and Materials

DMEM/F12 medium and keratinocyte serum-free medium (K-SFM) were purchased from Gibco; fetal bovine serum (FBS) from MDgenics; trypsin, EGF, and colchicine from Sigma; insulin-like growth factor-I (IGF-I) and hydrocortisone from Peprotech; penicillin sodium from Shandong Lukang Chenxin Pharmaceutical; streptomycin sulfate from Shandong Ruiyang Pharmaceutical; Giemsa stain from Fluka; cytokeratin-19 (CK-19) antibody from Acris; ready-to-use Biotin SP-HRP immunohistochemistry kit and diaminobenzidine (DAB) chromogenic kit from Beijing Dingguo Changsheng Biotechnology.

Complete culture medium: DMEM/F12 containing 10 ng/mL EGF, 10 ng/mL IGF-I, 0.1 ng/mL bFGF, 0.4 g/mL hydrocortisone, and 10% FBS.

Working culture medium: Serum-free keratinocyte medium containing 10 ng/mL IGF-I, 10 ng/mL EGF, 0.1 ng/mL bFGF, and 0.4 g/mL hydrocortisone.

Maintenance culture medium: DMEM/F12 containing 10 ng/mL IGF-I, 10 ng/mL EGF, 0.1 ng/mL bFGF, 0.4 g/mL hydrocortisone, and 2% FBS.

D-Hank' s solution: Composed of 8 g/L NaCl, 0.4 g/L KCl, 0.06 g/L $Na_2HPO_4 \cdot H_2O$, 0.06 g/L KH_2PO_4, 0.35 g/L $NaHCO_3$, and 0.4 g/L phenol red.

Phosphate-buffered saline (PBS): Composed of 8 g/L NaCl, 0.2 g/L KCl, 1.56 g/L $Na_2HPO_4 \cdot H_2O$, and 0.2 g/L KH_2PO_4.

Cryopreservation medium: DMEM/F12 containing 10% dimethyl sulfoxide (DMSO) and 30% FBS.

Carnoy' s fixative: Prepared fresh by mixing three volumes of methanol with one volume of glacial acetic acid, stored on ice.

1.2 Experimental Animals and Sample Collection

Pregnant ewes were purchased from Jiaxiang Breeding Sheep Farm in Jining City, Shandong Province, and raised at the Animal Husbandry Science and Technology Experimental Station of Shandong Agricultural University. Using surgical procedures, dorsal skin tissue blocks approximately 0.5 cm × 0.5 cm were randomly collected from 1–3-day-old lambs. The skin tissue blocks were disinfected with iodine and alcohol for about 30 seconds, then washed with physiological saline to remove the alcohol, and immediately placed in serum-free DMEM/F12 medium on ice for transport to the laboratory. Isolation and culture were performed within 12 hours.

1.3 Preparation of Cell Culture Plates for Primary ORSC Culture

Pre-cultured goat skin fibroblasts were inoculated into 6-well cell culture plates with 3 mL of DMEM/F12 containing 10% FBS per well, and cultured at 37°C

with 5% CO_2 in saturated humidity. After 3–5 days when cells reached confluence, the medium was removed. Approximately 3 mL of sterile 1% Triton X-100 was added to the monolayer and incubated at 37°C for 30 minutes. The Triton X-100 solution was then removed, the plates were rinsed thoroughly with sterile ultrapure water, and stored at 4°C for later use.

1.4 Primary Culture of Goat Hair Follicle Outer Root Sheath Cells

The collected skin tissue blocks were disinfected by soaking in 70% alcohol, washed with D-Hank' s solution to remove residual alcohol, and placed under a stereomicroscope. Curved ophthalmic scissors and dissecting needles were used to scrape off residual exposed hair shafts and subcutaneous fat in a culture dish. The tissue was rinsed with sterile physiological saline to remove surface debris, wiped with iodine, and deiodized with 75% ethanol. The skin blocks were then washed with D-Hank' s solution containing penicillin (400 U/mL), streptomycin (400 g/mL), and tylosin (250 g/mL) until the supernatant became clear, and transferred to fresh D-Hank' s solution for sterile processing.

In the sterile room, D-Hank' s solution was aspirated, and the pre-treated skin tissue blocks were immersed in approximately 10 mL of 0.25% neutral protease for overnight digestion at 4°C. The tissue was then placed in fresh D-Hank' s solution, and the dermis and subcutaneous tissue were separated with a scalpel. After removing the epidermal layer with sterile curved forceps, secondary hair follicles were grasped at their distal ends with sterile forceps under a stereomicroscope and pulled out along the follicle direction. The isolated follicles were digested in 0.25% trypsin for 5 minutes, then evenly adhered to 6-well plates precoated with goat skin fibroblast extracellular matrix. Complete culture medium (3 mL per well) was added, and cultures were maintained at 37°C with 5% CO_2. After substantial cell migration from the follicles, the medium was replaced with an equal volume of working culture medium to initiate primary culture at 37°C with 5% CO_2. When the migrated outer root sheath cells formed a monolayer, they were digested and transferred to culture flasks, with the working culture medium changed every 2–3 days.

1.5 Subculture of Cells

When outer root sheath cells reached confluence, subculture was performed. The old medium was discarded, cells were rinsed once with D-Hank' s solution, and approximately 1 mL of 0.25% trypsin was added to moisten the cells for several seconds. After removing the liquid, 4 mL of 0.25% trypsin was added and digestion was carried out at room temperature for about 5 minutes. When the cell-attached flask wall gradually turned white and small cell clumps detached upon gentle shaking, 6 mL of complete culture medium was added to terminate digestion. Cells were pipetted to suspend them, counted, and diluted to a density of 10 cells/mL. Five milliliters of cell suspension was added to a 25 cm^2 culture flask, 10 mL of working culture medium was added per flask, and cultures were returned to the incubator. The working culture medium was changed every

3 days thereafter. When outer root sheath cells grew stably to passages 8–10, an equal volume of maintenance culture medium was used to facilitate long-term subculture. Well-growing logarithmic phase outer root sheath cells were selected for morphological observation and growth measurement.

1.6 Giemsa Staining of Goat Hair Follicle Outer Root Sheath Cells

Sterilized coverslips were placed in disposable cell culture dishes, and 2 mL of outer root sheath cell suspension was added to the dishes for culture in a CO_2 incubator. After the outer root sheath cells formed a good monolayer on the coverslips, the slides were removed and fixed in anhydrous methanol for 15 minutes, then rinsed with fresh anhydrous methanol before being stained in pure Giemsa stain for 5–10 minutes. The slides were then dried, cleared with xylene, mounted with neutral resin, and observed under a microscope.

1.7 Measurement of Cell Growth Curve

Passage 5 outer root sheath cells in logarithmic growth phase were harvested and prepared as a cell suspension using the subculture method. After counting, the cell suspension was inoculated into 24-well plates at a density of 1.02×10^4 cells/mL, with 1 mL per well. Every 24 hours, cells from three wells were collected and counted separately, and the average value was taken. The growth curve of in vitro cultured hair follicle outer root sheath cells was plotted with culture time as the x-axis and average daily cell number as the y-axis.

1.8 Chromosome Karyotype Analysis of Goat Hair Follicle Outer Root Sheath Cells

When passage 40 outer root sheath cells reached logarithmic growth phase, they were transferred to 25 mL culture flasks and cultured in a CO_2 incubator for 54 hours. Colchicine (0.5 g/mL) was added to the maintenance culture medium, and culture was continued at 37°C for approximately 8 hours until most cells were in metaphase. Cells were digested with trypsin, transferred to 15 mL centrifuge tubes, and collected by centrifugation at 1000 r/min for 10 minutes at 4°C. The supernatant was aspirated, and 0.5 mL of pre-warmed (37°C) hypotonic solution (0.075 mol/L KCl) was added dropwise with mixing, followed by additional hypotonic solution to a total volume of 10 mL. The suspension was gently pipetted, incubated at 37°C for 30 minutes, then pre-fixed by adding 1 mL of fresh Carnoy' s fixative. After centrifugation and supernatant removal, cells were fixed with 10 mL of fresh Carnoy' s fixative, with the process repeated for secondary fixation. Clean slides were pre-cooled at 4°C, placed at a 45° angle, and 2–3 drops of fixed cell suspension were rapidly dropped from a height of approximately 1 m, then air-dried at room temperature. Finally, slides were stained with Giemsa, mounted with neutral resin, examined first under low magnification to find good metaphase spreads, then observed under high magnification.

1.9 Immunocytochemical Identification of Outer Root Sheath Cells

One and a half milliliters of passage 5 outer root sheath cell suspension was evenly inoculated onto clean sterile coverslips and cultured at 37°C with 5% CO_2. When outer root sheath cells covered more than 80% of the coverslip area, the coverslips were removed, washed with PBS, and fixed with pre-cooled (-20°C) acetic acid-ethanol fixative for 20 minutes, followed by three PBS washes (3 minutes each). Immunocytochemical detection of cytokeratin 19 (CK-19) was performed according to the instructions of the ready-to-use Biotin SP-HRP immunohistochemistry kit. Finally, DAB chromogenic reaction was carried out in the dark for 3–10 minutes, washed with distilled water, observed, and photographed.

1.10 Cryopreservation and Recovery of Outer Root Sheath Cells

Passage 5 logarithmic phase cells were selected, prepared as a cell suspension using the subculture method, and centrifuged at 1000 r/min for 10 minutes. The supernatant was removed, cryopreservation medium (DMEM/F12 containing 10% DMSO and 30% FBS) was added, and cell density was adjusted to 5×10^6 cells/mL. The suspension was aliquoted into 5 mL sterile cryovials, sealed, labeled, and sequentially placed at 4°C for 1 hour, -20°C for 1 hour, -70°C for 12 hours, and finally transferred to liquid nitrogen for long-term storage.

For cell recovery, cryovials were rapidly thawed in 40°C warm water, transferred to 15 mL centrifuge tubes, and slowly diluted dropwise with 10 mL of maintenance culture medium. After gentle pipetting, cells were centrifuged at 1000 r/min for 10 minutes, the supernatant was discarded, and cells were appropriately diluted with maintenance culture medium before being inoculated into culture flasks at a density of 5×10^5 cells/mL.

1.11 Measurement of Cell Viability and Attachment Rate

Using the trypan blue dye exclusion method, 1000 cells were counted to determine the average viability before cryopreservation and the average viability and attachment rate 24 hours after recovery.

Results

2.1 Morphological Observation of Primary Cultured Outer Root Sheath Cells

Using the adherent method to isolate hair follicle outer root sheath cells, individual cells were observed migrating outward from the outer root sheath edge after 4–5 days in complete culture medium. The cells were initially round, then extended into a polygonal morphology (Figure 1 [Figure 1: see original paper]-a). After 7 days of culture, substantial cell outgrowth from the outer root sheath

edge was clearly visible (Figure 1-b). As the number of migrating cells increased, the growth rate gradually accelerated, entering logarithmic growth phase by day 15. Under inverted phase-contrast microscopy, the migrated hair follicle outer root sheath cells were primarily short spindle-shaped and polygonal, with full cell bodies, high transparency, 2–4 lamellipodia extending outward, oval nuclei, and relatively little cytoplasm (Figure 1-c). Subsequently, cells grew clonally in all directions, forming a dense monolayer where cells became short spindle-shaped and arranged in parallel (Figure 1-d).

Figure 1 Primary outer root sheath cells of grey goats after in vitro culture (100×)

2.2 Observation of Giemsa-Stained Outer Root Sheath Cells

After Giemsa staining of in vitro cultured hair follicle outer root sheath cells, the cytoplasm appeared light blue, with oval nuclei stained dark blue containing 1–2 nucleoli visible near the center of the cell body (Figure 2 [Figure 2: see original paper]-a). Observation of logarithmic phase outer root sheath cells revealed numerous mitotic figures in the field of view, and this staining method clearly displayed different phases of cell division (Figure 2-b). When cells in the culture flask formed a good monolayer (i.e., reached confluence), staining showed only a very small number of cells in the M phase, with most cells exhibiting contact inhibition (Figure 2-c). Some individual cells showed nuclear division without cytoplasmic division, forming syncytium-like structures (passage 40, Figure 2-d).

Figure 2 Giemsa staining of outer root sheath cells of grey goats after in vitro culture (200×)

2.3 Growth Characteristics of Subcultured Outer Root Sheath Cells

Subcultured outer root sheath cells showed no significant differences in morphology or growth rate compared with primary cells, and cell growth and division remained vigorous. Monolayer cells could reform a monolayer approximately 7 days after subculture, and their sensitivity to trypsin digestion was significantly enhanced compared with primary cultured cells. The growth curve of in vitro cultured outer root sheath cells (Figure 3 [Figure 3: see original paper]) showed a lag phase during the first 2 days of adherent growth, a logarithmic growth phase from days 3–5, and entry into plateau and decline phases after 5 days. The population doubling time was 51.9 h, indicating that outer root sheath cells maintained vigorous growth and division capacity after subculture.

Figure 3 Growth curve of outer root sheath cells of grey goats

2.4 Chromosome Analysis of Outer Root Sheath Cells

Chromosome specimen counting was performed on cells at passage 40. Results showed that Jining grey goat chromosomes with 2n=60 constituted the major-

ity, but some cells exhibited chromosome loss and deletion, showing typical aneuploid characteristics of cell lines, indicating that the in vitro culture environment affected genetic stability. However, among the counted cells, those with 60 chromosomes still accounted for 58.3% of the total cell population, and these cells showed normal karyotypes (Figure 4 [Figure 4: see original paper]). Therefore, the established cell line was confirmed to be derived from grey goats.

a: chromosome count of passage 40 outer root sheath cells; b: passage 40 outer root sheath cells at metaphase of cell division.

Figure 4 Chromosome analysis of passage 40 outer root sheath cells of grey goats

2.5 Immunocytochemical Identification of Outer Root Sheath Cells

Immunocytochemical staining was used to detect expression of the cytoskeletal protein cytokeratin 19 in low-passage (passage 5) outer root sheath cells cultured in vitro. Microscopic observation showed that the cytoplasm of isolated and cultured cells appeared yellow-brown, indicating positive expression of cytokeratin 19 in outer root sheath cells (Figure 5 [Figure 5: see original paper]). Since cytokeratin 19 is a marker for outer root sheath cells and skin stem cells, this confirmed that the cells isolated and cultured in this study were indeed outer root sheath cells differentiated from hair follicle stem cells.

Figure 5 Immunocytochemical staining of outer root sheath cells of grey goats (100×)

2.6 Viability and Attachment Rate of Outer Root Sheath Cells

Using a hemocytometer, the average viability 24 hours before cryopreservation and the average viability and attachment rate 24 hours after recovery were measured. Statistical analysis showed that the average viability of goat hair follicle outer root sheath cells was 94.4% before cryopreservation and 92.1% after recovery, with approximately 85% of cells attaching to the flask bottom 24 hours post-recovery. These results indicate that cryopreservation and recovery caused minimal damage to cell viability, and cells maintained good growth status (Figure 6 [Figure 6: see original paper]).

a: outer root sheath cells before cryopreservation; b: outer root sheath cells after 24 h of recovery.

Figure 6 Outer root sheath cells of grey goats before cryopreservation and after recovery (100×)

2.7 Morphology Recovery of Outer Root Sheath Cells

Passage 40 outer root sheath cells were continuously cultured in working culture medium. After 7 days, cells in working culture medium gradually transformed from a fibroblast-like morphology to an endothelial-like morphology (Figure

7 [Figure 7: see original paper]-a), while outer root sheath cells cultured in maintenance culture medium retained a fibroblast-like morphology (Figure 7-b).

a: outer root sheath cells cultured in working culture medium; b: outer root sheath cells cultured in feeding medium.

Figure 7 Morphology recovery of passage 40 outer root sheath cells of grey goats (100×)

Discussion

Animal hair follicles are skin appendages composed of multiple cell types, and in vitro culture of various hair follicle cells is fundamental to revealing hair follicle development mechanisms. Hair follicle outer root sheath cells are closely related to hair follicle stem cells in terms of histogenesis and biological characteristics. Obtaining an outer root sheath cell line that can be cultured long-term in vitro can lay the foundation for studying interactions between outer root sheath cells and dermal papilla cells, ultimately leading to successful in vitro hair induction.

3.1 Promotional Effect of Skin Fibroblast Extracellular Matrix on ORSC Adhesion

Previous methods for culturing hair follicle outer root sheath cells commonly involved inoculating cells onto feeder layers treated with radiation or mitomycin, using serum-containing medium with additives. This approach not only required numerous preparation steps but also carried a high risk of contamination during operation [14].

In this study, free hair follicles were inoculated into culture flasks pre-coated with goat skin fibroblast extracellular matrix. After outer root sheath cells migrated out, rapid growth was observed, forming a membranous structure within 10–15 days. Analysis suggests that skin fibroblasts may promote adhesion and growth of hair follicle outer root sheath cells, and in vivo mature hair follicles have outer root sheath cells intimately associated with skin fibroblasts.

The method established in this study not only yields large quantities of monolayer outer root sheath cells in a short time but is also simpler and more convenient than the feeder layer method, effectively avoiding microbial contamination and mixing of miscellaneous cells during operation. Moreover, using extracellular matrix from grey goat skin fibroblasts as a coating for culture flasks provides a more in vivo-like extracellular environment for primary cultured cells, which helps stimulate hair follicle outer root sheath cells to resume division capacity —an important condition for successful subculture.

3.2 Combination of Enzymatic Digestion and Mechanical Separation

In 1982, Wells [15] used mechanical separation to obtain free hair follicles and directly cultured them in plastic dishes. Although this method could yield outer root sheath cells, free follicles required 15–20 days before cell outmigration, with low cell yield and poor regenerative capacity, making subculture impossible. In 1994, Kurata et al. [16] treated isolated follicles with trypsin digestion to obtain outer root sheath cell suspensions for culture, but this method was not only tedious and time-consuming in preparation but also prone to cell contamination. Subsequently, domestic and international researchers investigated isolation and culture of hair follicle outer root sheath cells. The mechanical isolation and adherent culture method, though relatively easy to succeed, was time-consuming and labor-intensive with slow cell proliferation. Pure enzymatic digestion methods could also succeed but carried risks of other cell contamination and microbial contamination [17-18]. This study improved and combined both methods, offering advantages of simple operation, high cell yield, and good cell adhesion while effectively avoiding shortcomings such as slow proliferation, difficult subculture, and cell/microbial contamination.

3.3 Regulatory Effects of Growth Factors on Outer Root Sheath Cells

This study added 10 ng/mL IGF-I, 10 ng/mL EGF, and 0.1 ng/mL bFGF to the culture medium, and these growth factors were key to successfully initiating primary culture and subculture of outer root sheath cells.

Numerous studies have shown that adding appropriate concentrations of growth factors to culture medium significantly promotes adhesion and proliferation of hair follicle outer root sheath cells [19-20]. Many researchers have used IGF-I and EGF in in vitro culture of mammalian hair follicle outer root sheath cells and found they promote cell growth [21-22]. IGF-I has insulin-like metabolic effects and can stimulate proliferation and differentiation of outer root sheath cells, dermal papilla cells, and melanocytes. Immunohistochemical studies have confirmed the presence of IGF-I in animal hair follicle outer root sheath cells and other follicular cells during different stages of the hair growth cycle, indicating that IGF-I plays extensive and essential roles in differentiation and growth of hair follicle cells. EGF is an exogenous growth factor that regulates the cell cycle and stimulates proliferation of various cell types. Researchers using immunohistochemistry and autoradiography have found EGF and EGF receptors in outer root sheath regions of hair follicles both in vitro and in vivo, suggesting that EGF binding to receptors on outer root sheath cells promotes synthesis of intracellular DNA, RNA, and hydroxyproline, regulates protein synthesis, and induces changes in growth, synthesis, and secretion activities of outer root sheath cells [23]. In recent years, some scholars have conducted in-depth studies on the effects of bFGF on in vitro culture of outer root sheath cells and dermal papilla cells, finding that both cell types express bFGF and its receptors, and that bFGF addition to culture systems has strong mitogenic effects [24].

Based on previous research and comparative experiments, this study added 10 ng/mL IGF-I, 10 ng/mL EGF, and 0.1 ng/mL bFGF to the culture medium, which successfully facilitated initiation of primary culture and subculture of outer root sheath cells.

3.4 Inhibition and Removal of Non-Outer Root Sheath Cells

Inhibition and removal of non-outer root sheath cell growth is one of the key factors ensuring primary initiation and successful subculture of outer root sheath cells. Studies have found that serum components in culture medium contain various adhesion factors that promote cell differentiation and morphological changes and favor fibroblast proliferation, thereby inhibiting growth of epithelial-like cells [25-26]. Jahoda et al. [27] used serum-free medium to culture primary epidermal stem cells and outer root sheath cells, observing after 96 hours that fibroblast proliferation was significantly inhibited while proliferation time was shortened and cell numbers increased for epidermal stem cells and outer root sheath cells. In recent years, serum-free keratinocyte medium has been increasingly applied to purification culture of hair follicle stem cells and outer root sheath cells. This medium is rich in trace elements such as copper, selenium, and zinc, and essential amino acids, making it the best choice for outer root sheath cell culture and purification. In addition to medium and serum effects on outer root sheath cell purity, isolation and inoculation methods for free hair follicles can also affect the ratio of outer root sheath cells to other cells. Limat et al. [28] first pulled hair follicles directly from freshly collected skin under a microscope, then prepared free follicle suspensions for inoculation onto culture substrates. Although some free follicles could adhere and produce outgrowing cells, most isolated follicles failed to adhere or adhered loosely and could not produce migrating cells. Moreover, because skin tissue samples were not digested beforehand, the obtained follicles had severely damaged outer root sheath portions, and the isolated cells were mostly sebaceous gland cells and fibroblasts, which was not conducive to further purification of outer root sheath cells. Hoeller et al. [29] treated isolated follicles with trypsin and EDTA solution before adding them to culture medium, obtaining relatively large quantities of outer root sheath cells. However, this method also left parts of the outer root sheath in the tissue during follicle isolation and easily mixed in many fibroblasts, affecting subsequent cell purification.

Based on isolation and inoculation methods from domestic and international researchers, this study first used 0.25% neutral protease to pretreat skin tissue at 4°C to ensure integrity of the isolated free secondary follicle outer root sheath portions. Second, isolated secondary follicles were first adhered to the culture substrate, then treated with 0.25% trypsin digestion and supplemented with a small amount of high-serum-concentration (10% FBS) medium to promote adhesion. These approaches improved the adhesion rate of free follicles and the proportion of outer root sheath cells in the obtained cell population, facilitating acquisition of pure outer root sheath cells.

This study successfully initiated primary culture of outer root sheath cells using skin fibroblast extracellular matrix induction combined with mechanical separation and enzymatic digestion, and established a goat hair follicle outer root sheath cell line by adding multiple growth factors. This cell line has been stably passaged to passage 40 with good growth and division status, and a population doubling time of approximately 51.9 h. Chromosome analysis and morphology recovery tests confirmed that the established hair follicle outer root sheath cell line has distinct characteristics and can provide a good model for studying hair follicle growth and differentiation mechanisms, particularly the interaction mechanisms among major hair follicle cell types.

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