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Original Research

Isolation, Characterization, and *In-Vitro* Establishment of Mammary Epithelial Stem Cells From Beetal Goats

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Abstract

Background: The mammary gland has a remarkable ability to renew and repair itself, making mammary epithelial stem cells (MESCs) critical targets for understanding lactation biology and developing therapeutic interventions for intramammary infections. **Objective**: The objective of this study was to isolate, culture, and characterize goat mammary epithelial stem cells (GMESCs) from Beetal goats to optimize growth conditions and establish a foundation for future biomedical research. **Methods**: Mammary tissues were harvested from Beetal goats and subjected to enzymatic digestion to isolate GMESCs. To identify the optimal culture environment, the cells were screened across multiple growth and differentiation media containing varying growth factors. Optimization was performed based on proliferation index and population doubling time. Purification of isolated cells was performed using the EasySep™ PE positive selection kit targeting the CD24 luminal marker. Characterization of stem cells, milk secretion, and proliferation markers was performed using phase-contrast microscopy, colony-forming unit (CFU) assays, qualitative real-time-polymerase chain reaction (qRT-PCR), and immune cytochemistry. **Results**: The optimal basal growth medium was Dulbecco modified eagle medium (DMEM)/F12 fortified with 10% fetal bovine serum (FBS), three essential growth factors (epidermal growth factor [EGF], basic fibroblast growth factor [bFGF], and insulin), and 1% antibiotics. Under these conditions, the GMESCs exhibited a characteristic cobblestone morphology and successfully differentiated into functional units, including domes, alveoli-like structures, and interconnecting tubules. Although CFU assays revealed relatively low colony-forming efficiency in these epithelial cells, the identity of GMESCs was validated by the presence of specific markers, including endogenous stem cell markers and milk-secreting proteins. Purified CD24+ luminal cells maintained stable morphological characteristics after 10 days of culture. **Conclusion**: This study successfully established a robust protocol for the isolation and expansion of GMESCs from Beetal goats. By defining the specific markers and media requirements of these cells, this study provides a valuable model for mammary tissue repair and broader applications in veterinary regenerative medicine and sustainable production.

Keywords: mammary epithelial cells; goat diseases; growth curve; immunocytochemistry; mammary luminal cells

1. Introduction

Mammary glands play an important role in the reproduction of mammals, and the milk of domesticated ruminants is an important source of nutrients for humans. Mammary morphogenesis is predominantly a postnatal process [1] and coordinated phases of mammary growth and regression facilitate offspring nourishment and are frequently observed in domestic species to regulate milk yield and reproductive efficiency. The proliferative potential of mammary epithelial cells is highlighted by their ability to undergo repeated cycles of expansion and functional restoration [2]. The long-term viability and expansion of mammary tissue rely on the regenerative contribution of both stem and progenitor cell lineages. The standard definition of "stem cells" centers on their proliferative self-maintenance and their potential to give rise to differentiated progeny [3]. Mammary gland stem cells constitute a dormant, self-maintaining reservoir capable of generating distinct lineages of luminal ductal, secretory acinar, and contractile myoepithelial cells [4]. Mammary or glandular parenchyma is the primary functional tissue of the udder and is dominated by mammary epithelial cells (MECs). MECs are the sites of milk synthesis; therefore, they remain a central subject in bovine lactation research. These cells form a single-layered secretory lining within alveoli, microscopic spheres ranging from 50 µm to 250 µm in diameter, depending on milk volume. In a typical bovine gland, the alveolar epithelium contains approximately 5 trillion of

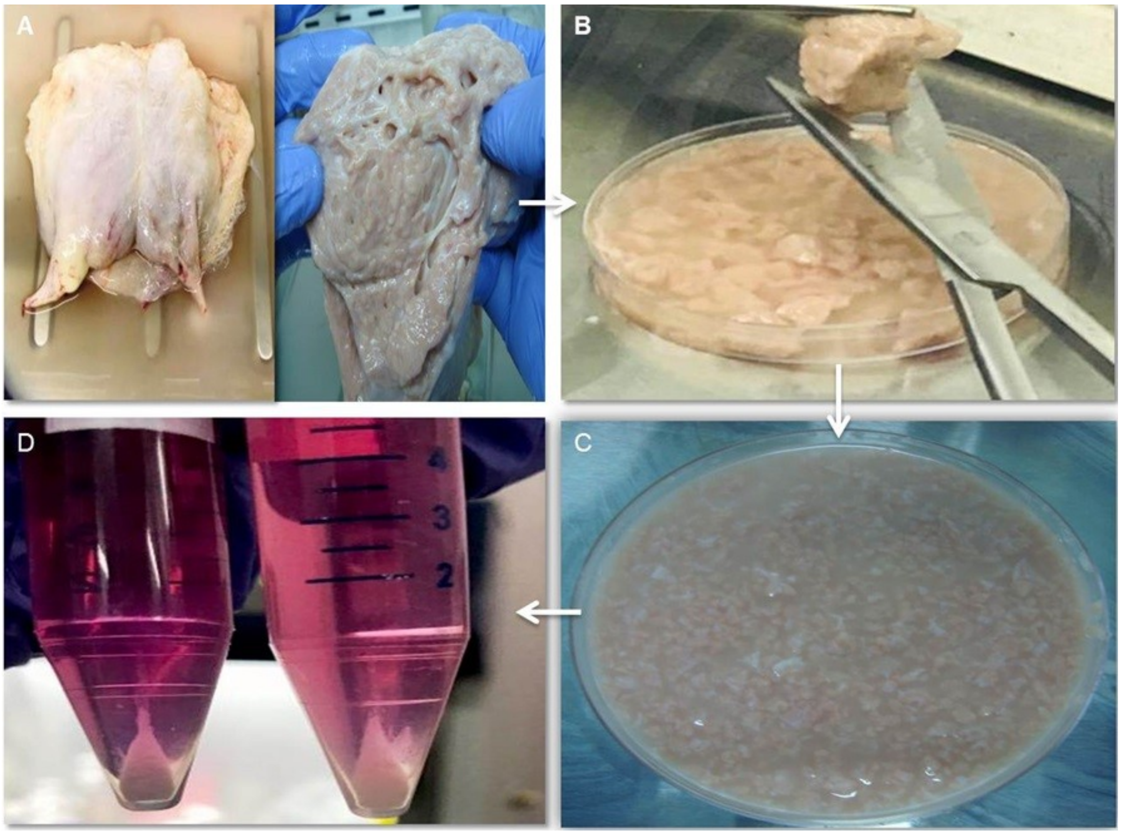

Fig. 1. Processing of goat mammary tissue samples. (A) Goat mammary gland. (B) Goat mammary tissue chopped with sterilized scissors. (C) Minced goat mammary parenchyma. (D) Cell pellet after centrifugation.

these secretory units. The central organization follows a bilayered ductal system in which the inner luminal (secretory) cells are enveloped by a basal layer of myoepithelial cells. These muscle-like cells contract in response to mechanical stimuli or to oxytocin, which drives the milk-ejection reflex. Beyond production, MECs are critical mediators of the innate immune response, particularly during inflammatory events such as goat mastitis [5].

Present study targeted the Beetal goats, because this is one of India's most significant long-eared, large-sized breeds, primarily found in the North India. Popular for its dual-purpose utility (milk and meat), superior lactation performance compared to other Indian breeds it serves as a critical genetic resource for improving local goat populations. Unlike many indigenous breeds, the Beetal exhibits superior dairy potential, with an average lactation yield ranging from 150 to 190 kg over a period of 170 to 190 days. Biologically, Beetal goats possess high thermotolerance and resilience to varied agro-climatic conditions, which justify its selection for this study. Their prolificacy is also a key trait, with a high frequency of twinning and triplets, contributing to their high economic value in sustainable livestock systems. Therefore, present study was planned to focus on the isolation and characterization of Beetal goat mammary epithelial stem cells for further biomedical application and genetic improvement.

2. Materials and Methods

2.1 Collection and Processing of Goat Mammary Tissue Samples

Goat mammary tissue (Fig. 1A) was collected from a government slaughter house in Jammu, Jammu and Kashmir, India. Following all ethical and animal welfare requirements. Collected mammary tissue was transferred to a chilled transportation medium comprising 1×phosphate buffer saline (PBS; Cat. TL1032, Himedia, Mumbai, India) fortified with 5% Fetal Bovine Serum (FBS; Cat.RM1112, Himedia, Mumbai, India), penicillin (5000 unit/mL) and streptomycin (5 mg/mL; Cat. A004, Himedia, Mumbai, India), amphotericin B (2.5 µg/mL; Cat.A011, Himedia, Mumbai, India), and gentamicin solution (100 µg/mL; Cat. A010, Himedia, Mumbai, India) and transported to the laboratory in the ice box. Mammary tissue samples were further processed in the laboratory within 1–2 h after goat slaughter and mammary tissue collection.

2.2 Mammary Gland Digestion Into Organoids

Goat mammary tissue organoids were prepared according to a previously described protocol [6]. Small pieces of the mammary parenchyma were prepared using sterilized scissors and blades (Fig. 1B). Furthermore, the mammary parenchyma was minced until paste formation (Fig. 1C). It underwent multiple rounds of purification with washing buffer (WB) containing PBS supplemented with 5% FBS, 1000 unit/mL penicillin, streptomycin (50 mg/mL), gentamicin (100 µg/mL), and amphotericin B (2.5 µg/mL) to remove milk and blood. Minced mammary tissue was digested using digestion medium (DM) containing Dulbecco's Modified Eagle's Medium (DMEM)-F12 (Invitrogen, USA) fortified with 5% FBS (Cat. AL066A, Himedia, Mumbai, India), penicillin (5000 unit/mL) and streptomycin (5 mg/mL; Cat. Cat. A004, Himedia, India), amphotericin B (2.5 µg/mL; Cat. A011, Himedia Laboratories, India), gentamicin solution (100 µg/mL; Cat. A010, Himedia, Mumbai, India), insulin (5 µg/mL; Cat. RM8507, Himedia, Mumbai, India), collagenase type II (300 U/mL; Cat. TC212, Himedia, Mumbai, India), hydrocortisone (1 µg/mL; Cat. TC344, Himedia, Mumbai, India), and hyaluronidase (0.5 mg/mL; Cat. TC331, Himedia, Mumbai, India). The DM was filtered and freshly prepared for use. Approximately 4–5 g of finely fragmented tissue was transferred into 50 mL sterile conical tubes and submerged in 30 mL of specialized enzymatic DM. The tubes were then gently shaken and incubated at 37 °C for 1–2 h. The resulting digest was filtered through sterilized muslin cloth and centrifuged at 800 rpm for 5 min at 4 °C (5430R, Eppendorf, India). Following removal of the supernatant, the pellet was resuspended in 35 mL of wash buffer and centrifuged again at 1200 rpm for 5 min. This washing cycle was repeated 4–5 times to ensure the isolation of high-purity organoids (Fig. 1D).

2.3 Organoid Processing to a Single Cell

Mammary organoids are rich reservoirs of epithelial stem cells and progenitor cells. To obtain a monodisperse cell suspension, the isolated organoids were first washed in WB and subjected to brief proteolysis with 0.5% trypsin-EDTA (Cat. TCL007, Himedia, Mumbai, India). Chemical dissociation was augmented by mechanical trituration via vigorous pipetting. To ensure complete release, the cells were further incubated in an enzymatic cocktail containing dispase (5 mg/mL) (Cat. 17105041, Gibco, USA) and DNase I (125 µg/mL) (Cat. 18068015, Invitrogen, USA) at 37 °C. The resulting isolate was purified by repetitive centrifugal washing (1200 rpm, 5 min) and filtered through a 40 µm mesh (BD Falcon) to eliminate any remaining aggregates or debris.

2.4 Isolation of Goat Mammary Epithelial Cells (GMECs) and Systematic Calibration of Cultivation

Following enzymatic liberation via collagenase digestion, the resulting monodisperse GMECs were strategically seeded into culture flasks containing basal growth medium composed of DMEM-F12 medium (Cat. 11320033, Thermo Fisher, USA) fortified with 10% FBS (Cat. AL066A, Himedia, Mumbai, India), penicillin (5000 unit/mL) and streptomycin (5 mg/mL; Cat. A004, Himedia, Mumbai, India), amphotericin-B (2.5 µg/mL; Cat. A011, Himedia, Mumbai, India), gentamicin solution (100 µg/mL; Cat. A010, Himedia, India), insulin (5 µg/mL; Cat. RM8507, Himedia, Mumbai, India), and epidermal growth factor (10 ng/mL; Cat. CF023, Himedia, Mumbai, India) to initiate primary expansion. The cultured flasks were incubated at 37 °C in a CO_2 incubator (Galaxy 170S; Eppendorf, India) with 5% CO_2 and 95% humidified air.

2.5 Removal of Fibroblasts and Enrichment of GMECs

During cell passaging, the fibroblasts were removed by selective trypsinization (2–3 passes). Cellular detachment was achieved by treating the monolayer with a 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution for 1–2 min after cleaning with PBS, and then incubating at 37 °C in a CO_2 incubator (Galaxy 170S; Eppendorf, India) with 5% CO_2 and 95% humidified air. The resulting change in fibroblast shape caused their detachment from the culture flask. DMEM with 10% FBS was added to neutralize trypsin, followed by removal of the detached fibroblasts. Subsequently, the cells were washed with PBS-containing antibiotics. This procedure was performed at least 3–4 times until the cells were devoid of fibroblasts, leaving only primary MECs in the culture.

2.6 Culture of Purified GMECs

Purified GMECs were cultured in a basal growth medium supplemented with growth factors and propagated for further use. Cultured cells were sub-passaged at 80–90% confluence following removal of the medium, rinsed with PBS, and trypsinized (0.25% trypsin-EDTA, Cat.TCL007, Himedia, Mumbai, India).

2.7 Proliferative and Morphological Profiling of GMECs on a Plastic Substrate

To assess the capacity for development and differentiation of GMECs on a plastic substrate, cells were grown in the culture flasks containing DMEM media supplemented with 10% FBS (Cat. AL066A, Himedia, Mumbai, India), insulin (5 µg/mL; Cat. RM8507, Himedia, Mumbai, India), epidermal growth factor (10 ng/mL; Cat. CF023, Himedia, Mumbai, India), gentamicin (100 µg/mL; Cat. A010, Himedia, Mumbai, India), amphotericin (2.5 µg/mL; Cat. A011, Himedia, Mumbai, India), penicillin (5000 unit/mL) and streptomycin (5 mg/mL; Cat. A004, Himedia, Mumbai, India). Cultivation was conducted under standardized atmospheric conditions (37 °C, 5% CO_2, and 95% humidity), with tri-weekly media exchange to ensure nutrient availability. Proliferative and morphotypic shifts were systematically tracked using an inverted phase-contrast microscope (Magnus, Olympus, India). Notably, GMECs exhibited advanced spatial organization, characterized by the emergence of protruding dome structures and the formation of interconnected tubular networks.

2.8 Growth Curve (Proliferation Index and Doubling Time)

Measuring proliferation rates is essential for characterizing stem cells, as it offers a direct window into their self-renewal efficiency and overall fitness *in vitro*. For proliferation and doubling time of cells, GMECs from P9_GMECs (passage nine) were seeded at a cell density of 1×10^5 (12,600 cells/cm^2) for 6 days in 6-well tissue culture plates. Two distinct media were used to cultivate the cells, viz., media I (media + growth factor) and media II (media – without growth factors). Media I consisted of basal growth media containing EGF (Cat. CF023, Himedia, Mumbai, India), bFGF (Cat. 13256029, Invitrogen, USA), and insulin (Himedia, India), whereas media II served as the growth factor-depleted control, utilizing the standard basal medium but explicitly excluding EGF, bFGF, and insulin. Three wells of each medium were cultured with GMECs and maintained at 37 °C with 95% humidified air and 5% CO_2. Cells from three wells of each medium were trypsinized with 0.25% trypsin-EDTA solution (1×; Himedia, Mumbai, India) and counted at 48 h (2 days), 96 h (4 days), and 144 h (6 days) using an automated cell counter (Model: T20, Bio-Rad Lab, India). All experiments were performed in triplicate.

Growth parameters, specifically the proliferation index and population doubling time, were calculated following the standardized frameworks of Eqns. 1,2:

$$\mathrm{PI} = \alpha/\delta \tag{1}$$

where δ is the initial seeding density on day 0, and α is the terminal cell count recorded at the conclusion of the analysis period.

The following formulas were used to quantify the minimum population doubling time and fold expansion of the total number of nucleated cells [7]:

$$\text{Population doubling time} = \sigma/\mu \tag{2}$$

where σ represents the temporal duration of growth and μ is the cumulative number of cellular divisions.

Here, the number of cellular divisions μ = log N1-log N0/log2, whereas N0 is the number of cells seeded and N1 is the number of cells at passage.

2.9 Clonal Colony-Forming Unit (CFU) Assay

The *in vitro* growth characteristics and colony-forming ability of GMECs were also assessed using CFU assays on soft agar, as reported by Sun *et al*. [8] and Anand *et al*. [9], with slight modifications. In brief, 1.2% agar (Cat. 214010, Bacto-Agar; Difco Becton, USA) was prepared in basal growth medium at 40 °C, and 1.5 mL growth medium-agar was added to 35 mm culture dishes and left to solidify. A GMEC suspension at a density of 2500 cells/well in a 6-well culture plate in growth medium with 0.4% agar (at 40 °C) was poured on base agar and incubated at 37 °C, and 5% CO_2, and 95% humidified air for 20 days.

Liquid medium was added after 3 days of inoculation, and fresh growth medium was changed every 3 days. GMESC colony-forming ability, growth characteristics, and daily inverted phase-contrast microscopy (Olympus IX70, Japan) were used to evaluate cells' morphology.

To observe colony formation and count, cultures were fixed with ethanol and acetone (1:1, v/v) for 10 min at 4 °C and stained with Wright's Giemsa stain. The colony was defined as a cluster of >50 cells [10], counted using an inverted phase-contrast microscope at 100× objective lens magnification.

Colonies were counted and the cloning efficiency (CE) was determined using the following formula:

$$\mathrm{CE}\ (\%) = n/\mu \times 100$$

Where *n* is the number of colonies and *μ* is the number of cells seeded.

2.10 Isolation and Purification of Goat Mammary Epithelial Luminal Cells

EasySep™ Release PE Positive CD24 Selection Kit (Cat. 17614A, Stem cell Technologies, Canada) was used to isolate highly purified luminal cells. A GMEC sample was prepared at the indicated cell density of 1.5×10^7 cells/mL, and then the FcR blocker was added. The cells were then incubated with PE-conjugated CD24 antibodies. After the selection cocktail was added, the sample was allowed to sit at room temperature for 3 min. Rapid spheres were then added, and the solution was placed in a magnet. The supernatant was discarded by inverting the magnet. Desired cells, targeted with antibodies and magnetic particles, were separated without the use of columns using an EasySep™ magnet. Unwanted cells were removed, whereas the desired cells remained in the tube. Isolated pure CD24-positive luminal cells were cultured in two wells of a 6-well culture plate consisting of basal media composed of DMEM/F12 supplemented with EGF (Cat. CF023, Himedia, Mumbai, India), bFGF (Cat. 13256029, Invitrogen, USA), and insulin (Cat. RM8507, Himedia, Mumbai, India) and maintained at 37 °C with 95% humidified air and 5% CO_2.

2.11 Ultrastructural Characterization

GMECs were characterized by transmission electron microscopy (TEM). Post-trypsinization, the cell pellet was fixed for 1 h at 4 °C in a 0.1 M phosphate buffer (pH 7.2) containing 2.5% glutaraldehyde and 1% paraformaldehyde. Secondary fixation was performed using 1% osmium tetroxide (2 h at 4 °C), followed by dehydration through an ascending acetone gradient and embedding in Epoxy Medium (Cat. 45359, Sigma, India). Ultrathin sections were cut using a diamond knife and mounted on a 300-mesh copper grid (EMS). For the final comparison, carbon-coated grids were treated with 2% uranyl acetate and dried. All imaging was performed at 80 kV using a JEOL JEM 1400 Plus Transmission Electron microscope.

2.12 Total RNA Extraction From GMESCs and cDNA Synthesis

The protocol for total RNA extraction from GMECs was optimized using TRIzol reagent (Cat. 15596026, Thermo Fisher, USA). Briefly, 1 mL of TRIzol was added to 30–40 mg of cells. The sample was incubated for 5 min at room temperature to permit complete dissociation of the nucleoprotein complexes. The solution was pipetted for 4–5 min and centrifuged to remove the cell debris. The supernatant was transferred to a new tube. Chloroform (Cat. MB109, Himedia Laboratories, India) was added at 0.2 mL per mL of TRIzol reagent. The samples were mixed, vortexed for 15 s, and incubated at room temperature for 2–3 min. Further, it was centrifuged at 12000 rpm for 10 min at 4 °C, and the upper aqueous phase was transferred into a fresh tube. A volume of 0.5 mL of isopropanol (Cat. MB063, Himedia Laboratories, India) was added per mL of TRIzol reagent. The aqueous sample was gently mixed with isopropanol by inverting the tube to precipitate the RNA. The tubes containing RNA were incubated on ice for 15 min and centrifuged at 12000 rpm for 10 min at 4 °C. The supernatants were discarded. The RNA pellet was washed with at least 1 mL of 70% ethanol. The samples were mixed by vortexing and centrifuged at 7500 rpm for 5 min. This washing step was repeated. RNA pellet was dissolved in DEPC water (20 µL) after being air dried for 5–10 min. The concentration and quality of the purified RNA (OD 260/280 ratio) was evaluated using a BioSpectrophotometer (Model. Biospectrometer Kinetic, Eppendorf, India). RNA samples were subjected to electrophoresis to check RNA quality.

RNA samples were subjected to cDNA synthesis using the Improm-II transcription system (Cat. A3800, Promega, Sverige). Autoclaved and diethylpyrocarbonate (DEPC)-treated PCR tubes were placed on ice and the mixture of RNA sample, oligo (dT), and nuclease-free water was incubated at 70 °C for 5 min in a thermal cycler (Model. T100, Bio-Rad, India). After that, the RNA sample mix was incubated in a thermal cycler for 5 min at 25 °C, 1 h at 42 °C, and 25 min at 70 °C for the final termination (T100, Bio-Rad).

2.13 Expression of Markers of Stemness, Endogenous, Cell Proliferation, and Milk Synthesis-Related Genes

GMESs were subjected to total RNA extraction after 7 days of culture using the TRIzol method, in accordance with the recommended protocol for the systematic evaluation of gene expression (**Supplementary Table 1**). Characterization was performed using surface marker identification. The isolated RNA was checked on a 2% agarose gel, and cDNA was synthesized using a cDNA synthesis kit (Cat. MBT076, Himedia, Mumbai, India), as mentioned above.

The genes were optimized in a thermal cycler under varying cycling conditions and annealing temperatures, as shown in **Supplementary Table 2**.

2.14 mRNA Expression of Stemness Genes

Aldehyde dehydrogenase 1 (ALDH1), hepatocyte nuclear factor 4A (HNF4A), fibronectin type III domain-containing 3B (FNDC3B), and notch 1 (NOTCH1) are stem cell markers whose gene expression was used to assess the stemness features of GMECs.

2.15 mRNA Expression of Cell Proliferation Markers

Cell proliferation of GMECs was evaluated by measuring the expression of the genes Ki-67 (MKI67) and tumor protein translationally-controlled 1 (TPT1).

2.16 mRNA Expression of Endogenous Reference Genes

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as endogenous control genes [11]. A gradient PCR was set up for these genes, and specific bands of the desired length were observed.

2.17 mRNA Expression of Milk Synthesis-Related Genes

Alpha S2-casein (CSN1S2), fatty acid binding protein 3 (FABP3), and CD36 molecule (CD36) markers were the genes linked to milk production, and their expression was assessed.

2.18 Immunocytochemistry of GMESCs

Primary GMECs were seeded on sterile cover slips at a cell density of 1×10^5 cells/well in 6-well plates. After overnight incubation, the cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. The cells were then rinsed twice with PBS. The cells were permeabilized using 0.5% triton X-100 in PBS at room temperature for 10 min. Blocking was performed using 5% goat serum in TBST for 1 h at room temperature. Antibody incubation was performed using the goat-specific primary goat anti-CD24, anti-ALDH1, anti-HNF4A, and anti-ITGA6 antibodies at 10 µg/mL in 5% goat serum and was kept overnight at 4 °C. The cover slips were gently washed three times with PBS for 5 min each. Alexa Flour 594-labelled donkey affinity anti-rabbit IgG (H+L) secondary antibodies, in 5% goat serum, at a concentration of 2 µg/mL, were used for 1 h in the dark at room temperature. After three consecutive PBS washes, the coverslips were incubated with 4′,6-diamidino-2-phenylindole (DAPI) for 10min at 37 °C. Confocal microscopy was used to view the tagged cells after three additional washes in PBS to eliminate any remaining stain.

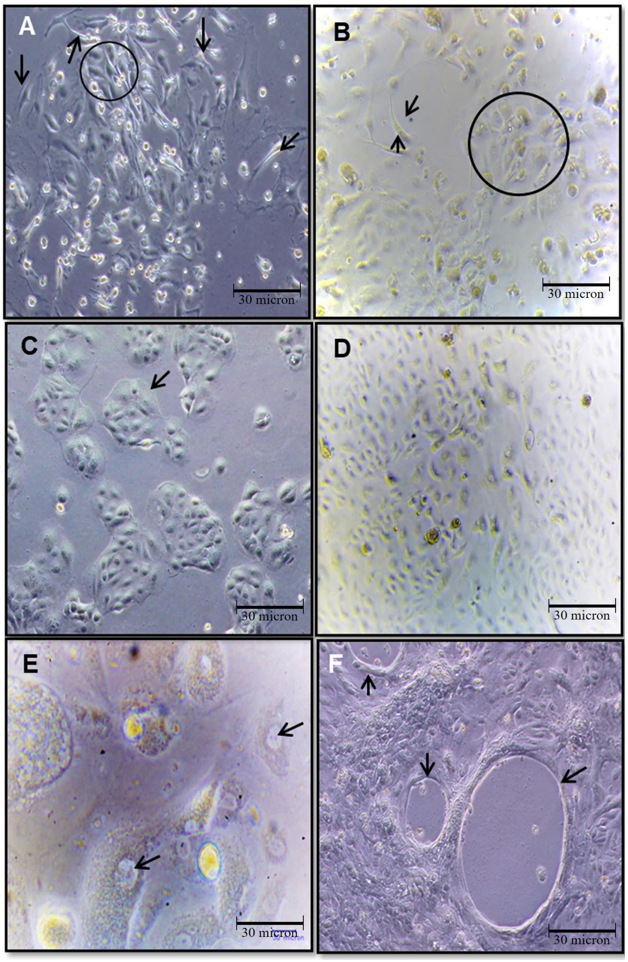

Fig. 2. GMEC isolation and morphological traits in the basal growth medium. (A) GMECs (circle) and mesenchymal cells/fibroblasts (arrow) in symbiosis following a 24-h culture period (100×). (B) Fibroblasts' declining tendency following a single passage (Arrow: fibroblast cells; Circle: pure mammary epithelial cells, 100×). (C) Population of purified, closely spaced GMECs. Lamellipodia (arrow) were often seen (100×), and subconfluent GMECs formed as clusters (islands). (D) GMECs with a typical cobblestone shape developed as a monolayer (100×). (E) GMECs with a conspicuous nucleus and nucleoli (arrow, 400×). (F) After 5 to 6 days of third-passaged cell culture, confluent culture created a lumen-like structure with elongated cells in the margins (arrow) (100×). GMEC, Goat Mammary Epithelial Cell (Scale bar = 30 micron).

2.19 Senescence-Associated *β*-Galactosidase (SA-*β*-Gal) Activity of Luminal Cells

The Senescence Cells Histochemical Staining Kit (Sigma, USA) was used to assess the senescent activity of luminal cells. Isolated luminal cells of GMECs at passages 2 and 20 were washed thrice with 1× PBS. The cells were then carefully washed to prevent detachment. Fixation buffer (1×) was added, and the cells were incubated with the solution at room temperature for 6–7 min. During the fixation process, a staining solution was prepared following the manufacturer's instructions. After rinsing the cells again with 1× PBS, the staining solution was added, and the plate was incubated at 37 °C for 12 h. The staining was pH-dependent. Therefore, the cells were incubated in the absence of CO_2. These cells were analyzed the next day under a phase-contrast microscope, and the senescent activity was calculated.

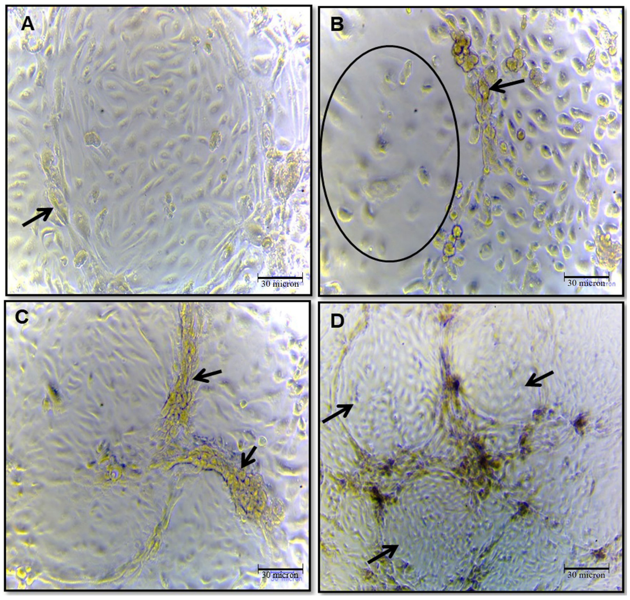

Fig. 3. Differential characteristics of 4th-passaged GMECs on a plastic substratum. (A) Phase contrast photomicrograph of the dome structure's early growth (arrow, ×100) on the 7th day of culture. (B) Image of dome structure with emphasis on the monolayer of GMECs (circle) and spatial arrangement of cells around the dome structures as elongated myoepithelial like cells (arrow, 100×). (C) Spatial localization and arrangement of the cells became thicker and more prominent and started to form interconnecting structures (arrow) at the 9th day (100×). (D) Development of alveoli-like lumen with interconnecting network at the 15th day of culture (arrow, 100×) (Scale bar = 30 micron).

2.20 Statistical Analysis

All experiments were performed with three independent biological replicates. Data are presented as the mean ± standard deviation (SD). Quantitative real-time PCR data were analyzed using the ΔCq method, where ΔCq = Cq (gene) – Cq (GAPDH). For comparisons between undifferentiated and differentiated GMECs, an unpaired two-tailed Student's *t*-test was performed on the ΔCq values. Fold-change values ($2^{-\Delta\Delta Ct}$) were calculated, and statistical significance was assessed using a one-sample *t*-test comparing the fold-change to a theoretical mean of 1. All statistical analyses were conducted using GraphPad Prism (version 9.0, GraphPad Software, Inc., La Jolla, CA, USA). A *p*-value < 0.05 was considered statistically significant. Sig-

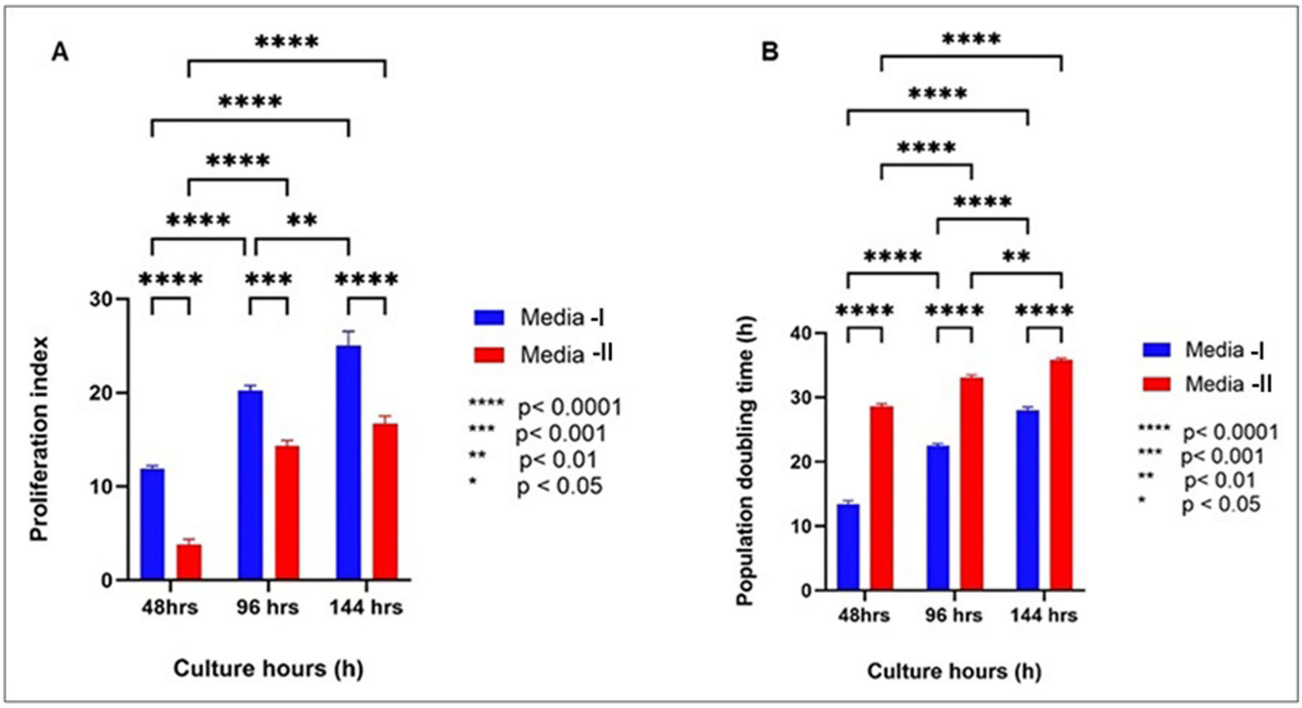

Fig. 4. Growth rate and proliferation index of 9th day-passaged GMECs propagated in two different media for 6 days. (A) GMEC growth rate in media-I (basal growth media with EGF, bFGF, and bovine insulin) and media-II (basal growth medium without growth factors). (B) Population doubling time is trending upward from media-I to media-II. EGF, epidermal growth factor; bFGF, basic fibroblast growth factor.

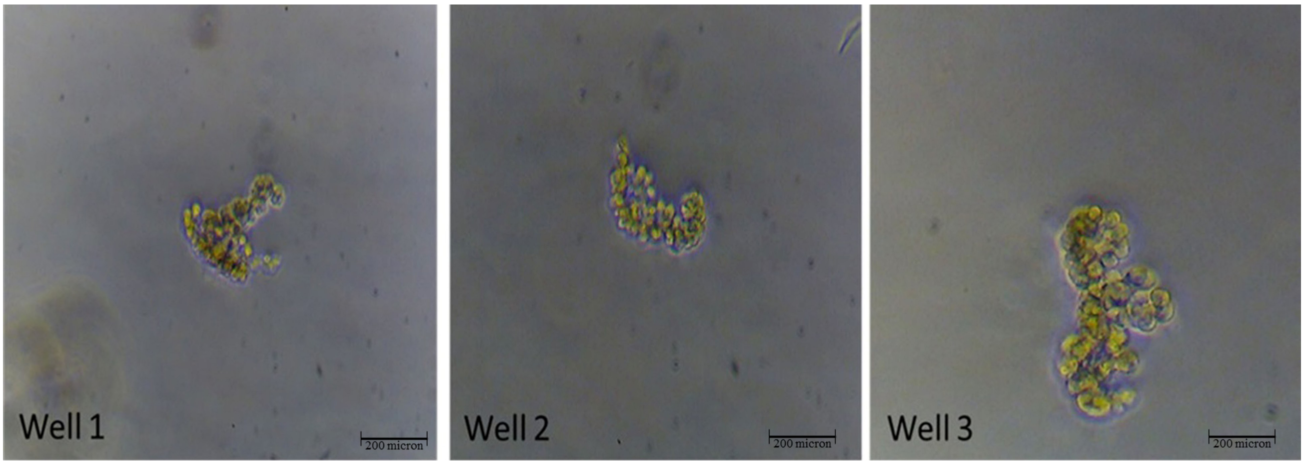

Fig. 5. Colony formation after staining colonies with Giemsa. At 100× magnification under a phase-contrast microscope, very small colonies are observed in wells 1, 2, and 3, analyzed after 20 days. (Scale bar = 200 micron).

nificance levels are denoted as: *$p < 0.05$; **$p < 0.01$ and Non-significant comparisons are labelled as "ns".

3. Results

3.1 Isolation and Purification of Primary GMECs

The protocols for the digestion of mammary parenchyma were optimized using various methods, such as digestion media and 0.5% trypsin-EDTA. Different incubation times were used for digestion, such as 30 min, 1 h, 1.5 h, 2 h, 2.5 h, and 3 h, with gentle shaking of the mammary tissue with the DM. The best digestion results were found after 1–2 h of incubation. Although no digestion was observed at 30 min, it was over digested at 3 h. No satisfactory results were obtained for digestion with 0.5% trypsin-EDTA. Following digestion of the mammary parenchyma, the organoids were successfully processed for single-cell isolation.

After digestion of organoids, single GMECs were cultured in basal growth medium supplemented with growth factors and incubated at 37 °C with 5% CO_2 and 95%

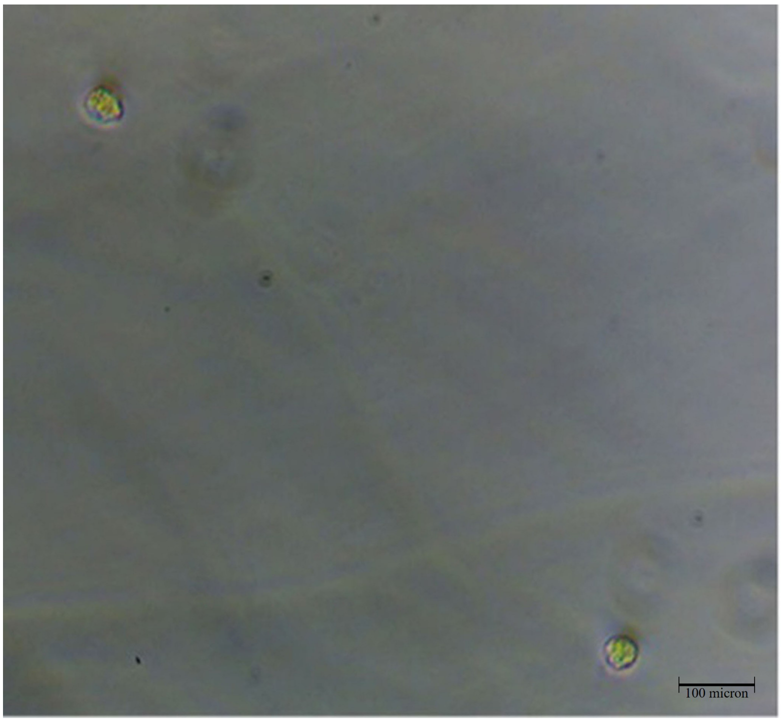

Fig. 6. Isolated CD24 targeted cells (luminal cells) using the EasySep™ kit form a mixture of cell suspension. Morphological characteristics of the isolated CD24-coated cells in 6-well plates under phase contrast microscopy at ×100 magnification (Scale bar = 100 micron).

humidity. After 24 h of incubation, the cultured primary GMECs comprised a morphologically heterogeneous population characterized by an architectural mix of elongated, spindle-shaped mesenchymal cells and cuboidal epithelial clusters in symbiosis (Fig. 2A). The purified GMECs were successfully recovered by selective trypsinization with 0.25% trypsin-EDTA solution and subsequent subculture. At the 2nd passage, the number of fibroblasts decreased (Fig. 2B). It was observed that less than 2–3 min required for fibroblast detachment. Fibroblasts exhibit tenuous intercellular and substrate-bound interactions, leading to accelerated enzymatic dissociation compared with more cohesive epithelial clusters [12]. This disparity in adhesion kinetics can be exploited to enrich the epithelial population through stratified enzymatic dissociation, thereby effectively partitioning the more labile stromal elements.

After the 3rd round of fibroblast removal, purified homogenous colonies of GMECs were obtained; in the subconfluent stage, cells grew as adherent clusters (islands), and distinctive lamellipodial extensions (arrow) were observed (Fig. 2C). Once >80–90% confluence was reached, the cells showed the typical characteristics of the cobblestone morphology of mammary epithelial cells (Fig. 2D). A pure cell line of GMECs exhibited the prototypical ‘cobblestone’ morphology consistent with previous studies [13–15]. GMECs showed a clear nucleus and 2–3 nucleoli in each cell under an inverted microscope at 400× (Fig. 2E). Following a 10- to 12-day cultivation period in basal medium, GMECs underwent spontaneous morphogenesis, resulting in the formation of pseudo luminal cavities demarcated by a peripheral layer of attenuated, elongated cells (Fig. 2F). Similar findings were reported in previous studies [16].

Every third day, the medium was replaced, and the GMECs were cultured as monolayers. The cells were cryopreserved in a liquid nitrogen container using DMEM supplemented with 20% FBS and 10% dimethyl sulfoxide (DMSO) (Cat. TC185, Himedia, Mumbai, India).

3.2 Growth Characteristics of Differentiating GMECs on a Plastic Substratum

To assess the GMECs’ capacity for growth and differentiation on a plastic substratum, 4th passaged cells were grown on the flasks containing DMEM media supplemented with 10% FBS, 5 µg/mL insulin, 10 ng/mL EGF, 100 µg/mL gentamicin, 2.5 µg/mL amphotericin, 100 units/mL penicillin, and 100 µg/mL streptomycin. When GMECs were cultured on a plastic substrate without a matrix, the cells initially localized spatially, forming typical mammary gland-like structures after 6–7 days of culture.

After 7–8 days of culture of GMECs in 5% CO_2 and 95% humidity, the cells showed structural changes from a monolayer towards differentiated dome-like structures (Fig. 3A) on day 7th of culture and started to arrange in a spatial manner, with elongated myoepithelial-like cells around the dome structure at the margins (Fig. 3B). Similar findings have been reported in previous studies [15].

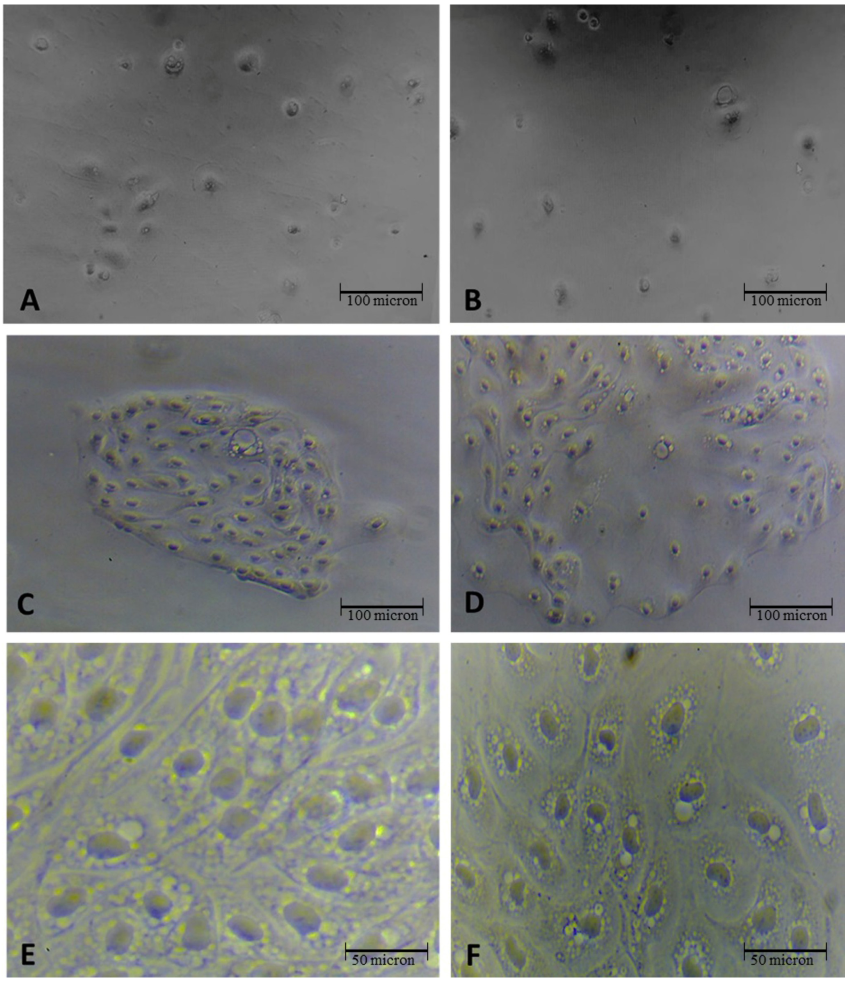

Fig. 7. Morphological differentiation of luminal GMESCs on a plastic substratum. (A,B) Phase contrast microphotograph showing GMECs (circular morphology) (100×). (Scale bar = 100 micron) (C,D) Phase contrast microphotograph showing GMEC islands after 10 days of culture on a plastic substratum (×100). (Scale bar = 100 micron) (E,F) Phase contrast microphotograph showing GMECs after 10 days of culture on a plastic substratum (400×) having a prominent nucleus. GMESCs, goat mammary epithelial stem cells; (Scale bar = 50 micron).

On the 9th day of growth, the spatial localization and organization of the cells became thicker, more noticeable, and started to form interconnected structures (Fig. 3C). At the 15th day, formation of a more prominent alveoli-like lumen with an interconnected network of tubes was observed (Fig. 3D). To connect with every dome construction, these connecting tubes created a branching network. This was the primary culture up to the 15th day of growth, and cells were maintained for further development of stronger structures. Accordingly, this study indicated that a plastic substrate was sufficient for GMEC differentiation, but it took a little longer for all forms to emerge *in vitro*.

3.3 Growth Curve (Proliferation Index and Doubling Time)

The objective of this study was to determine the doubling time and proliferation rate of GMESCs and to identify the most effective medium for *in vitro* cultivation. Furthermore, we evaluated the influence of growth factors, including EGF, bFGF, insulin, and FBS, on cellular expansion. The findings indicated that the specific medium composition and added supplements significantly affected growth patterns and final biomass density at confluence. When GMESCs were seeded at a density of 12,600 cells/cm^2 in 6-well plates, they exhibited a markedly higher proliferation index in the basal growth medium (medium-I), which included growth factor supplementation, compared to the growth factor-deficient medium-II (Fig. 4A).

In addition to the negative correlation, the pattern of doubling times was examined. The doubling time in medium-I was much shorter than that in medium-II (Fig. 4B).

As the duration of culture increased, the doubling time also increased. These results showed that growth factors, including EGF, bFGF, insulin, and FBS, were important for the development and multiplication of GMESCs.

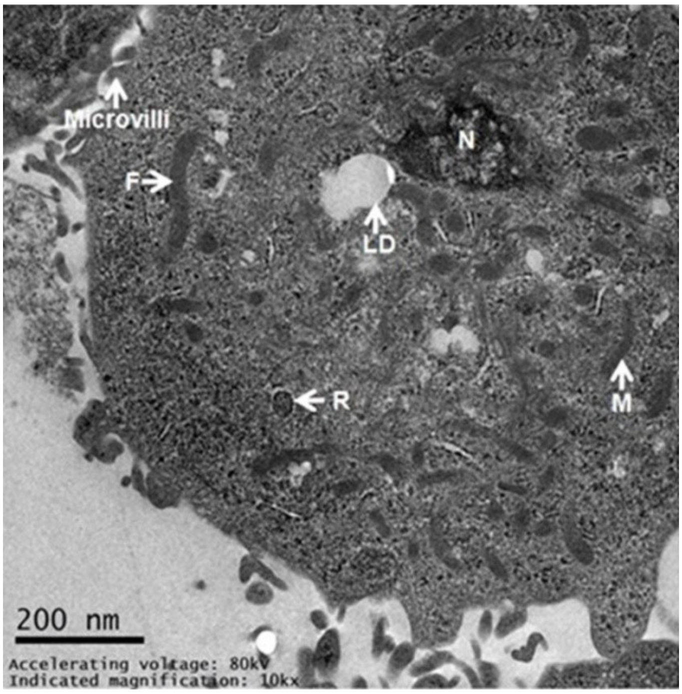

Fig. 8. Transmission electron micrographs of goat mammary gland-derived differentiated mammary epithelial cells (GMECs) grown *in vitro*. The casein micelle (shown by an arrow) is present in the GMECs' distinctive secretory vesicles (SV). Nucleus (N), lipid droplets (LD), mitochondria (M), filaments (F), ribosomes (R), and microvilli are all found in the cytoplasm. (Scale bar = 200 nm).

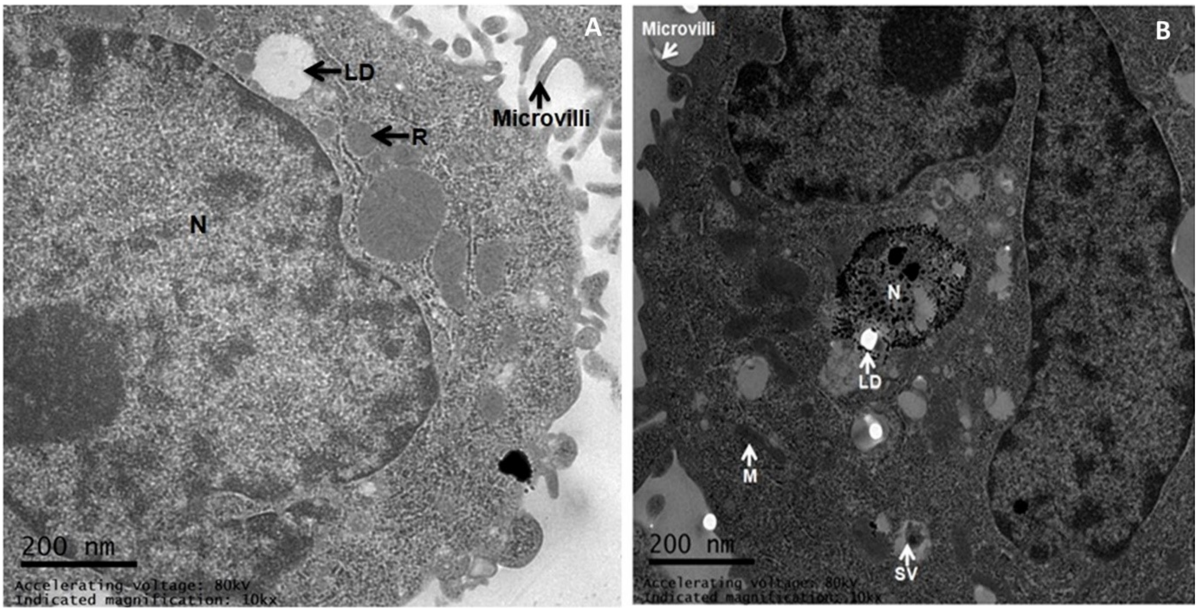

Fig. 9. Transmission electron micrographs of goat mammary gland-derived mammary epithelial cells (GMECs) grown *in vitro*. (A) Undifferentiated cell having progenitor cell traits, such as widely distributed free ribosomes and cytoplasmic lipid droplets. (B) Cell with a higher nucleus-to-cytoplasm ratio than differentiated cells. One common feature of GMECs is their ability to generate milk lipids. Nucleus (N), lipid droplet (LD), ribosome (R), mitochondria (M), lipid droplets (LD), secretory vesicles (SV) (Scale bar = 200 nm).

In summary, DMEM/F12 supplemented with 10% FBS, EGF, bFGF, insulin, and 1% antibiotics was the optimal basal growth medium for the cultivation and expansion of GMECs.

3.4 Clonal/CFU Assay

The soft agar assay/CFU assay is an anchorage-independent growth assay in soft agar used to detect the malignant transformation capacity of cells. Transformation capability was very low for colony formation in soft agar

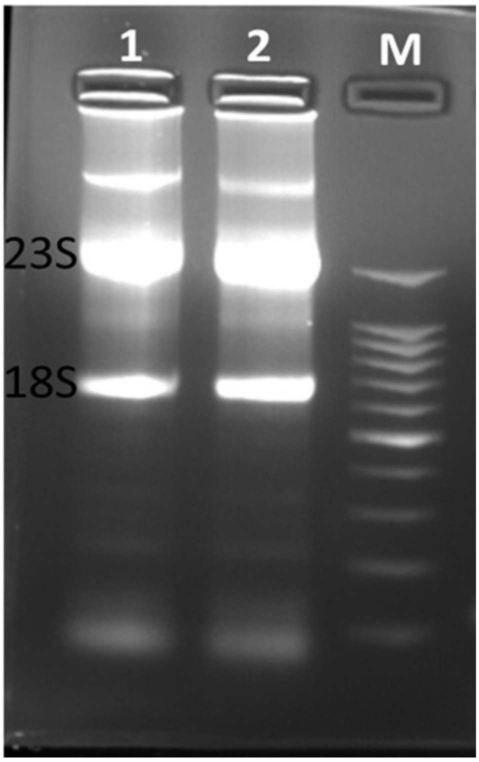

Fig. 10. Gel electrophoresis of purified total RNA samples on a 2% agarose gel. Lane M: 100 bp DNA ladder (marker); lanes 1 and 2: RNA samples extracted from GMECs (23S and 18S rRNA bands).

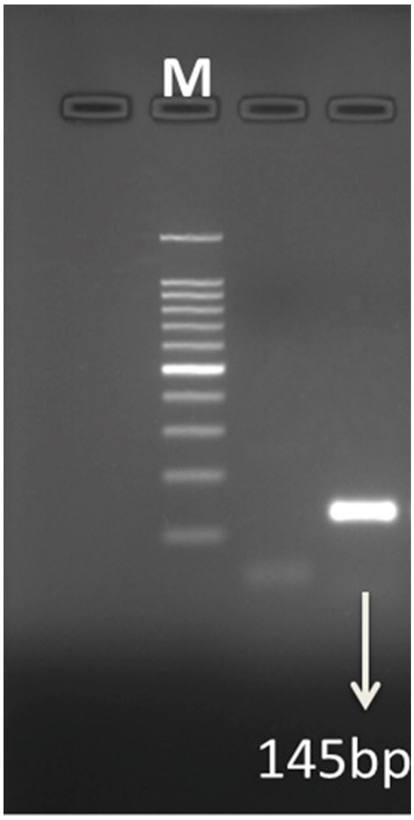

Fig. 11. PCR product of 145 bp obtained with GAPDH primers (internal control) using cDNA as a template and analyzed with 2% agarose gel electrophoresis.

over 20 days, although the cells maintained all the phenotypic characteristics of normal cells, with no signs of transformation. In contrast, RAW 264.7 cells (the positive control) exhibited strong transformation capacity, as evidenced by colony formation after 4–5 days of culture, and reached confluence by the 9th day (Fig. 5).

$$\mathrm{CE}\ (\%) = n/\mu \times 100$$

Where n is the number of colonies and μ is the number of cells seeded.

$$\mathrm{CE}\ (\%) = 3.8$$

3.5 Purification of Isolated GMECs Using the EasySep™ Mouse PE Positive Selection Kit

EasySep™ Mouse PE Positive selection kit was used to isolate luminal cells from a suspension of GMESCs by positive selection. Luminal cells were targeted using an anti-CD24 antibody, which formed a complex with rapid spheres (magnetic particles). The cocktail also contained an FcR blocker to minimize non-specific binding. The CD24-labelled cells were then separated using the EasySep™ magnet.

Cells were seeded into two wells of a 6-well culture plate and maintained at 37 °C, 95% humidity, and 5% CO_2 (Fig. 6). After 10 days of culture of 2nd-passaged luminal cells, GMESCs exhibited typical growth characteristics (Fig. 7).

3.6 Ultrastructural Characterization

TEM of GMECs revealed typical traits, including a large number of microvilli on the apical membrane (Fig. 8). At high magnification, cytoplasmic organelles, including secretory vesicles, mitochondria, ribosomes, lipid droplets, and numerous microvilli, were visible (Fig. 8). Similar findings have been reported in MECs. Compared with differentiated cells, undifferentiated progenitor cells had a higher nuclear-to-cytoplasmic ratio (Fig. 9) and all cellular organelles were found in the cytoplasm. The developed cells, on the other hand, showed condensed cytoplasmic organelles and a modest nucleus-to-cytoplasm ratio (Fig. 8).

3.7 Quality Check and Gel Electrophoresis of RNA Extracted From GMECs

Total RNA extracted from purified GMECs using TRIzol reagent was analyzed by gel electrophoresis on a 2% agarose gel. The quantification was carried out using an aspectrophotometer, which indicated a concentration of

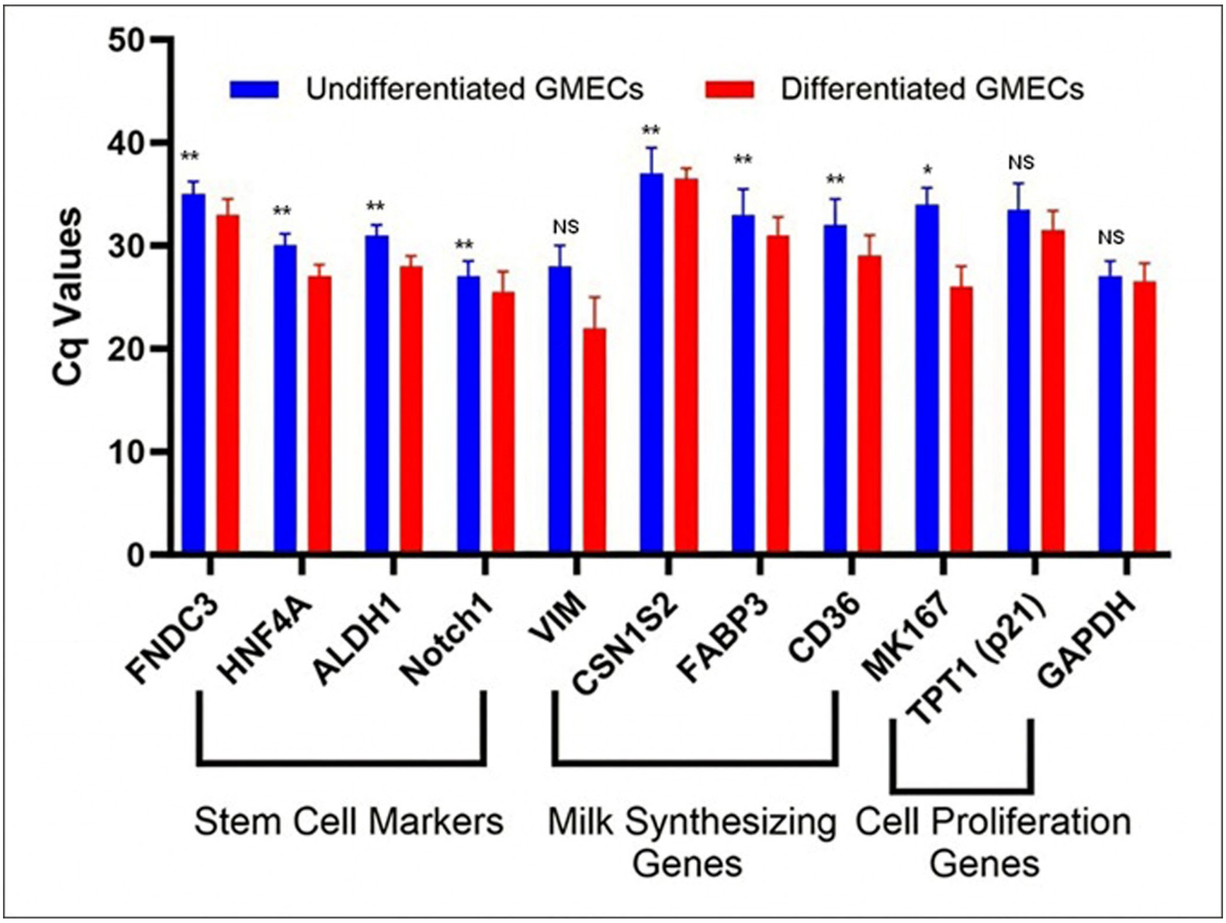

Fig. 12. Comparative analysis of cycle threshold (Ct) values (mean ± SEM) for stemness markers (FNDC3, HNF4A, ALDH1, and NOTCH1), milk-synthesizing genes (VIM, CSN1S2, FABP3, and CD36), and cell proliferation markers (MKI67 and TPT1) in undifferentiated and differentiated GMECs. Statistical analysis was performed using two-way ANOVA, followed by Tukey's post-hoc multiple comparison test. A p-value < 0.05 was considered statistically significant. Significance levels are denoted as: *$p < 0.05$; **$p < 0.01$ and Non-significant comparisons are labelled as "NS". Error bars represent SEM.

4.8 µg/mL of RNA with a 260/280 ratio of 1.97. The quality was confirmed by electrophoresis on a 2% agarose gel, and two bands corresponding to 23S and 18S rRNA were observed (Fig. 10). The RNA was subjected to cDNA synthesis, which was then used for PCR with GAPDH (Fig. 11).

3.8 Gene Expression of Stem Cell Markers, Milk Synthesis Protein Markers, and Cell Proliferation Markers Using RT-qPCR

To evaluate the expression of GMESCs using RT-qPCR, cDNA was generated from GMECs (passages 5 and 6), and it was found that the expression of stemness-related genes in GMESCs was higher in differentiated cells than in early-passaged GMECs. RT-PCR conditions were optimized (**Supplementary Table 3**).

The gene expression levels for the molecular markers of stemness fibronectin type III domain containing 3B (FNDC3B), hepatocyte nuclear factor 4A (HNF4A), aldehyde dehydrogenase 1 (ALDH1), and notch 1 (NOTCH1); milk synthesis-related genes markers of basal and myoepithelium (vimentin, VIM), alpha S2-casein (CSN1S2), fatty acid binding protein 3 (FABP3), and CD36 molecule (CD36); and cell proliferation genes Ki-67 (MKI67) and tumor protein translationally-controlled 1 (TPT1) were determined by RT-PCR.

The identification of stemness markers, including ALDH1, FNDC3B, HNF4A, and NOTCH1, confirmed the multipotency and undifferentiated nature of these cells. In this study, GMESCs exhibited robust expression of these markers within fully differentiated stem cell populations, particularly at later passages, compared with standard GMECs (Figs. 12,13).

3.9 Immunocytochemistry of GMESCs

Strong CD24 expression in GMESCs was demonstrated by immuno-cytochemistry, suggesting stem cell-like characteristics. In addition, a similar expression pattern was observed in other stem cell markers, including ALDH1, HNF4A, and ITGA6, further confirming the stemness characteristics of goat mammary stem/progenitor

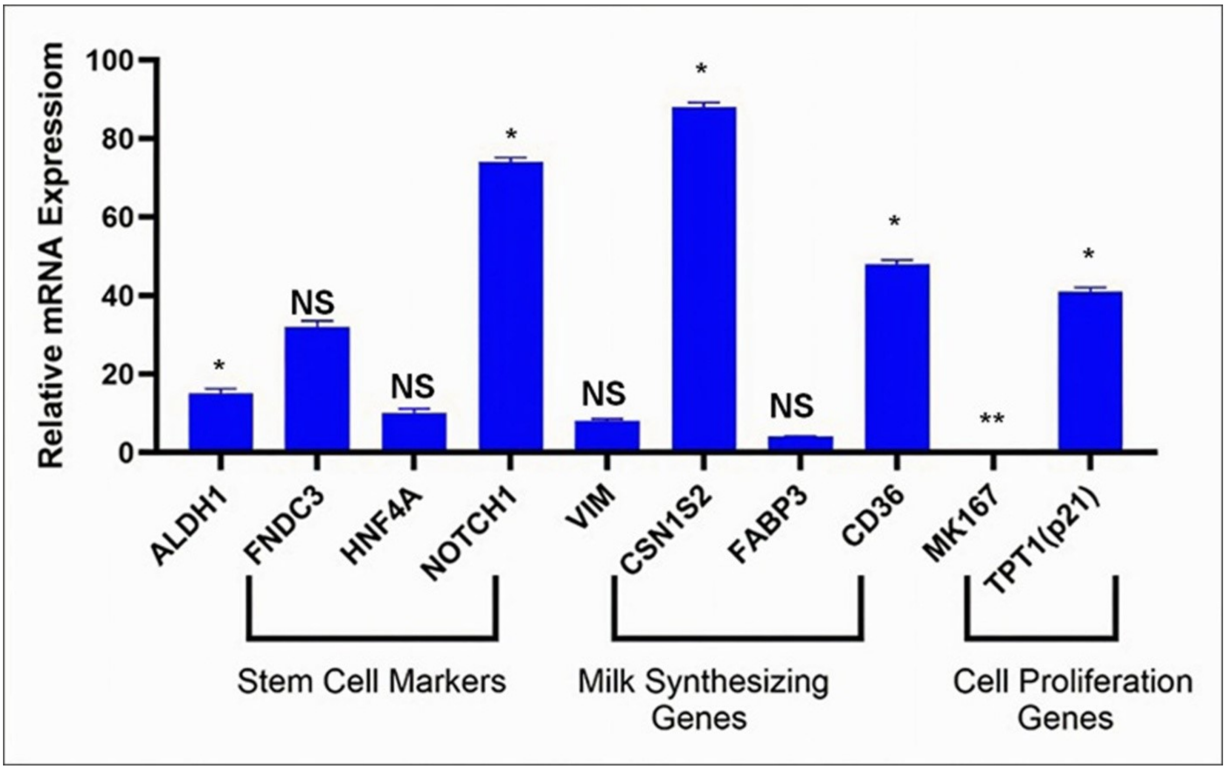

Fig. 13. Relative mRNA expression profile (mean ± SEM) of stemness, milk-synthesizing, and cell proliferation genes in goat mammary epithelial stem cells (GMESCs). Gene expression levels were quantified by RT-qPCR and calculated using the $2^{-\Delta\Delta Ct}$ method, normalized to the endogenous reference gene (GAPDH). Statistical analysis was performed using one-way ANOVA, followed by Tukey's post-hoc test. A p-value < 0.05 was considered statistically significant. Significance levels are denoted as: *$p < 0.05$; **$p < 0.01$ and Non-significant comparisons are labelled as "NS". Error bars represent SEM.

cells. Cytoplasmic staining of CD24 (Fig. 14A1–A3), ITGA6 (Fig. 14B1–B3), HNF4A (Fig. 14C1–C3), ALDH1 (Fig. 14D1–D3) were abundantly present in GMESCs. The strong expression of these markers suggests the stemness of GMESCs. To ensure the accuracy of the results, negative control staining with the corresponding IgG isotypes showed no specific cellular staining (Fig. 14E1–E3).

3.10 SA-β-gal Activity of Luminal Cells

SA-β-gal was performed on early- and late-passaged luminal cells to assess senescence. Only 5% of cells were stained (Fig. 15). The cytoplasm of senescent cells was stained blue.

To date, we have maintained GMECs in continuous culture for more than 34 passages without evidence of senescence, and we will repeat the experiment to assess for senescence at higher passage numbers.

4. Discussion

The physiological functions of the mammary glands have been extensively studied using GMECs as models [17]. In this work, we have isolated, described, and examined the dynamics of GMECs. Mammary stem cells that can self-renew and differentiate into the basal and luminal lineages that make up the functional mammary epithelium have been reported in several studies [18,19]. The isolated pure CD24-labeled cells were luminal cells. A pure population of luminal and myoepithelial cells was simultaneously identified and isolated using the CD24 marker [20]. Lineage-negative cells isolated from Korean Holstein dairy cattle mammary epithelial cells using a magnetic separator defined the characteristic properties of stem cells [6]. Lineage-negative mouse mammary epithelial cells have stem cell properties, and a single cell may regrow a complete mammary organ [21,22]. GMECs were effectively cultivated in primary culture under ideal *in vitro* conditions, and the resulting cells could be cultured *in vitro* for several passages while remaining hormone-sensitive and capable of synthesizing milk proteins [23]. Cell progression through the cell cycle from G1 to S was stimulated by growth factors to promote cell division and growth. Mammary tissue was collected from a slaughterhouse (Jammu, India). It was digested, and to maximize the ideal media culture conditions, GMECs were separated and cultivated in a variety of growth and differentiation media. Based on these two factors, namely, the proliferation index and population doubling time, basal growth media were optimized, with DMEM/F12, FBS, EGF, and 1% antibiotic as the best media for the growth and culture of GMECs. Similar findings

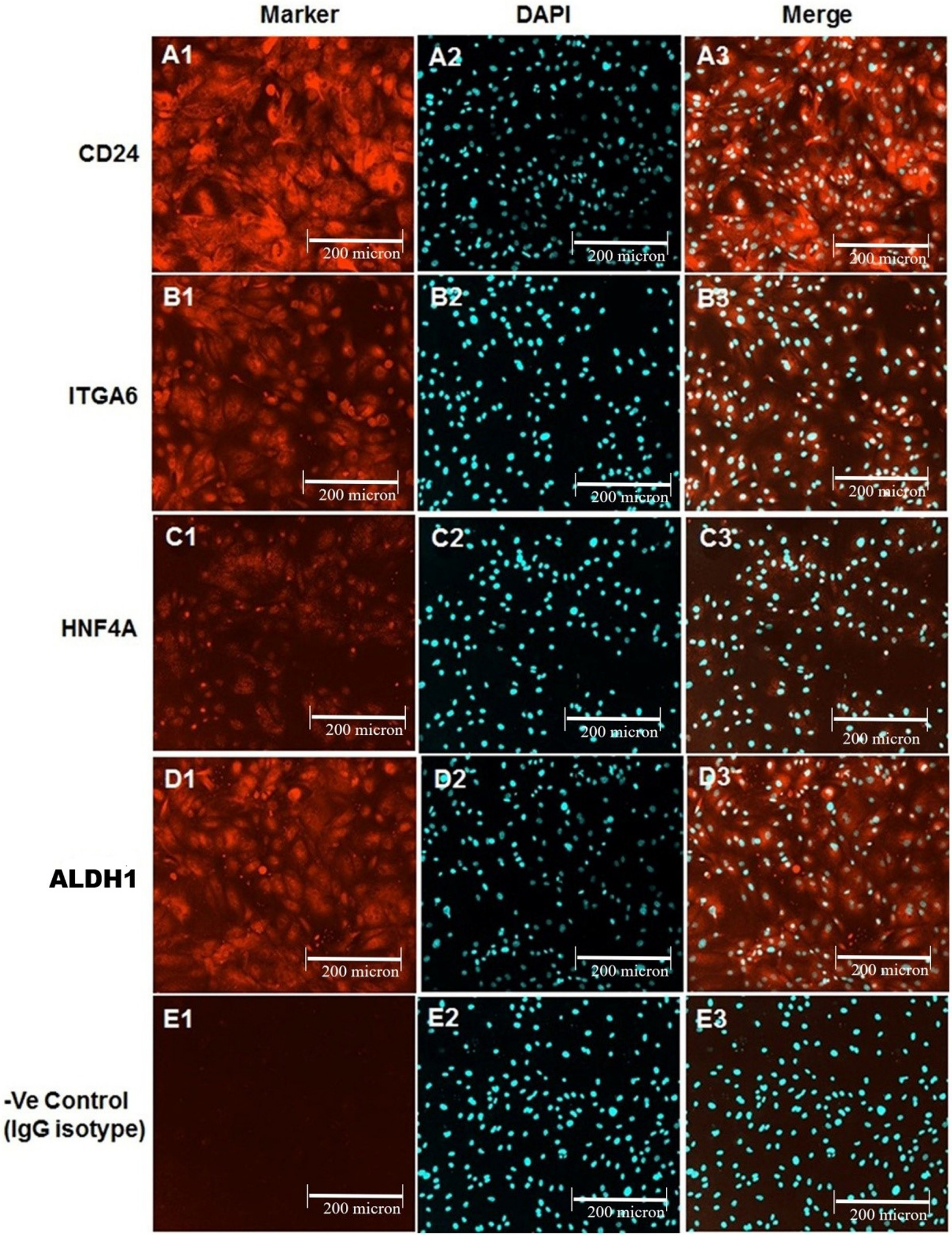

Fig. 14. Characterization of goat mammary epithelia stem/progenitor cells using immunochemistry. Goat anti-CD24 primary antibody causes red fluorescence (Alexa fluor 594) to be expressed in (A1–A3), whereas goat anti-ITGA6 primary antibody causes red fluorescence (Alexa fluor 594) to be present in (B1–B3). (C1–C3) Goat anti-HNF4A primary antibody shows strong fluorescence (Alexa fluor 594). (D1–D3) Goat anti-ALDH1 primary antibody-induced, strong fluorescence (Alexa fluor 594). (E1–E3) Mouse IgG isotype control with Alexa fluor 594-conjugated secondary antibodies showed no particular affinity. DAPI, 4,6-diamidino-2-phenylindole; (Scale bar = 200 micron).

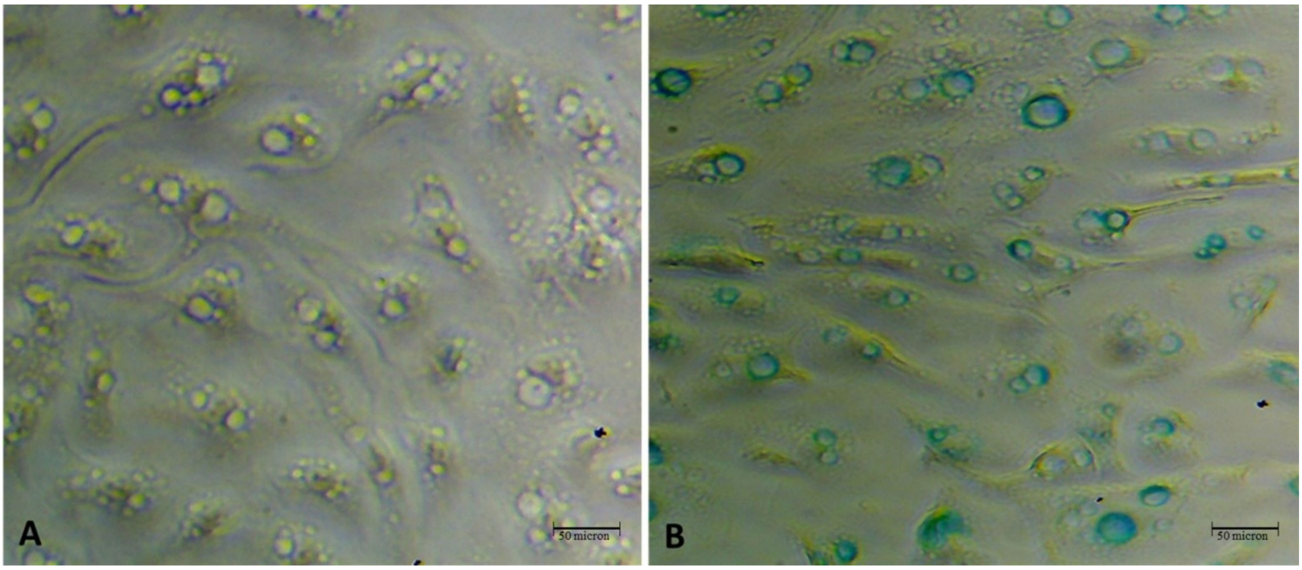

Fig. 15. Cell senescence assay (β-galactosidase activity) in GMECs. (A) Cells at an early passage (2) ($\times$400) after β-gal staining. (B) Stained cells at a late passage (20) showing a blue cytoplasm after incubating the cells in staining solution for a period of 12 h. Passaged-20 Cells after 12 h of culture showed senescence 5% cells (Scale bar = 50 micron).

have been reported in other studies [24]. Under optimized media, GMECs showed a pure cell line with characteristic "cobblestone appearance", mammospheres, which eventually underwent differentiation into structures resembling domes and alveoli with a network of tubules linking them. Additionally, previous studies have shown that when cells are cultivated at low densities on plastic substrates, they typically form island monolayer aggregates with a distinctive cobblestone appearance in epithelial cells [14,15,25].

We observed a relatively less CFU efficiency, a result consistent with existing literature on primary mammary epithelial cell (MEC) cultures. This outcome likely stems from several biological and technical variables. The mammary epithelium is characterized by a sophisticated dual-layered architecture, primarily composed of cells that have reached terminal differentiation [26]. During the initial isolation phase, MECs often undergo "culture shock", characterized by a swift decline in stemness and an inclination toward senescence or differentiation, which diminishes the pool of clonogenic cells [24]. Additionally, the lack of 3D structural cues—specifically a laminin-dense basement membrane—may hinder the expansion potential of primary MECs [27].

GMESCs' characterization and establishment as a stable cell line underscores the necessity of validating their protein synthesis capabilities, as this more accurately reflects their behavior within a living system. In the present study, ultrastructural analysis identified typical progenitor traits in undifferentiated GMECs, specifically, a high nucleus-to-cytoplasm ratio and dispersed free ribosomes. In contrast, differentiated cells were distinguished by a lower nucleus-to-cytoplasm ratio and increased condensation of cytoplasmic organelles, indicating a clear transition from a precursor state to a specialized functional state. Similar observations have been reported in other studies [28], in which the ultrastructural features of cells determined stem cell characteristics. Morphologically undifferentiated bovine mammary small light cells that lacked or had few cellular organelles without any specialized function were characterized as stem cell populations. These small, stable stem cells have been suggested to be epithelial cell precursors or progenitors.

Their multipotency and stem cell properties were demonstrated by the expression of stemness markers, including ALDH1, FNDC3B, HNF4A, and NOTCH1. GMESCs strongly expressed stemness markers along with molecular markers for cell proliferation and milk-synthesizing genes in fully differentiated stem cells, as seen in the higher passaged number compared to GMECs. Similar studies have been conducted using molecular markers related to stemness, cell proliferation, and milk-synthesizing genes, in which it was reported that these molecular markers could be induced and hence promote mammary-specific gene expression and stem transcripts [6].

Immunocytochemistry is used to identify mammary stem cells *in situ* in slides of mammary gland tissues. Through immunocytochemistry, CD24 showed a strong expression level in GMESCs, which depicted their stem cell like characters. In addition, a similar expression pattern was observed for other stem cell markers, such as ALDH1, HNF4A, and ITGA6, further confirming the presence of goat mammary stem/progenitor cells. CD24, a single marker (P-selectin ligand) expressed on luminal cells, has been used in conjunction with additional markers, such as CD29 (beta 1-integrin) or ITGA6 (CD49f), to isolate mouse mammary stem cells. This is a simple and efficient means of separating mouse epithelial cells from non-epithelial cells [20]. A single cell from a $CD29^{hi}/CD24^{+}$ or $CD49f^{hi}/CD24^{+}$ population can reconstitute a functional mammary gland *in vivo* [22]. However, CD24, CD29, CD24, and ITGA6 are not exclusive markers of MESCs. Our findings regarding $CD24^{+}$ expression suggest a highly specialized role for this glycoprotein in the caprine mammary hierarchy. In the goat mammary gland, $CD24^{+}$ serves not merely as a surface marker but as a functional mediator of cell-cell adhesion and signaling. The localization of $CD24^{+}$ primarily within the luminal layer suggests its involvement in maintaining the secretory architecture of the alveoli. High expression ($CD24^{high}$) in specific clusters may demarcate progenitor niches that drive lobulo-alveolar expansion during pregnancy, a critical phase for dairy goats. In mice, the $CD24^{high}/CD49f^{low}$ phenotype is the classic hallmark of luminal progenitors. Our results in the goat model align with this to an extent; however, the caprine CD24 profile appears more heterogeneous.

Other studies have also used ALDH1toidentify mouse and human mammary stem cells. It is an adult stem/progenitor cell marker expressed in various cell types, including cancer stem cells [29]. Mammary stem cells are functionally characterized by ALDH1 activity, which can be detected by immunocytochemistry. Moreover, $ALDH^{high}$ cells exhibited luminal features, whereas $ALDH^{low}$ cells produced colonies with myoepithelial features [30]. However, ALDH1 expression is not exclusive to mammary stem cells; progenitor cells also express this enzyme. Therefore, they should always be used in combination with cell surface markers to increase the purity of the mammary stem cell population [31]. Hepatocyte nuclear factor 4 alpha (HNF4A) is a transcription factor that plays a crucial role in the specification of hepatic progenitor cells [32]. Additionally, it is a potential marker of putative mammary stem cell populations in the buffalo and bovine mammary stem/progenitor cells [32].

The development and validation of a Beetal goat mammary epithelial stem cell line provides a robust, biologically accurate *in vitro* framework for investigating the mechanisms of intramammary infections (mastitis). These cells may offer a superior alternative to immortalized cell lines, which often suffer from distorted signaling pathways; instead, they retain the plasticity required to differentiate into functional secretory cells providing a high-fidelity model for the various possible applications such as host-pathogen interactions and internalization, cytokine profiling, blood-milk barrier integrity, evaluation of novel antimicrobials etc. [33].

5. Limitations

Despite the successful isolation, characterization, and culture of goat MESCs, there are a few limitations. Obtaining a pure population of MECs from fibroblasts without using a flow cytometer is difficult. Primary mammary epithelial cell lines may take longer to grow in subsequent experiments.

6. Conclusion

In conclusion, this study evaluated various isolation and culture methodologies and demonstrated their significant impact on the *in vitro* growth of Beetal GMESCs. We successfully established rare GMESC lines, revealing that the suspension cultures retained substantial developmental potential, differentiating into myoepithelial and epithelial-like lineages. Conversely, adherent cultures yielded a homogeneous population of epithelial-like cells that aligned with established differentiation models. Although we observed hierarchical lineage development consistent with previously reported morphogenetic properties, our findings emphasize that no single surface marker is sufficient to identify MESCs exclusively. However, the functional roles of these markers remain unclear, highlighting the need for further investigation. Finally, the minimal presence of senescent cells observed via SA-β-gal assays across multiple passages confirms the successful establishment of a robust, continuous GMESC culture.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

NS: Writing—original draft, Conceptualization, Project administration, Funding acquisition, Supervision. SD: Writing—original draft, Methodology, Formal Analysis. Sandeep K: Writing—review and editing, Methodology. SSP: Writing—review and editing, Methodology. BN: Writing—review and editing, Formal analysis. Shaguneet K: Writing—review and editing, Methodology. DAOAP: Writing—review and editing, Methodology. SJL: Conceptualization, formal analysis, Writing—review and editing, Funding acquisition. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Ethical approval was not required for this study as all mammary tissue samples were obtained from a commercial slaughterhouse post-mortem. The animals were slaughtered for human consumption in accordance with [Government of India] food safety and animal welfare regulations. No live animals were used for the purposes of this research.

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL49338.

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