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Cellular characteristics and milk component productivity of primary bovine mammary cells for cell-cultured milk component production

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13 **Abstract**

14 Despite the increasing demand for milk, there is a simultaneous growth in awareness regarding
15 sustainable dairy farming and concerns about environmental issues. The concept of generating milk
16 components without traditional dairy farming has been introduced through the utilization of bovine
17 mammary cells. However, the establishment of a robust primary bovine mammary alveolar cells for cell-
18 cultured milk component production remains a challenge. Hence, the aim of this study was to assess the
19 cellular attributes and milk component productivity of primary bovine mammary cells through various
20 stages of cell subculture. The 1 cm^3 pieces of mammary tissues were incubated onto a 10-cm cell culture
21 dish until the cells grow out from the tissues. After the removal of mammary tissues, primary bovine
22 mammary cells (fibroblasts, FBs; myoepithelial cells, MCs; epithelial cells, ECs) were isolated and purified
23 through their different trypsin sensitivity. The primary bovine mammary cells were cultured with control
24 culture media (CCM; without hormones) and differentiation culture media (DCM; with prolactin, insulin,
25 cortisol, progesterone, 17β-estradiol, and epidermal growth factor). At passage 1, FBs, MCs, and ECs
26 cultured with CCM displayed the highest levels of vimentin, α-smooth muscle actin, and cytokeratin 18/19
27 expression, respectively ($p < 0.001$). These cellular characteristics were not consistently maintained across
28 subsequent passages, with a notable reduction in cell numbers ($p < 0.001$). At passage 1, ECs cultured in
29 DCM exhibited higher milk component productivity in comparison to those cultured in CCM ($p < 0.05$).
30 However, the synthesis of milk components exhibited a gradual decline as vacuoles increased in ECs
31 throughout consecutive passaging. ECs cultured with CCM were unable to synthesize milk components
32 due to the loss of tight junctions caused by matrix metalloproteinase activation. Conversely, ECs cultured
33 with DCM boosted milk component production by intact tight junctions and low matrix metalloproteinase
34 activity ($p < 0.05$). Our findings demonstrated the requirement for various hormones to maintain the
35 productivity of primary bovine mammary cells over successive passages. These results highlight the
36 importance of hormonal optimization to establish the stable primary cells in cell-cultured milk production.
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38 **Keywords**: Milk protein, Milk fat, Cell proliferation, Cell differentiation, Primary bovine mammary cells,
39 Cell-cultured milk production
40
41
42

Introduction

43 Milk is consumed for human health, nutrition, and immune function because of its essential nutrients and
44 natural bioactive components [1]. Global milk production is forecast to steadily increase from 887 metric
45 tons in 2021 to 1,060 metric tons in 2031 [2]. However, the global population of 8.0 billion in 2022 is
46 projected to increase to 8.7 billion by 2032 and 10 billion by 2050 [3,4,5]. While the current population of
47 cows utilized for milk production stands at nearly 234 million [6], the demand for milk production is
48 projected to persistently rise.

49 Over the past 70 years, the dairy industry has been guided by dairy research and policies aimed at
50 enhancing the economic efficiency of milk production [7]. Consequently, dairy cows in current dairy
51 farming have been raised to increase milk production in concentrated animal-feeding operations [8].
52 However, the dairy farming has negatively impacted the environment and animal welfare [7]. To mitigate
53 the negative environmental impacts of dairy farming, a Dairy Sustainability Framework has been
54 established by the global dairy sector [9]. Also, in alignment with sustainable dairy farming practices,
55 several researchers in the field are dedicated to developing cow milk proteins through precision
56 fermentation technology and cell culture. [10,11].

57 The milk proteins and fats are synthesized within the epithelial cells of the mammary gland [12] The
58 mammary gland is comprised of luminal epithelium and basal epithelium. The luminal epithelium consists
59 of epithelial cells (ECs), while the basal epithelium consists of fibroblasts (FBs), myoepithelial cells (MCs),
60 and adipocytes [13]. According to a previous study, milk productivity is directly related to the number of
61 mammary ECs [14]. This indicates that the establishment of a bovine mammary ECs is essential for the
62 production of milk proteins and fats in *in vitro* cell culture systems. However, in fact, primary bovine
63 mammary ECs exhibit cellular instability because of their finite lifespan [15,16].

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64 Previously, primary bovine mammary EC models have been established to study the synthesis of milk
65 components and mammary gland function [17-19]. Most of these studies have evaluated cellular
66 characteristics at early passages via the analysis of morphology, cell-specific markers, growth patterns, and
67 secretion of milk components. Nevertheless, the development of a reliable primary bovine mammary
68 alveolar cell model remains a challenge, especially as passages progress. A prior study suggested that
69 enhancing cellular stability can be achieved by assessing cellular characteristics during cell growth and
70 bioproduction across successive passages. [20]. Hence, it is important to conduct a comprehensive
71 assessment of cellular characteristics and milk component productivity across successive passages to ensure
72 the stability of primary bovine mammary ECs.
73 Overall, the cellular characteristics of the primary bovine mammary ECs during serial passage have not
74 been comprehensively studied, mainly due to cellular instability. Therefore, this study aimed to assess the
75 cellular characteristics and milk component productivity of primary bovine mammary ECs throughout cell
76 subculturing, with the goal of identifying areas for enhancing the stability of cells responsible for the
77 sustainable production of cell-cultured milk.
78
79

80 Materials and Methods

81 Chemicals and reagents

82 Dulbecco's modified Eagle medium/nutrient mixture F12 (DMEM/F12) was obtained from Gibco
83 (Grand Island, NY, USA). Fetal bovine serum (FBS), penicillin/streptomycin (P/S), and trypsin-
84 ethylenediaminetetraacetic acid (EDTA) were supplied by WELGENE Inc. (Gyeongsan, Daegu, Korea).
85 Gentamicin sulfate was obtained from Sigma-Aldrich (St. Louis, MO, USA). Amphotericin B was
86 purchased from Gibco. Insulin (INS), cortisol (CORT), progesterone (P4), 17-β estradiol (E2), and
87 epidermal growth factor (EGF) were purchased from Sigma-Aldrich. Prolactin (PRL) was obtained from
88 ProSpec (Ness-Ziona, Israel). Phosphate-buffered saline (PBS) was purchased from Lonza (Walkersville,
89 MD, USA). Hank's balanced salt solution (HBSS) was provided by Gibco. The control culture media (CCM)

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were freshly prepared with DMEM/F12 containing 10% FBS, 1% P/S, 100 ug/mL gentamycin, and 5 ug/mL amphotericin B. The differentiation culture media (DCM) were prepared with CCM added with 1 ug/mL PRL, 5 ug/mL INS, 1 ug/mL CORT, 5 ug/mL P4, 5 ug/mL E2, and 10 ng/mL EGF.

Isolation, purification, culture, and differentiation of bovine mammary cells

Primary bovine mammary cells were isolated from the bovine mammary parenchymal tissues of one lactating Holstein dairy cows (53-month-old and 306 kg body weight) after slaughter. Fresh pieces of the mammary tissue were placed in the sterilized specimen cups with HBSS supplemented with penicillin (200 U/mL), streptomycin (200 ug/mL), gentamycin (200 ug/mL), and amphotericin B (10 ug/mL) and immediately transported to the laboratory. Five grams of mammary tissue was cut into 1 cm^3 pieces, centrifuged at 1,500 × g for 5 min, and washed three times with HBSS to remove blood and milk. The rinsed pieces of tissues were transferred onto a 10-cm cell culture dish using 1 mL sterile tips (SPL, Pocheon, Korea) and were carefully incubated at 37° in a humidified 5% CO2 incubator using CCM. The CCM was replaced every 2 d until the cells grow out from the tissues to the bottom of the 10-cm cell culture dish. After the removal of mammary tissues, FBs, MCs, and ECs were isolated and purified using trypsin-EDTA solution based on their different trypsin sensitivity of 2, 2, and 10 min, respectively. ECs were differentiated using DCM for 5 d, and DCM was replaced every 2 d. The morphology of the cells was imaged and captured using Nikon Eclipse Ti2-U and Nikon Eclipse Ts2R cameras (Nikon Co., Ltd., Tokyo, Japan).

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Immunofluorescence

Cytoskeletal protein levels were determined by immunofluorescence staining. FBs, MCs, and ECs were cultured with CCM at a density of 0.05 × 10^6 cells per well in 12-well cell culture plates for 7 d and fixed with 4% paraformaldehyde for 15 min. Then, cells were treated with 0.1% Triton X-100 in PBS for permeabilization for 10 min. The cells were then blocked using 3% bovine serum albumin for 90 min and incubated with anti-mouse cytokeratin 18 (CK18; 1:200, Santa Cruz, CA, USA), anti-mouse CK19 (1:200, Progen Biotechnik GMBH, Heidelberg, Germany), anti-rabbit vimentin (1:500, Thermo Fisher Scientific,

116 PA, USA), and anti-rabbit α-smooth muscle actin (1:500, α-SMA; Invitrogen, MA, USA) diluted in 3%
117 bovine serum albumin at 4 ℃ for 15 h. Subsequently, the cells were washed three times with 0.1% tween
118 20 in PBS and incubated with DyLight 488 conjugated-donkey anti-rabbit IgG H&L (1:1000, Bethyl
119 Laboratories, TX, USA) and goat anti-mouse IgG H&L FITC (1:1000, Abcam, MA, UK) at 25 ℃ for 90
120 min. After washing three times with 0.1% tween 20 in PBS, the cells were fixed with 4% paraformaldehyde.
121 The nuclei were stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; 1 μg/mL) for 10 min
122 and washed three times with 0.1% tween 20 in PBS. The stained cells were imaged and captured using a
123 Nikon Eclipse Ti2-U and Nikon Eclipse Ts2R camera.
124

125 **Protein extraction and Western blot**

126 FBs, MCs, and ECs were grown with CCM for 7 d at a density of 0.1×10^6 cells per well in 6-well cell
127 culture plates. ECs were cultured with CCM and DCM for 5 d at a density of 0.1×10^6 cells per well in 6-
128 well cell culture plates. Radioimmunoprecipitation assay buffer (Elpis Biotech, Daejeon, Korea)
129 supplemented with a protease inhibitor cocktail (Abbkine Inc., CA, USA) was used to lyse the cells. The
130 cell lysates were collected in a 1.7 mL microtube and centrifuged at 17,000 ×g at 4 ℃ for 20 min. Proteins
131 were quantified using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, IL, USA). Protein
132 samples were loaded into sample wells of stacking (5%) and separating (10%) acrylamide gels and
133 separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The loaded proteins were
134 transferred onto nitrocellulose membranes (GE Healthcare Biosciences, PA, USA) using a Semi-Dry
135 Electrophoretic Transfer Cell (Bio-Rad Laboratories, CA, USA). The nitrocellulose membrane was blocked
136 with 5% nonfat milk buffer dissolved in Tris-buffered saline with Tween 20 buffer at 25 ℃ for 90 min and
137 incubated with anti-mouse CK 18 (1:500), anti-mouse CK19 (1:500), anti-rabbit vimentin (1:3000), anti-
138 rabbit α-SMA (1:3000), anti-rabbit E-cadherin (1:20000, Merck Millipore, Darmstadt, Germany), anti-
139 rabbit occludin (1:3000, Invitrogen), anti-rabbit claudin-1 (1:3000, Merck Millipore), anti-rabbit α-tubulin
140 (1:5000, Cell Signaling Technology, MA, USA), and anti-glyceraldehyde 3-phosphate dehydrogenase
141 (GAPDH; Merck Millipore) at 4 ℃ for 1–3 d. After washing three times with PBS for 15 min, the

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142 membranes were incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (Enzo Life
143 Sciences, Lausen, Switzerland) and goat anti-mouse IgG H&L conjugated with horseradish peroxidase
144 (Abcam, MA, UK). The signals of proteins were visualized using enhanced chemiluminescence detection
145 reagents (Thermo Fisher Scientific). α-Tubulin and GAPDH were used as internal controls for
146 normalization. The intensities of protein bands were quantified using Image J software (National Institute
147 of Health, MD, USA).
148

149 **Growth characteristic using trypan blue assay**

150 The growth characteristics of FBs, MCs, and ECs cultured with CCM and DCM were evaluated using
151 trypan blue solution (Gibco). The cells were seeded at a density of 0.05×10^6 cells per well in a 12-well
152 cell culture. The cells were dissociated using trypsin-ethylenediaminetetraacetic acid solution for 1, 3, 5, or
153 7 d. After staining the cells with trypan blue solution, viable cells were counted using a hemocytometer
154 (Hausser Scientific, PA, USA).
155

156 **RNA extraction and real-time polymerase chain reaction**

157 Gene expression levels related to milk proteins (CSN1S1, CSN2, CSN3, ALA, and BLG) were evaluated
158 using real-time polymerase chain reaction (RT-PCR). ECs were cultured and differentiated with CCM and
159 DCM at a density of 0.05×10^6 cells per well in 12-well cell culture plates for 5 d. Total RNA was extracted
160 using TRIzol (Ambion, TX, USA) and cDNA was synthesized using the TOPscript RT DryMIX kit
161 (Enzynomics, Daejeon, Korea). The mRNA expression levels were analyzed using 2× RT-PCR Smart mix
162 (BIOFACT CO., Ltd., Daejeon, Korea) and an RT-PCR system (Roche LightCycler® 96 System, Basel,
163 Switzerland) with thermal cycling conditions of 95 ℃ for 15 min and 60 cycles of denaturation at 95 ℃
164 for 10 s, annealing at 60 ℃ for 10 s, and extension at 72 ℃ for 10 s. The mRNA expression levels were
165 quantified using the $2^{-\Delta\Delta Ct}$ method and GAPDH was used as an internal control for normalization. The
166 primer sequence (BIONICS Co., Ltd., Seoul, Korea) designed using the AmplifX software version 1.7.0 as
167 follows: CSN1S1, (F) 5'- ACT GAG GAT CAA GCC ATG GAA G-3, (R) 5'-GAA TGT GCT TCT GCT

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168 CAA CAC T-3'; CSN2, (F) 5'-CTG GAA TTA ACT GCT TCT ACC T-3, ' (R) 5'-TAC TCT GCG ATT
169 TGT CTT ATT GA-3'; CSN3, (F) 5'-GGC GAG CCT ACA AGT ACA CCT A-3, ' (R) 5'-GGA CTG TGT
170 TGA TCT CAG GTG G-3'; ALA, (F) 5'-CCT GAA TGG GTC TGT ACC ACG TTT-3, ' (R) 5'-ATG TTG
171 CTT GAG TGA GGG TTC TGG-3'; BLG, (F) 5'-AGG CCT CCT ATT GTC CTC GT-3, ' (R) 5'-GCA
172 AAG GAC ACA GGG AGA AG-3. ' GAPDH (F) 5'-ATG ATT CCA CCC ACG GCA AGT T-3,' and (R)
173 5'-ATC ACC CCA CTT GAT GTT GGC A-3.'
174

175 **Oil red O staining**

176 Oil red O dye (Sigma-Aldrich) was used to evaluate the levels of intracellular triglycerides, major
177 components of milk fat. ECs were cultured with CCM and DCM at a density of 0.05×10^6 cells per well in
178 12-well cell culture plates for 5 d. The cells were fixed in 10% formalin at 25 ℃ for 1 h. After fixation,
179 cells were washed with 60% isopropanol and allowed to dry completely. Subsequently, the cells were
180 stained with the oil red O working solution for 10 min. After washing three times with deionized distilled
181 water to remove the unbound dye, the stained cells were imaged and captured using Nikon Eclipse Ti2-U
182 and Nikon Eclipse Ts2R cameras. The intensities of the oil red O-stained areas were quantified using
183 ImageJ software.
184

185 **Enzyme-linked immunosorbent assay and triglyceride assay**

186 α-Casein and triglycerides contents in culture media were analyzed using Bovine Casein Alpha (CSN1)
187 enzyme-linked immunosorbent assay (ELISA) Kit (Bioss Antibodies, MA, USA) and Triglyceride Assay
188 Kit (Abcam). In brief, ECs were cultured with CCM and DCM at a density of 0.05×10^6 cells per well in a
189 12-well cell culture plate for 5 d. After that, CCM and DCM were collected in 1.7 mL micro tubes and
190 centrifuged at 17,000 ×g at 4 ℃ for 20 min. The supernatants of CCM and DCM were preserved at –80 ℃
191 until use. The ELISA was performed according to the instructions of the manufacturers.
192

193 **Matrix metalloproteinase activity using gelatin zymography**

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194 The matrix metalloproteinase (MMP) activity was determined as described previously, with slight
195 modifications [21]. ECs were cultured with CCM and DCM at a density of 0.05×10^6 cells per well in 12-
196 well cell culture plates for 5 d. Subsequently, CCM and DCM were collected in 1.7 mL micro tubes and
197 centrifuged at 17,000 ×g at 4 ℃ for 20 min. Proteins in the supernatants were quantified using a
198 bicinchoninic acid protein assay kit. The CCM and DCM were analyzed using 8% sodium dodecyl sulfate-
199 polyacrylamide gel electrophoresis containing 0.1% gelatin as the MMP substrate. After that, the gel was
200 washed twice for 30 min with a washing buffer containing 12.5% Triton™ X-100. The gel was then
201 incubated with reaction buffer solution supplemented with Tris-HCl of 40 mM, calcium chloride of 5 mM,
202 and sodium azide of 3 mM at 37 °C for 15 h. The activities of MMP-2 and -9 were determined by negative
203 staining with Coomassie Brilliant Blue R 250 (Sigma-Aldrich).
204
205 **Statistical analysis**
206 All experiment were conducted at least three times independently. The experimental data were presented
207 as mean ± standard error of the mean (SEM). Statistical significance was analyzed by Student's t-test using
208 SPSS-PASW statistics software (version 22.0 (SPSS Inc., IL, USA). Statistical differences were considered
209 significant at $p < 0.05$.
210
211
212
Results
213 **Isolation of primary bovine mammary cells from bovine mammary gland**
214 The parenchymal tissues of bovine mammary gland were dissected from the bovine udder (Fig. 1A). The
215 1 cm^3 pieces of bovine mammary parenchymal tissues were placed on the bottom of 10-cm cell culture dish
216 for inducing the growth of bovine mammary cells. After 12 d of tissue incubation in CCM, FBs were firstly
217 isolated from the mammary parenchymal tissue (Fig. 1B). MCs and ECs were isolated from mammary
218 tissue after 24 d of tissue incubation, where dome-like structure was observed. In addition, ECs were

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219 enveloped by MCs with the outer layer consisting of FBs (Fig. 1B). The isolated FBs, MCs, and ECs were
220 purified using different trypsin digestion time, i.e., 2 and 10 min, respectively.
221
222 **Characteristics of cytoskeleton fluorescence in primary bovine mammary cells**
223 The fluorescence expression levels of CK18 and CK19 were markedly higher in ECs at passage 1 than
224 in FBs and MCs (Fig. 2A). In contrast, the vimentin and α-SMA in FBs and MCs at passage 1 had higher
225 fluorescence expression levels than ECs (Fig. 2A). CK18 and CK19 in ECs at passage 5 showed higher
226 fluorescence expression levels than those in FBs and MCs. FBs and MCs at passage 5 had relatively higher
227 fluorescence expression levels of α-SMA than ECs (Fig. 2B). However, there were no differences in the
228 expression levels of vimentin among FBs, MCs, and ECs at passage 5 (Fig. 2B). At passage 10, fluorescence
229 expression levels of CK18, CK19, vimentin, and α-SMA were slightly higher in ECs than in FBs and MCs
230 (Fig. 2C).
231
232 **Characteristics of cytoskeleton protein in primary bovine mammary cells**
233 At passage 1, CK18 and CK19 showed significantly higher protein expression levels than in FBs and
234 MCs (Fig. 3A, $p < 0.01$). Also at passage 1, vimentin and α-SMA in FBs and MCs had significantly higher
235 protein expression levels than ECs (Fig. 3A, $p < 0.001$). At passage 5, the protein expressions of CK18 and
236 CK19 showed significant increases in ECs compared to FBs and MCs ($p < 0.05$, Fig. 3B). In contrast, the
237 protein expression of α-SMA showed a significant increase in FBs and MCs at passage 5 compared to ECs
238 ($p < 0.01$, Fig. 3B) and there was no significant difference among FBs, MCs, and ECs at passage 5 in the
239 protein expression level of vimentin ($p > 0.05$, Fig. 3B). All protein expressions of CK18, CK19, vimentin,
240 and α-SMA were significantly higher in ECs at passage 10 than FBs and MCs ($p < 0.01$, Fig. 3C).
241
242 **Characteristics of growth curve in primary bovine mammary cells**
243 At passage 1, FBs and MCs grew from 2.33×10^4 at 1 d to 1.80×10^5 at 7 d (Fig. 4A). In contrast, ECs
244 had cell numbers of 2.83×10^4 at 1 d and 1.90×10^5 at 7 d (Fig. 4B). However, at passage 15, the growth

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245 of FBs and MCs significantly decreased from 2.33×10^4 at 1 d to 1.41×10^5 at 7 d compared to that at
246 passage 1 ($p < 0.001$, Fig. 4A). The growth of ECs significantly decreased from 2.58×10^4 at 1 d to $1.48 \times$
247 10^5 at 7 d ($p < 0.001$, Fig. 4B).
248
249 **Characteristics of milk component production in primary bovine mammary epithelial cells**
250 Culturing ECs with DCM for 5 d resulted in significant morphological changes compared with cells
251 cultured with CCM (Fig. 5A–C). Alveoli-like structural forms were observed in ECs cultured in DCM.
252 However, a decrease in alveoli-like structures and an increase in vacuoles in ECs were observed in
253 accordance with an increase in passage number (Fig. 5A–C). At passage 1, ECs cultured in DCM exhibited
254 significantly elevated expression levels (8.08-, 10.71-, 4.92-, 11.82-, 9.47-, and 13.12-fold) of milk protein-
255 related genes (CSN1S1, CSN2, CSN3, ALA, and BLG) and displayed increased oil red O staining
256 compared to those cultured in CCM ($p < 0.05$, Fig. 5A). At passage 5, the levels of milk protein-related
257 genes and oil red O staining in ECs cultured with DCM were elevated to 3.39-, 4.757-, 2.639-, 2.621-, 1.55-,
258 and 4.86-fold compared to the CCM ($p < 0.05$, Fig. 5B). However, at passage 10, ECs cultured in DCM
259 did not show significant increases in the expression of milk protein-related genes or oil red O staining
260 compared to those cultured in CCM ($p > 0.05$, Fig. 5C).
261
262 **Comparison between control culture media and differentiation culture media in primary bovine**
263 **mammary epithelial cells**
264 The CCM increased the number of ECs, whereas the DCM maintained the cell count (Fig. 6A,B). CCM
265 exhibited morphological characteristics resembling those of vacuoles, whereas DCM displayed an alveolar
266 structure (Fig. 6A). Moreover, ECs cultured in DCM significantly elevated the protein expression level of
267 αS1-casein compared to those cultured in CCM ($p < 0.05$, Fig. 6C,D). ECs cultured with DCM had
268 significantly higher α-casein and triglyceride levels in media than those cultured with CCM ($p < 0.001$,
269 Fig. 6E). The green fluorescence and protein expression levels of tight junctions (TJs) including E-cadherin
270 and occludin were significantly enhanced in ECs cultured with DCM than those cultured with CCM ($p <$

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271 0.05, Fig. 6F–H). The activity of MMP-2 and -9 in cells cultured in DCM was significantly lower than
272 those in CCM ($p < 0.05$, Fig. 6I).
273
274
275 # Discussion

276 Interest in sustainable dairy farming is continuously increasing because of concerns about greenhouse
277 gas emissions, nitrogen and phosphorus excretion from manure, and animal welfare resulting from intensive
278 dairy farming [22]. In recent years, companies have used fermentation-based cellular agriculture to
279 manufacture milk components like CSN and BLG [10]. However, this innovative precision fermentation
280 technology has raised public concerns, particularly in relation to the utilization of genetically modified
281 organisms [23]. Cellular agriculture in the dairy sector is driven by the goal of utilizing animal cells,
282 specifically ECs derived from the alveoli of the mammary gland [24, 25]. These cells have the capacity to
283 generate essential milk components, including milk proteins and fats through secretory differentiation.
284 Consequently, our research has focused on examining the cellular properties of primary bovine mammary
285 cells and establishing an EC model for the production of milk constituents.

286 Mammary alveoli are fundamental components of the mammary glands that are responsible for milk
287 production and secretion [24]. The parenchyma of alveoli is composed of inner milk secretory ECs that
288 surround the lumen, outer MCs that attach to the base of the mammary epithelium, and the basement
289 membrane that contacts the MCs. In addition, the stromal compartment comprises various stromal cells,
290 such as FBs, adipocytes, endothelial cells, and the extracellular matrix [26]. Therefore, from the perspective
291 of cellular structure, FBs and MCs are located on the outer side and ECs are positioned on the inner side of
292 the mammary alveolus. In accordance with the structure of the alveolus, our results showed that FBs and
293 MCs located on the outer side of the alveolus were first isolated after 12 d of tissue incubation, whereas
294 ECs located on the inner side were isolated after 24 d (Fig. 1). A previous study reported that FBs and ECs
295 were elongated after culturing for 5 and 10 d, respectively, in bovine mammary tissue from Chinese
296 Holstein dairy cows [17].

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297 The cytoskeleton plays an important role in maintaining cellular integrity, structure, and function and has
298 been reported to express different cytoskeletal proteins depending on cell type [26]. Therefore, cytoskeletal
299 protein markers such as CK18, CK19, vimentin, and α-SMA were used to establish the primary bovine
300 mammary cells [24, 26]. According to previous studies, CK18 and CK19 are characteristic markers for
301 mammary secretory ECs and vimentin and α-SMA are typical markers for mammary FBs and MCs,
302 respectively [28,29]. Consistent with the previous studies, our results showed that CK18 and CK19 were
303 primarily expressed in ECs, and vimentin and α-SMA were mainly expressed in FBs and MCs. However,
304 the differences in the fluorescence and protein levels of cytoskeletons among FBs, MCs, and ECs gradually
305 decreased over the serial passages (Fig. 2 and 3). In fact, primary bovine mammary cells have the finite
306 lifespan along with the instability of passage [15,16,20]. This cellular senescence of primary bovine
307 mammary cells induces an irreversible arrest of cell growth and proliferation, ultimately resulting in cell
308 death along with the occurrence of vacuoles [16]. Therefore, the growth curves of FBs, MCs, and ECs were
309 investigated to evaluate the correlation between cytoskeletal protein and cell numbers and our finding
310 showed that the cell number of these cells gradually decreased over the serial passages (Fig. 4). These
311 results suggest that the cause of the decreased cytoskeletal proteins is directly related to the replicative
312 senescence. Taken together, our data indicate that the cytoskeleton characteristics were not maintained due
313 to a decrease in cell numbers resulting from cellular senescence over serial passages.
314 Milk synthesis and secretion in ECs are primarily regulated by the coordinated action of reproductive
315 hormones (PRL, P4, and E2) and metabolic hormones (INS and CORT) [29]. PRL is a key hormone that
316 promotes alveolar differentiation and milk component production [30]. Therefore, in the current study, ECs
317 were differentiated using DCM supplemented with various hormones, including PRL, to evaluate the milk
318 component productivity through serial passages. The concentration of hormones in DCM was established
319 based on previous studies reporting the production of milk components in ECs [17,31,32]. ECs
320 differentiated with DCM at passage 1 significantly increased milk protein-related genes and milk fat-related
321 triglycerides compared to those that proliferated with CCM (Fig. 5). However, the synthesis of milk proteins
322 and fats gradually decreased with an increase in vacuoles in ECs over serial passaging. According to a

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323 previous study, the occurrence of large cytoplasmic vacuoles and the decrease of milk component
324 productivity were the major feature of involution in ECs of mammary gland [33]. In particular, involution
325 of the mammary gland is known to associated with the impairment of TJs integrity as a process of returning
326 milk-secreting ECs to their non-lactating state [26,33]. Thus, the correlation between milk component
327 productivity and TJs integrity, depending on hormone addition, was further investigated using CCM and
328 DCM. Previous studies have reported that mammary gland involution occurs in two distinct physiological
329 phases in the absence of lactogenic hormone secretion [30,34]. Early apoptosis and the loss of TJs occurred
330 in the first phase, and anoikis was irreversibly induced with a massive loss of mammary ECs after disruption
331 of the basement membrane through the activation of MMPs in the second phase [35]. In contrast, the
332 presence of hormones, such as PRL and CORT, enhanced the TJs formation and milk production by
333 mammary ECs [36]. Indeed, culture media supplemented with various hormones such as PRL, INS, CORT,
334 P4, E2, and EGF induced and maintained milk component production and cellular characteristics in
335 spontaneously immortalized primary yak and buffalo mammary ECs up to passages 50 and 60 [29,37]. In
336 line with these earlier findings, the present study demonstrated that CCM induced involution and the
337 formation of cellular vacuoles, whereas DCM led to differentiation and the development of alveolar
338 morphology. Moreover, ECs cultured with CCM could not synthesize milk components because of the
339 impairment of TJs caused by MMP activation, whereas DCM elevated milk component production by
340 enhancing TJs and decreasing MMPs activity. Taken together, the absence of hormones in CCM resulted
341 in MMP-induced impairment of TJs integrity, and the presence of hormones enhanced milk component
342 productivity and TJs protein expression via a decrease in MMP activity in ECs. Therefore, our data suggest
343 that hormones are essential for maintaining both cellular characteristics and milk component productivity
344 in ECs.

345 Several prior studies have successfully established mammary gland EC models to investigate their
346 functions and milk synthesis [17,18,38]. Nonetheless, these studies have predominantly focused on
347 assessing the cellular characteristics of ECs during their early passages. Consequently, our research holds
348 significance in elucidating the impact of hormones and the factors contributing to cellular instability as we

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349 are aiming to establish and maintain cellular stability across successive passages. Additionally, among the
350 array of hormones, while PRL plays a pivotal role in determining cellular stability in ECs [39], its
351 application is constrained by the prohibitive cost of recombinant PRL [40,41]. Consequently, there is a need
352 for further research into alternative PRL substitutes to facilitate the production of cell-cultured milk
353 components by ensuring the establishment of cellular stability in ECs.

354 In conclusion, our study demonstrated that primary bovine mammary cells in their early passages have
355 high expression in cytoskeleton (CK18, CK19, vimentin, and α-SMA) and milk components (CSN1S1,
356 CSN2, CSN3, ALA, BLG, and triglycerides)-related markers. These results indicate that primary bovine
357 mammary cells have the cellular stability in the structure, function, bioactivity, and bioproduction at early
358 passages. However, these cellular characteristics and functions gradually declined in subsequent passages.
359 Additionally, we observed that primary bovine mammary ECs exhibited decreased milk component
360 production in the absence of hormones, attributed to the damage induced in TJs by MMP. Conversely, the
361 introduction of various hormones into primary bovine mammary ECs resulted in increased milk component
362 productivity and preserved TJs integrity by inhibiting MMP activity. In summary, the establishment of
363 cellular stability in primary bovine mammary cells depends on the coordinated action of diverse hormones.
364 To facilitate the production of cell-cultured milk in cellular agriculture based on animal cells, the
365 optimization of hormone use and exploration of potential PRL substitutes are imperative.

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368 **Acknowledgments**

369 Not applicable.

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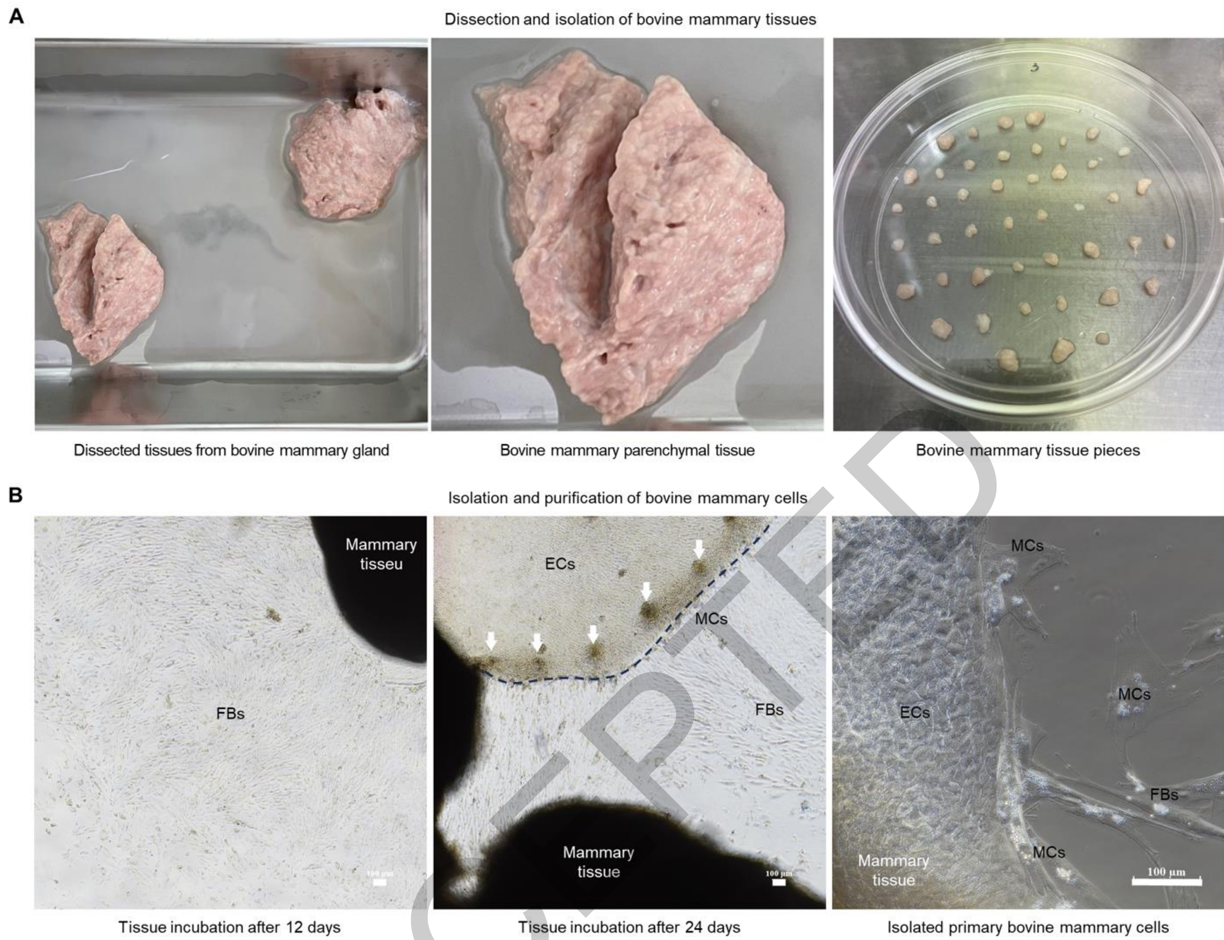
485 # Tables and Figures



486

487 **Fig. 1.** Isolation and purification of primary bovine mammary cells from parenchymal tissues of bovine
488 mammary gland. (A) Dissected parenchymal tissues from bovine mammary gland and attachment of
489 mammary tissues of 1 cm^3 on 10-cm cell culture dish for the incubation. (B) Isolation and purification of
490 primary bovine mammary fibroblasts (FBs), myoepithelial cells (MCs), and epithelial cells (ECs). Dome-
491 like structures are observed in ECs (white arrows). The scale bar indicates 100 µm.

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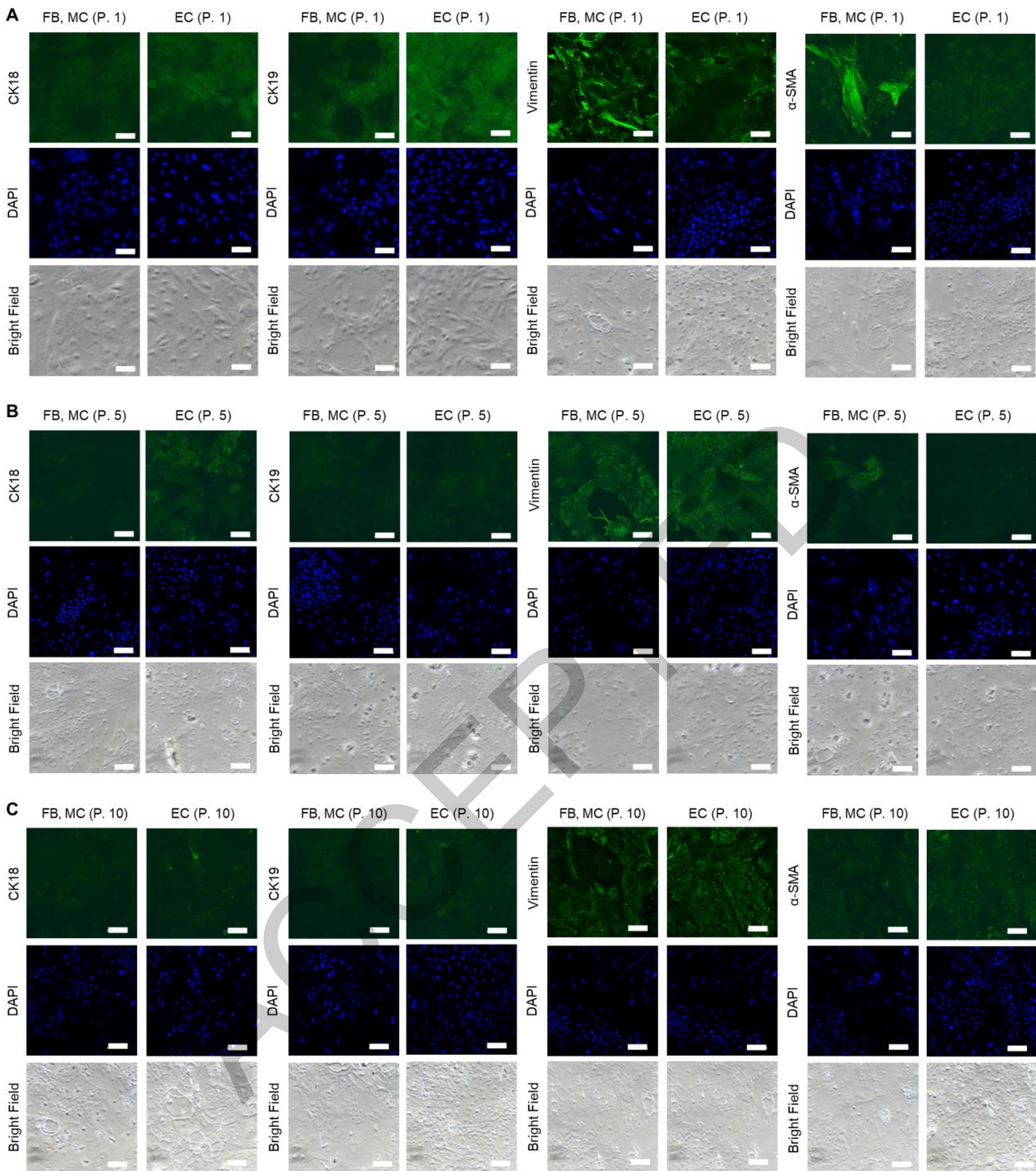

493

494 **Fig. 2.** Fluorescent characteristics of cytoskeleton proteins in primary bovine mammary cells.
495 Immunofluorescence images of cytokeratin 18 (CK18), CK19, vimentin, and α-smooth muscle actin (α-
496 SMA) at passage (A) 1, (B) 5, and (C) 10. The primary bovine mammary fibroblasts (FBs), myoepithelial
497 cells (MCs), and epithelial cells (ECs) were seeded at a density of 0.05×10^6 in 12-well cell culture plate

498 and cultured with control culture media for 7 d (n = 3 wells per group). The scale bar indicates 100 μm.

499 Representative images are selected from three independent replicates.

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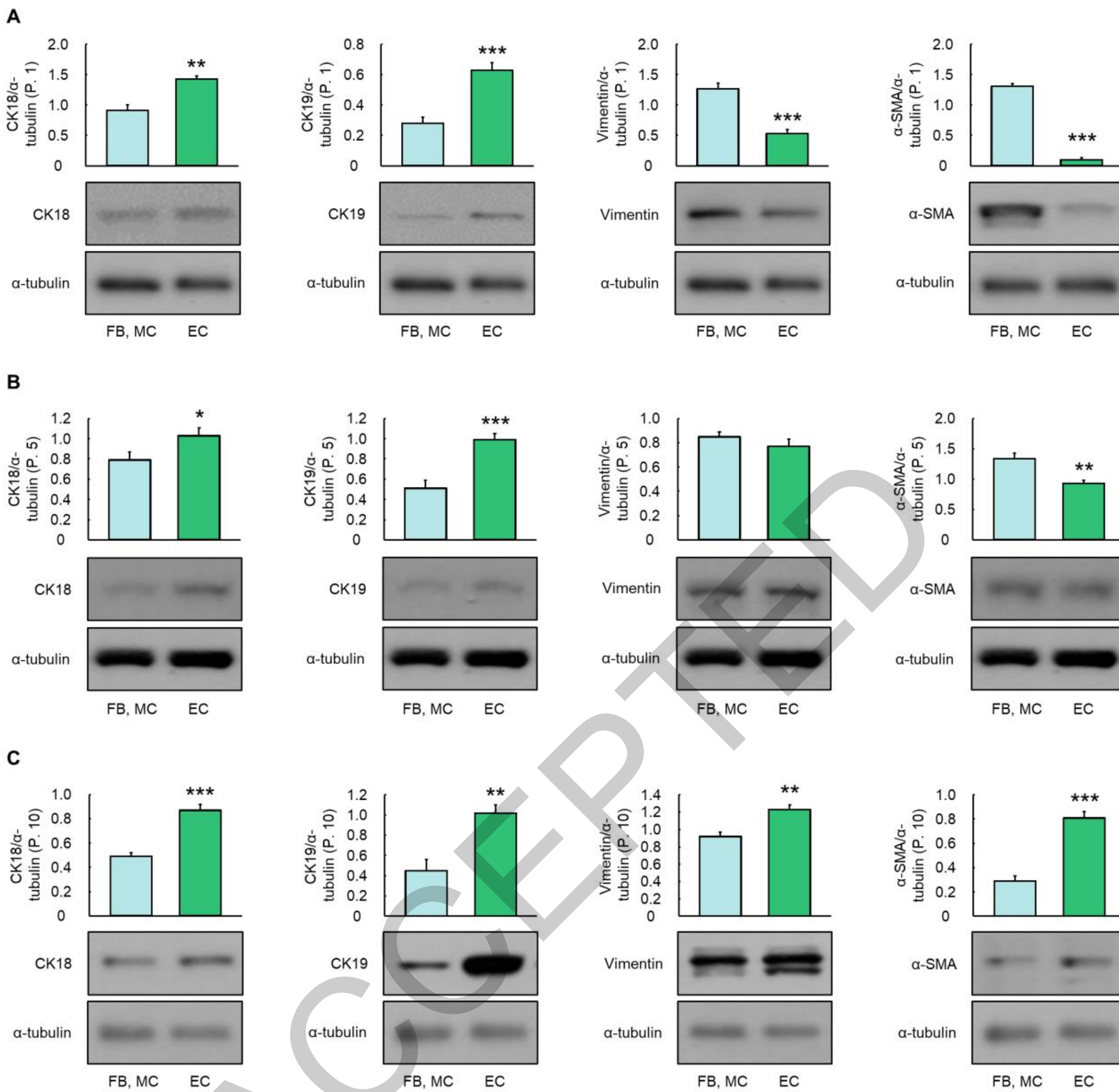

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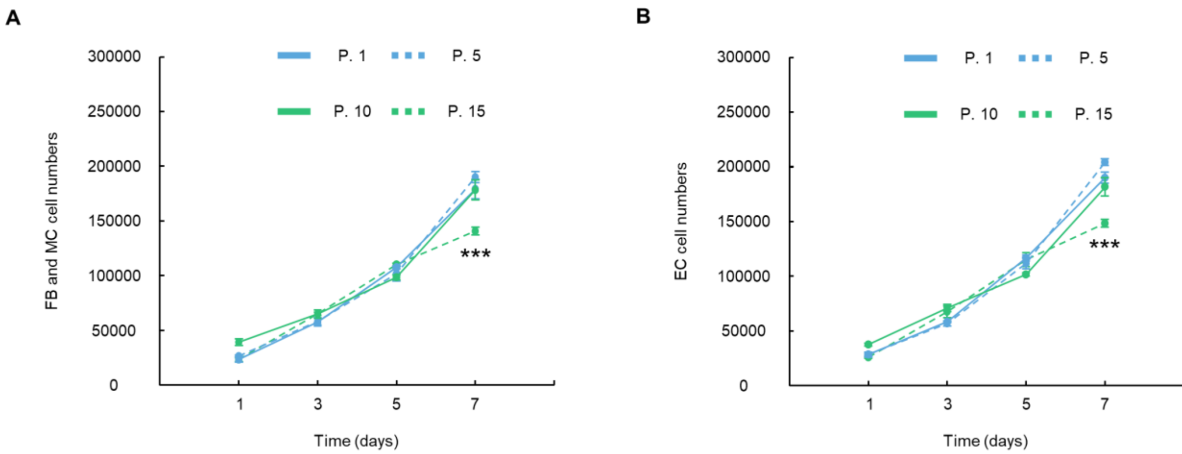
Fig. 3. Protein characteristics of cytoskeleton in primary bovine mammary cells. Protein expression levels of cytokeratin 18 (CK18), CK19, vimentin, and α-smooth muscle actin (α-SMA) at passage (A) 1, (B) 5, and (C) 10. The primary bovine mammary fibroblasts (FBs), myoepithelial cells (MCs), and epithelial cells (ECs) were seeded at a density of 0.1×10^6 in 6-well cell culture plate and cultured with control culture media for 7 d ($n = 5$ wells per group). α-Tubulin was used as housekeeping protein. Representative images are selected from five independent replicates. The data are presented as mean ± SEM. Statistical significances were expressed as * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$) compared to FBs and MCs.



509

510 **Fig. 4.** Growth characteristics of primary bovine mammary cells. (A) Cell numbers of primary bovine
511 mammary fibroblasts (FBs) and myoepithelial cells (MCs). (B) Cell numbers of primary bovine mammary
512 epithelial cells (ECs). FBs, MCs, and EC at passage 1 (blue solid line), 5 (blue dot line), 10 (green solid
513 line), and 15 (green dot line) were seeded at a density of 0.05×10^6 in 12-well cell culture plates and cultured
514 with control culture media for 7 d ($n = 3$ wells per group). The data are presented as mean ± SEM. Statistical
515 significances were expressed as *** ($p < 0.001$) compared to passage 1.

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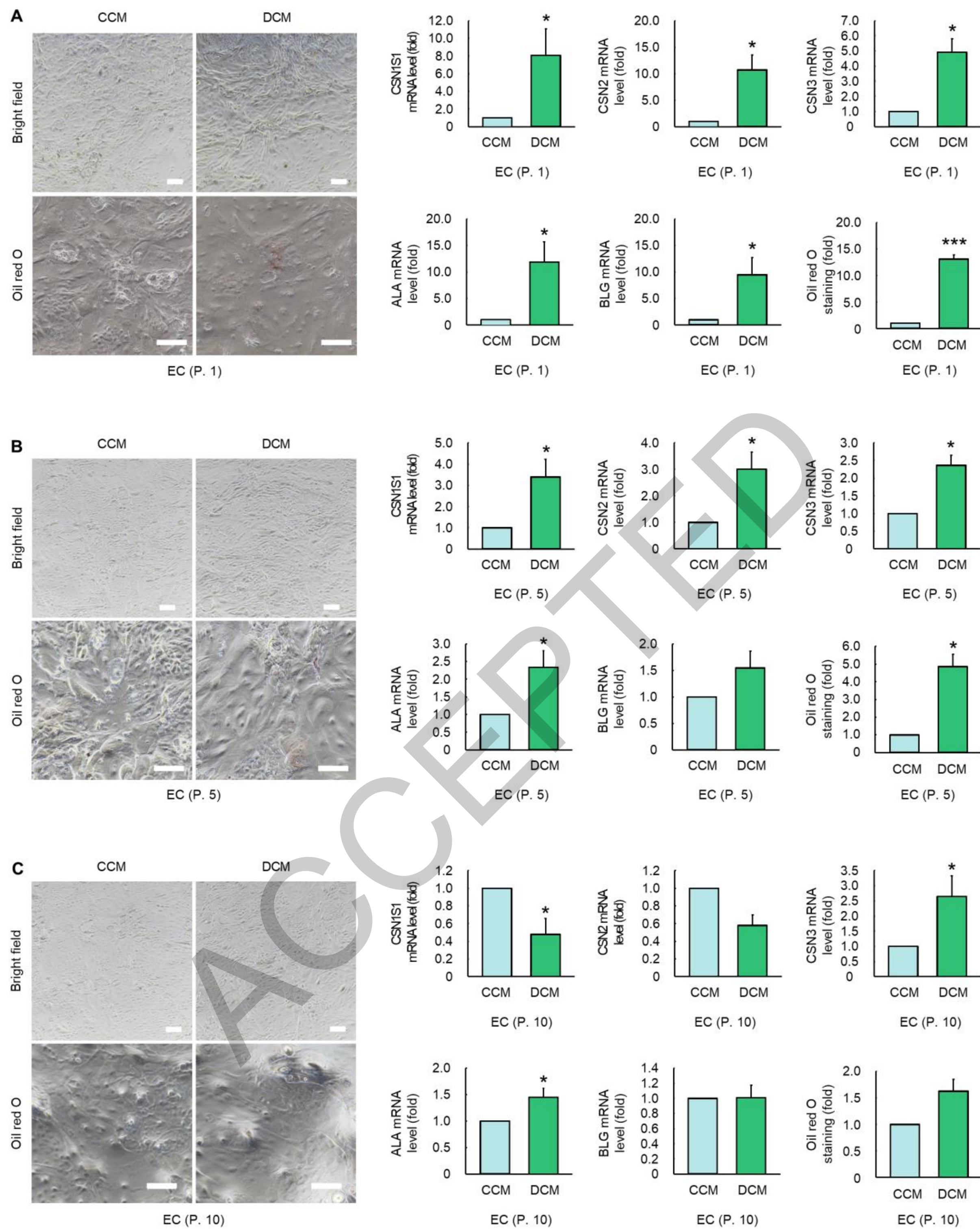

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Fig. 5. Milk components productivity in primary bovine mammary epithelial cells cultured with differentiation culture media. Microscopy images, gene expression levels (CSN1S1, αS1-casein; CSN2, β-casein; CSN3, κ-casein; ALA, α-lactalbumin; BLG, β-lactoglobulin), and oil red O staining area levels at

521 passage (A) 1, (B) 5, and (C) 10. The primary bovine mammary epithelial cells (ECs) were seeded at a

522 density of 0.05×10^6 in 12-well cell culture plate and cultured with control culture media (CCM) and

523 differentiation culture media (DCM) for 5 d ($n = 4 – 5$ wells per group). The scale bar indicates 100 μm.

524 Representative images are selected from three independent replicates. The data are presented as mean ±

525 SEM. Statistical significances were expressed as * ($p < 0.05$) and *** ($p < 0.001$) compared to CCM.

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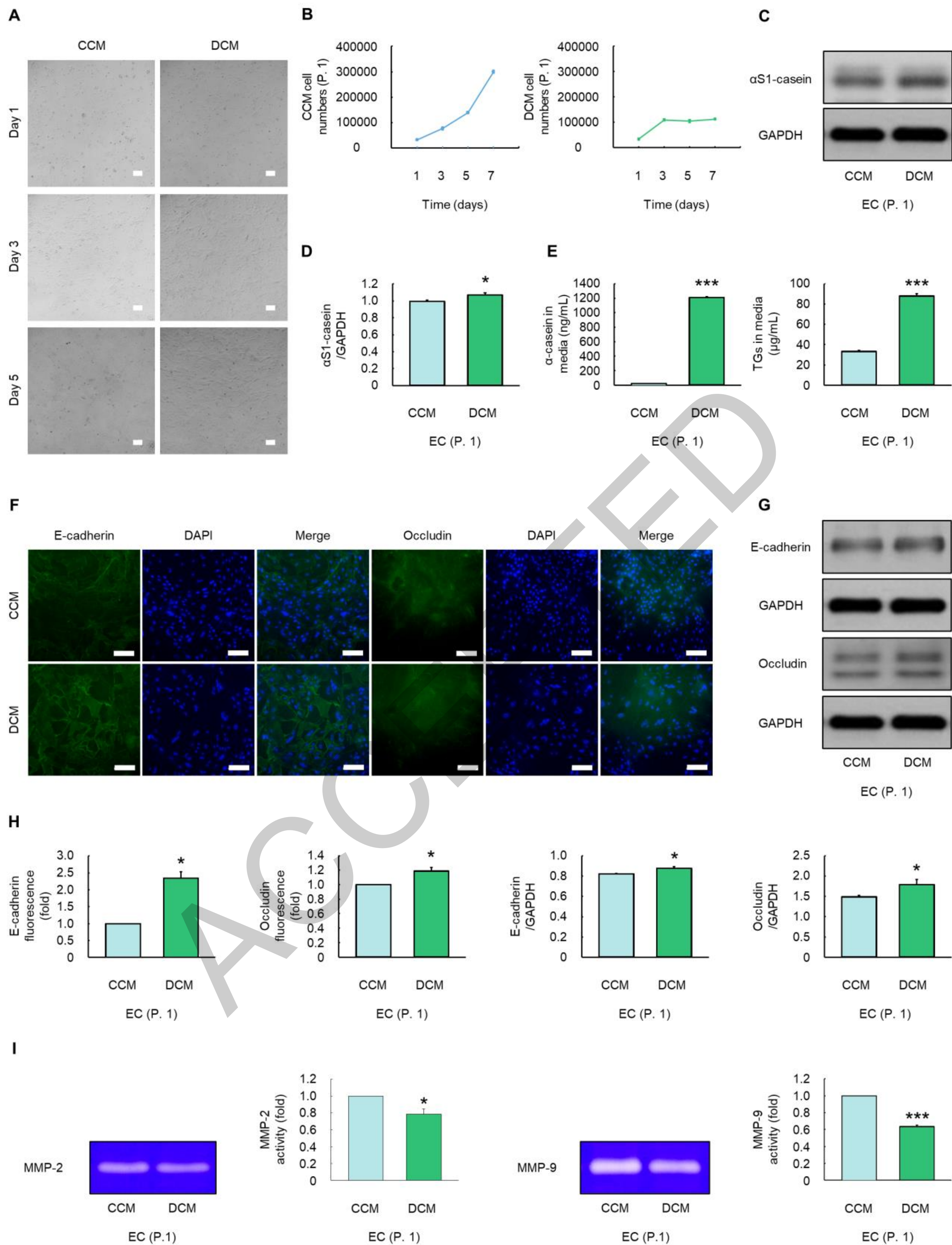

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527

528 **Fig. 6.** Milk components productivity and tight junction integrity in primary bovine mammary epithelial

529 cells cultured with control culture media and differentiation culture media. (A) Microscopy images (B) cell

530 numbers, (C) protein expression of αS1-casein, and (D) protein quantification level of αS1-casein. (E) α-
531 Casein and triglycerides (TGs) contents in media. (F) Green immunofluorescence, 4,6-diamidino-2-
532 phenylindole dihydrochloride (DAPI), (G) protein expression, and (H) quantification levels of E-cadherin
533 and occludin. (I) MMP-2 and -9 activity in media. The primary bovine mammary epithelial cells (ECs)
534 were seeded at a density of 0.05×10^6 in a 12-well cell culture plate and cultured with control culture media
535 (CCM) and differentiation culture media (DCM) for 5 – 7 d ($n = 3 – 5$ wells per group). Glyceraldehyde-
536 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping protein. The scale bar indicates 100 μm.
537 Representative images are selected from three independent replicates. The data are presented as mean ±
538 SEM. Statistical significances were expressed as * ($p < 0.05$) and *** ($p < 0.001$) compared to CCM.
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