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ARTICLE INFORMATION	
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8 **Optimization of culture conditions for maintaining pig muscle stem cells *in vitro***

9

10 **Abstract**

11 Muscle stem cells isolated from domestic animals, including cows and pigs, were
12 recently spotlighted as candidates for the production of alternative protein resources, so-called
13 cultured meat or lab-grown meat. In the present study, we aimed to optimize the *in vitro* culture
14 conditions for the long-term expansion of pig muscle stem cells via the screening of various
15 signaling molecules. Pig muscle stem cells were collected from the *biceps femoris* muscles of
16 3-d-old crossbred pigs (LYD; Landrace × Yorkshire × Duroc) and cultured in minimum
17 essential medium (MEM)-based growth media. However, the pig muscle stem cells gradually
18 lost their proliferation ability and featured morphologies during the long-term culture over two
19 weeks. To find suitable *in vitro* culture conditions for an extended period, skeletal muscle
20 growth medium-2 (SkGM-2), including epidermal growth factor (EGF), dexamethasone, and a
21 p38 inhibitor (SB203580), was used to support the stemness of the pig muscle stem cells.
22 Interestingly, pig muscle stem cells were stably maintained in a long-term culture without loss
23 of the expression of myogenic marker genes as determined by PCR analysis. Immunostaining
24 analysis showed that the stem cells were capable of myogenic differentiation after multiple
25 passaging. Therefore, we found that basal culture conditions containing EGF, dexamethasone,
26 and a p38 inhibitor were suitable for maintaining pig muscle stem cells during expanded culture
27 *in vitro*. This culture method may be applied for the production of cultured meat and further
28 basic research on muscle development in the pig.

29

30 **Keywords**: pig, muscle stem cells, EGF, dexamethasone, p38 inhibitor

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Introduction

The muscle stem cells that are responsible for the regeneration of muscle tissues include quiescent satellite cells and its progeny, such as activated satellite cells, the so-called myoblasts (Motohashi et al., 2012). Quiescent satellite cells reside beneath the basal lamina of myofibers, upon injury, they are activated into proliferating myoblasts via growth factor stimulation and differentiation forward to myocytes and myotubes. The quiescent satellite cells highly express Pax7, but not Myf5 or MyoD (Kuang et al., 2007). Stimulations, such as muscle injury, induce myogenic commitment via the activation of Myf5 and MyoD and inactivation of Pax7, which results in the activation of satellite cells into proliferating myoblasts. With the downregulation of Pax7, MyoG is subsequently expressed in myogenic precursors and completes the terminal differentiation (Jin et al., 2016). Muscle stem cells are generally cultured as proliferating myoblasts because the satellite cells cannot be maintained *in vitro* without losing their properties (Gilbert et al., 2010; Montarras et al., 2005). The *in vitro* culture of stem cells is accomplished by mimicking the *in vivo* cellular niche (Choi and Lee, 2019). To trigger proliferation *in vitro*, the isolated satellite cells are cultured with various cytokines and signaling molecules, which are secreted from the surrounding cells, such as stromal and interstitial cells, and muscle stem cells themselves (Yin et al., 2013).

The isolation procedure from muscle tissues is a type of muscle injury that triggers the activation of quiescent satellite cells into proliferating myoblasts within 24 hours via the upregulation of MyoD expression (Zammit et al., 2004). The proliferating myoblasts are most widely cultured with fibroblast growth factor 2 (FGF2)-supplemented media *in vitro*. FGF2 is secreted from stromal cells, such as fibroblasts, and it plays a role in the proliferation of the myoblasts *in vivo* (Groux-Muscatelli et al., 1990; Quinn et al., 1990). For *in vitro* culture, FGF2 treatment has a higher mitogenic effect on myoblasts compared to other growth factors, including insulin, insulin-like growth factors (IGFs), and leukemia inhibitory factor (LIF)

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57 (Gospodarowicz et al., 1976; Rando and Blau, 1994). IGFs and inflammatory cytokines also
58 enhance proliferation and suppress myogenic differentiation during muscle stem cell culture.
59 IGF-1 and IGF-2 play pivotal roles in the expansion of muscle stem cells *in vivo* and *in vitro*
60 (Florini et al., 1984; Jennische et al., 1987). Pro-inflammatory cytokines produced from the
61 infiltrated immune cells into the damaged area of muscle, such as interleukins (ILs), tumor
62 necrosis factor (TNFs), and interferons (IFNs), support the maintenance of the stemness of
63 muscle stem cells *in vitro* for an extended period (Fu et al., 2015). Several molecules that
64 regulate the signaling pathways involved in the stemness and aging of muscle stem cells were
65 also used, such as inhibitors of p38, Wnts, and dexamethasone (Bentzinger et al., 2013; Ding
66 et al., 2018; Dodson et al., 1985).

67 Because the pig is a valuable candidate as a preclinical model of human cell therapy
68 and an important food source, an understanding of the physiology of pig myogenic progenitors,
69 such as skeletal muscle satellite cells and myoblasts, is required to cure muscular diseases and
70 improve meat production. Muscle stem cells recently isolated from domestic animals, including
71 cows and pigs, were spotlighted as candidates for the production of alternative protein resources,
72 so-called cultured meat or lab-grown meat. Cultured meat is an artificial meat that imitates fresh
73 meat using the *in vitro* production of muscle tissue, which would solve the problems derived
74 from traditional meat production, including animal welfare and environmental pollution (Post,
75 2012). Various researchers used fetal bovine serum (FBS)-containing minimum essential
76 medium (MEM) to culture pig muscle stem cells for a long time (Doumit and Merkel, 1992;
77 Miersch et al., 2018). However, FBS-containing MEM-based culture conditions do not support
78 the stemness of pig muscle stem cells for an extended period. Therefore, the present study
79 optimized the *in vitro* culture conditions for the long-term expansion of pig muscle stem cells
80 via the screening of various signaling molecules.

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82 **Materials and Methods**

83 **Animal care**

84 The Institutional Animal Care and Use Committee (IACUC) at Seoul National
85 University approved the care and experimental use of pigs (approval no.: SNU-180612-2). The
86 experiments were performed according to the standard protocol of the Institute of Laboratory
87 Animal Resources at Seoul National University.

88

89 **Isolation and culture of pig muscle stem cells**

90 Pig muscle stem cells were isolated from the *biceps femoris* muscle of 3-d-old
91 crossbred pigs (LYD; Landrace × Yorkshire × Duroc), which were euthanized via CO_2
92 inhalation and exsanguination. The *biceps femoris* muscles were collected and washed with
93 Dulbecco's phosphate-buffered saline (DPBS; Welgene, Gyeongsan, Korea) containing 2×
94 antibiotic–antimycotic (AA; Gibco, Gaithersburg, USA), and excessive connective tissues and
95 blood vessels were removed. The collected tissues were minced in a meat grinder and digested
96 using 0.8 mg/mL Pronase (Sigma-Aldrich, St. Louis, USA) for 40 min at 37°C with vortexing
97 every 10 min. The resultant was harvested via centrifugation at 1200×g for 15 min, and the
98 pellets were resuspended in MEM containing 10% fetal bovine serum (FBS; Gibco). For
99 separation of undigested tissues from the digested cells containing the muscle stem cell
100 population, the digested muscle tissues were centrifuged at 300×g for 5 min, and the supernatant
101 was collected. The supernatant was filtered through a 100-μm cell strainer and harvested via
102 centrifugation at 1200×g for 15 min. The resulting cells were cultured in the basic growth media
103 (GM) described below or cryopreserved in GM containing 10% dimethyl sulfoxide (DMSO)
104 until used.

105 The isolated muscle stem cells were cultured on gelatin-coated dishes in basic GM,

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106 which consisted of MEM containing 10% (v/v) FBS, 1× glutamax, 1× AA, and 0.1 mM β-
107 mercaptoethanol (All from Gibco) or Skeletal Muscle Cell Growth Medium-2 BulletKit™
108 (SkGM-2; Lonza, Basel, Switzerland) supplemented with 20 μM SB203580 (Cayman
109 Chemical, Ann Arbor, USA) according to manufacturer's instructions. Pig muscle stem cells
110 were subcultured every 3 d. When the cells reached approximately 90% confluence, the
111 cultured cells were dissociated using TrypLE™ Express (Gibco). The dissociated cells were
112 transferred onto new gelatin-coated culture dishes at a 1:10 split ratio. The medium was
113 changed every 24 h, and the cells were cultured under humidified conditions in an atmosphere
114 containing 5% CO_2 at 37°C.

115

116 **Myogenic differentiation of pig muscle stem cells**

117 After three days of subculture, the muscle stem cells at confluence were used for
118 myogenic differentiation. The cells were cultured in a differentiation media consisting of MEM
119 containing 2% (v/v) horse serum (Biowest, Nuaillé, France), 1× glutamax, 1× AA, and 0.1 mM
120 β-mercaptoethanol for 2 d without media changes. After myofiber formation from muscle stem
121 cells, the cells were fixed with 4% paraformaldehyde for further analysis.

122

123 **Quantitative real-time polymerase chain reaction (qPCR)**

124 Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, USA), and cDNA
125 was synthesized using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City,
126 USA). cDNA was amplified using a DyNAmo HS SYBR Green qPCR Kit (Thermo Fisher
127 Scientific, Waltham, USA) containing 1–2 pmol of each primer set listed below in a 10 μL
128 reaction volume: 5'-GTGCCCTCAGTGAGTTCGAT-3' (forward) and 5'-
129 TCCAGACGGTTCCCTTTGTC-3' (reverse) for *PAX7*; 5'-CTGCCCAAGGTGGAAATCCT-

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130 3' (forward) and 5'-GGGGGCCGCTATAATCCATC-3' (reverse) for *MYOD1*; 5'-
131 AGTTCGGGGACGAGTTTGAG-3' (forward) and 5'-TCAAACGCCTGGTTGACCTT-3'
132 (reverse) for *MYF5*; 5'-GAGCTGTATGAGACATCCCCC-3' (forward) and 5'-
133 GTGGACGGGCAGGTAGTTTT-3' (reverse) for *MYOG*; 5'-TGCTCCTCCCCGTTCGAC-3'
134 (forward) and 5'-ATGCGGCCAAATCCGTTC-3' (reverse) for *GAPDH*. Amplification and
135 detection were performed using the ABI 7300 Real-Time PCR System (Applied Biosystems)
136 under the following conditions: one cycle at 50°C for 2 min and 95°C for 10 min, followed by
137 40 cycles of denaturation at 95°C for 15 s and annealing/extension for 1 min (60°C for *MYF5*,
138 *MYOD1*, *MYOG*, and *GAPDH*; 58°C for *PAX7*). The dissociation curves were analyzed, and
139 the amplified products were loaded onto gels to confirm the specificity of the PCR products.
140 Relative expression levels were calculated by normalizing the threshold cycle (Ct) values of
141 each gene to the reference gene, *GAPDH*, using the delta-delta Ct method.

142

143 **Immunocytochemistry**

144 Cell samples were preincubated for 10 min at 4°C and fixed in 4% (w/v)
145 paraformaldehyde for 30 min. After washing twice with DPBS (Welgene), the samples were
146 treated for 15 min with 0.2% (v/v) Triton X-100 (Sigma-Aldrich) and blocked for 1 h with 10%
147 (v/v) goat serum in DPBS to prevent nonspecific binding. Serum-treated cells were incubated
148 overnight at 4°C with primary antibodies against the myosin heavy chain (1:200; 05-716,
149 Sigma-Aldrich). After incubation with the primary antibody, the cells were treated overnight at
150 4°C with the appropriate Alexa Fluor-conjugated secondary antibodies. Nuclei were stained
151 with Hoechst 33342 (Molecular Probes, Eugene, USA). Images of stained cells were captured
152 using an inverted fluorescence microscope (Eclipse TE2000-U, Nikon, Konan, Japan).

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Statistical analysis

The data obtained in this study are presented as means ± standard error of the mean (SEM) and were analyzed using Prism 6 software (GraphPad Software, San Diego, USA). The significance of differences was determined using two-way analyses of variance followed by Fisher's least significant difference test. Differences were considered significant at $p<0.05$ ($^*p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ in Figures).

Results and Discussion

The total number of muscle stem cells per muscle mass having the same weight decreases during postnatal development (Campion et al., 1981), and the proliferation ability of the muscle stem cells is reduced with aging (Chakravarthy et al., 2000). When we compared muscle stem cells from 3-d-old and 25-d-old pigs, the cells from 3-d-old pigs were more numerous and proliferative than cells from the 25-d-old pigs (data not shown). Based on this preliminary observation, the cells were collected from *biceps femoris* muscles of 3-d-old LYD pigs to establish the *in vitro* culture conditions to maintain pig muscle stem cells. Because the muscle tissues digested by Pronase contained connective tissues, muscle fibers and quiescent satellite cells, the stem cell population was separated from tissue debris via differential centrifugation and filtration. The isolated muscle stem cells were cultured in MEM-based growth media. Various studies widely used FBS-containing MEM to culture pig muscle stem cells *in vitro* (Jeong et al., 2013; Miersch et al., 2018). Doumit et al. showed that pig muscle stem cells had a higher proliferation rate in MEM-based media compared to other media, including McCoy's 5A, Ham's F12, DMEM, and DMEM/F12. The high concentration of FBS, which contains several unknown growth factors, supports the proliferation rate and myogenic potential of the pig stem cells during *in vitro* culture (Doumit and Merkel, 1992). However, pig

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178 muscle stem cells gradually lose their proliferation ability and featured morphology during
179 long-term culture over two weeks (Fig. 1A). Quantitative PCR analysis showed that the marker
180 genes for satellite cells and myoblasts (*PAX7*, *MYF5*, and *MYOD1*)(Kuang et al., 2007) and
181 myogenesis-related gene (*MYOG*)(Cao et al., 2006) were dramatically decreased after
182 passaging (Fig. 1B), which indicates that FBS-containing MEM cannot support the stemness of
183 pig muscle stem cells for an extended period *in vitro*.

184 To find suitable *in vitro* culture conditions for pig muscle stem cells, we first tested the
185 commercially available media skeletal muscle growth medium-2 (SkGM-2), which includes
186 epidermal growth factor (EGF) and dexamethasone, for human myoblasts. EGF and
187 dexamethasone enhance the proliferation and differentiation capacity of myoblasts *in vitro* (Roe
188 et al., 1995; Syverud et al., 2016). The proliferation rate of pig muscle stem cells cultured in
189 SkGM-2 was highly increased compared to MEM-based growth media (Fig. 2A), and the cells
190 were maintained for an extended period (Fig. 2B). Although the myogenic cell marker genes
191 were significantly increased in the SkGM-2-treated group as measured using qPCR analysis,
192 the genes gradually declined with passaging (Fig. 2C). We next applied SkGM-2 as a basal
193 medium and examined the effect of the p38 inhibitor SB203580 on maintaining the
194 undifferentiated state of pig muscle stem cells. Activation of the p38 signaling pathway inhibits
195 proliferation and causes myogenic differentiation in muscle stem cells (Bernet et al., 2014; Troy
196 et al., 2012). Treatment with SB203580 prevents the downregulation of *PAX7* and maintains the
197 stemness of muscle stem cells *in vitro* (Ding et al., 2018). Although the number of cells was
198 slightly increased, no significant differences were observed between the SB203580 treatment
199 and control groups in morphological features (Fig. 2D). Notably, the myogenic marker genes
200 were significantly upregulated and constantly expressed during long-term culture in the
201 SB203580-treated group (Fig. 2E). The cells cultured in media supplemented with SB203580
202 were capable of myogenic differentiation after multiple passages, as determined by the

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203 immunostaining of myosin heavy chain (Fig. 3). We verified that these culture conditions also
204 maintained muscle stem cells from other breeds, including Berkshire and Korean traditional pig
205 (data not shown).

206 EGF is a well-known mitogen that stimulates the proliferation of pig muscle stem cells
207 cultured in serum-free media (Doumit et al., 1993). EGF enhances the nutrient uptake and
208 protein synthesis of ovine muscle stem cells (Roe et al., 1995). The p38 signaling pathway is
209 involved in the differentiation and aging of muscle stem cells *in vivo*. Muscle injury upregulates
210 the p38 pathway, which leads to the differentiation of quiescent satellite cells (Troy et al., 2012).
211 The p38 pathway was upregulated in the satellite cells of aged mice, which suppressed the
212 proliferation of satellite cells (Bernet et al., 2014). Dexamethasone is a synthetic glucocorticoid
213 that enhanced the proliferation ability of muscle stem cells via regulation of catabolism
214 (Guerriero and Florini, 1980). Dexamethasone also increased the mitogenic effect of growth
215 factors, such as IGF-1 and IGF-2, on satellite cells (Dodson et al., 1985) and promoted
216 myogenic maturation (Syverud et al., 2016). Similarly, our results demonstrated that these
217 signaling pathways also played pivotal roles in maintaining the stemness of pig muscle stem
218 cells. Accordingly, we found that basal culture conditions containing EGF, dexamethasone, and
219 a p38 inhibitor were suitable for maintaining pig muscle stem cells during an expanded culture
220 *in vitro*.

221

222 **Conflict of interest**

223 No potential conflicts of interest relevant to this article are reported.

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233

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315 **Figure legends**

316 **Fig. 1. Pig muscle stem cells cultured in 10% fetal bovine serum (FBS)-containing**
317 **minimum essential medium (MEM)**

318 Muscle stem cells isolated from the *biceps femoris* muscle of 3-d-old LYD pigs were cultured
319 with 10% FBS-supplemented MEM. (A) The morphological changes of the pig muscle stem
320 cells during the *in vitro* long-term culture. Scale bar = 400 μm. (B) The expression pattern of
321 myogenic marker genes during the *in vitro* long-term culture as measured using qPCR.

322

323 **Fig. 2. Optimizing culture conditions for supporting the stemness of pig muscle stem cells**
324 ***in vitro***

325 To find suitable *in vitro* culture conditions for pig muscle stem cells, various culture components
326 were tested. (A) Comparative analysis of the proliferation rate of pig muscle stem cells cultured
327 with 10% FBS-supplemented MEM and SkGM-2. (B) The effect of basal media on pig muscle
328 stem cells during the *in vitro* long-term culture. (C) The effect of basal media on the expression
329 of myogenic marker genes during the *in vitro* long-term culture using qPCR. (D) The effect of
330 SB203580 on pig muscle stem cells during the *in vitro* long-term culture. (E) The effect of
331 SB203580 on the expression of myogenic marker genes during the *in vitro* long-term culture
332 using qPCR. A, B and D: Scale bar = 400 μm; C and E: passage is abbreviated to 'P'. The
333 significance of differences was determined between the control and treated groups.

334

335 **Fig. 3. The myogenic potential of pig muscle stem cells cultured in SkGM-2-supplemented**
336 **SB203580**

337 The myogenic ability of pig muscle stem cells cultured in SkGM-2-supplemented SB203580

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338 was examined and defined using immunostaining of myosin heavy chain (MHC). Red and green

339 fluorescence represent nuclei and myosin heavy chain, respectively. Scale bar = 400 μm.

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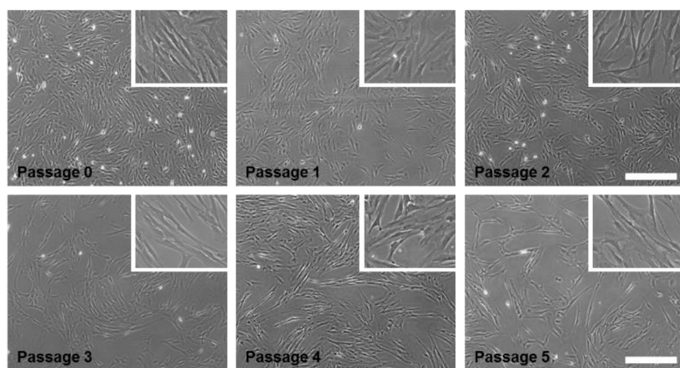
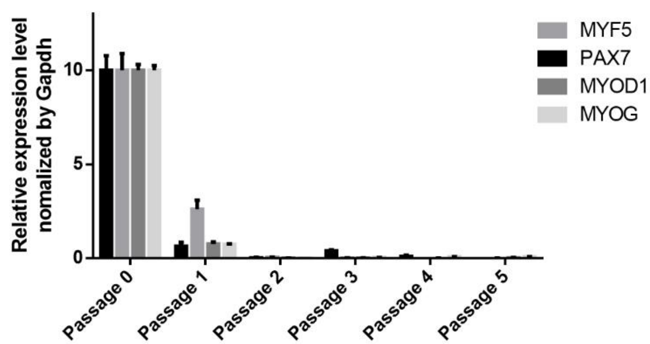
340 **Fig. 1**





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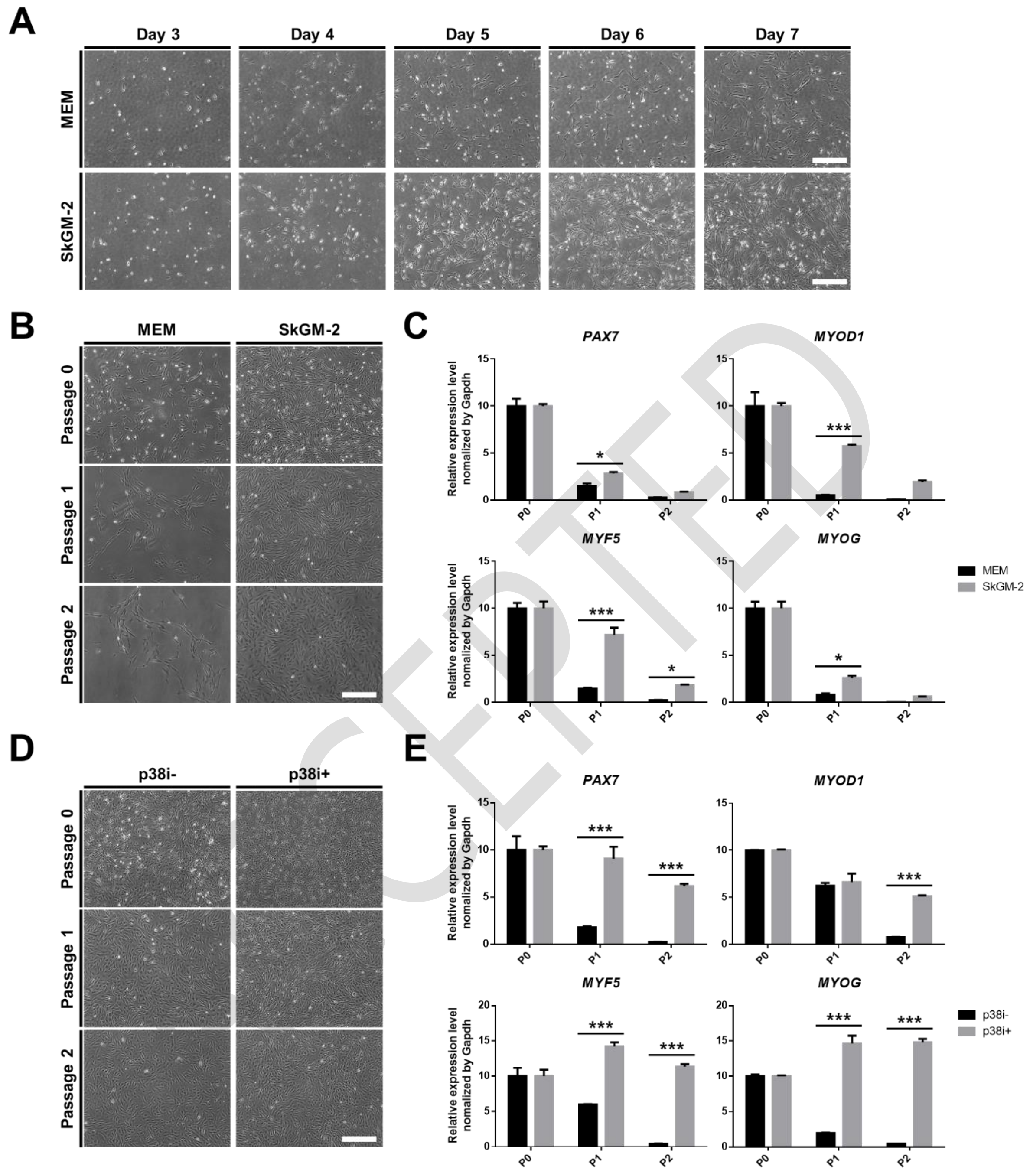
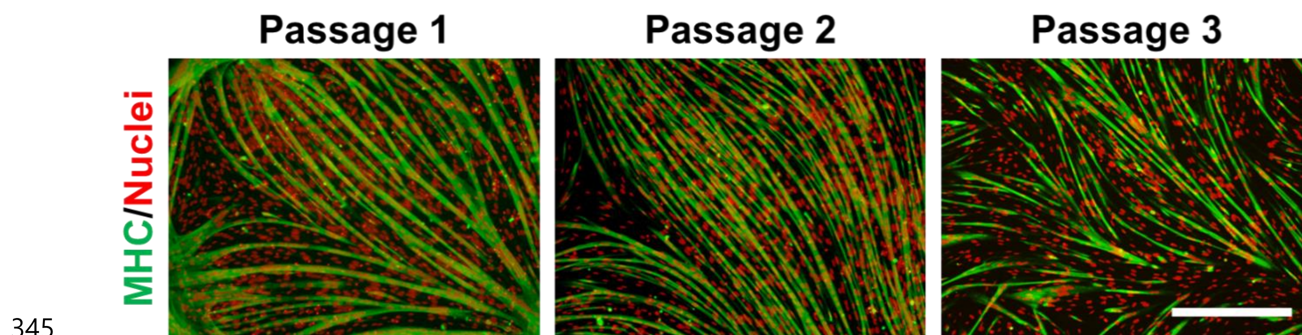
342 **Fig. 2**



343

344 **Fig. 3**



345

ACCEPTED