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# Optimization of culture conditions for maintaining pig muscle stem cells *in vitro*


## Abstract

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



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

## 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)

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

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

## Materials and Methods

### Animal care

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

### Isolation and culture of pig muscle stem cells

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

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

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

**Myogenic differentiation of pig muscle stem cells**

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

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

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

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

**Immunocytochemistry**

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

## 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

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

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

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

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



## Conflict of interest

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

## Acknowledgments

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## References

Bentzinger CF, Wang YX, Von Maltzahn J, Soleimani VD, Yin H, Rudnicki MA. 2013. Fibronectin regulates wnt7a signaling and satellite cell expansion. Cell Stem Cell 12:75-87.

Bernet JD, Doles JD, Hall JK, Kelly Tanaka K, Carter TA, Olwin BB. 2014. P38 mapk signaling underlies a cell-autonomous loss of stem cell self-renewal in skeletal muscle of aged mice. Nat Med 20:265-271.

Campion DR, Richardson RL, Reagan JO, Kraeling RR. 1981. Changes in the satellite cell population during postnatal growth of pig skeletal muscle. J Anim Sci 52:1014-1018.

Cao Y, Kumar RM, Penn BH, Berkes CA, Kooperberg C, Boyer LA, Young RA, Tapscott SJ. 2006. Global and gene-specific analyses show distinct roles for myod and myog at a common set of promoters. Embo Journal 25:502-511.

Chakravarthy MV, Davis BS, Booth FW. 2000. Igf-i restores satellite cell proliferative potential in immobilized old skeletal muscle. J Appl Physiol (1985) 89:1365-1379.

Choi KH, Lee CK. 2019. Pig pluripotent stem cells as a candidate for biomedical application. Journal of Animal Reproduction and Biotechnology 34:139-147.

Ding SJ, Swennen GNM, Messmer T, Gagliardi M, Molin DGM, Li CB, Zhou GH, Post MJ. 2018. Maintaining bovine satellite cells stemness through p38 pathway. Scientific Reports 8.

Dodson MV, Allen RE, Hossner KL. 1985. Ovine somatomedin, multiplication-stimulating activity, and insulin promote skeletal muscle satellite cell proliferation in vitro. Endocrinology 117:2357-2363.

Doumit ME, Cook DR, Merkel RA. 1993. Fibroblast growth factor, epidermal growth factor, insulin-like growth factors, and platelet-derived growth factor-bb stimulate proliferation of clonally derived porcine myogenic satellite cells. J Cell Physiol 157:326-332.

Doumit ME, Merkel RA. 1992. Conditions for isolation and culture of porcine myogenic satellite cells. Tissue Cell 24:253-262.

Florini JR, Ewton DZ, Evinger-Hodges MJ, Falen SL, Lau RL, Regan JF, Vertel BM. 1984. Stimulation and inhibition of myoblast differentiation by hormones. In Vitro 20:942-958.

Fu X, Xiao J, Wei Y, Li S, Liu Y, Yin J, Sun K, Sun H, Wang H, Zhang Z, Zhang BT, Sheng C, Wang H, Hu P. 2015. Combination of inflammation-related cytokines promotes long-term muscle stem cell expansion. Cell Res 25:655-673.

Gilbert PM, Havenstrite KL, Magnusson KE, Sacco A, Leonardi NA, Kraft P, Nguyen NK, Thrun S, Lutolf MP, Blau HM. 2010. Substrate elasticity regulates skeletal muscle stem cell self-renewal in culture. Science 329:1078-1081.

Gospodarowicz D, Weseman J, Moran JS, Lindstrom J. 1976. Effect of fibroblast growth factor on the division and fusion of bovine myoblasts. J Cell Biol 70:395-405.

Groux-Muscatelli B, Bassaglia Y, Barritault D, Caruelle JP, Gautron J. 1990. Proliferating satellite cells express acidic fibroblast growth factor during in vitro myogenesis. Dev Biol 142:380-385.

Guerriero V, Jr., Florini JR. 1980. Dexamethasone effects on myoblast proliferation and differentiation. Endocrinology 106:1198-1202.

Jennische E, Skottner A, Hansson HA. 1987. Satellite cells express the trophic factor igf-i in regenerating skeletal muscle. Acta Physiol Scand 129:9-15.

Jeong JY, Kim JM, Rajesh RV, Suresh S, Jang GW, Lee KT, Kim TH, Park M, Jeong HJ, Kim KW, Cho YM, Lee HJ. 2013. Comparison of gene expression levels of porcine satellite cells from postnatal muscle tissue during differentiation. Reproductive & developmental biology 37:219-224.

Jin W, Peng J, Jiang S. 2016. The epigenetic regulation of embryonic myogenesis and adult muscle regeneration by histone methylation modification. Biochem Biophys Rep 6:209-219.

Kuang S, Kuroda K, Le Grand F, Rudnicki MA. 2007. Asymmetric self-renewal and commitment of satellite stem cells in muscle. Cell 129:999-1010.

Miersch C, Stange K, Rontgen M. 2018. Separation of functionally divergent muscle precursor cell populations from porcine juvenile muscles by discontinuous percoll density gradient centrifugation. BMC Cell Biol 19:2.

Montarras D, Morgan J, Collins C, Relaix F, Zaffran S, Cumano A, Partridge T, Buckingham M. 2005. Direct isolation of satellite cells for skeletal muscle regeneration. Science 309:2064-2067.

Motohashi N, Alexander MS, Kunkel LM. 2012. Skeletal muscle regeneration and muscle progenitor cells. The Journal of Physical Fitness and Sports Medicine 1:151-154.

Post MJ. 2012. Cultured meat from stem cells: Challenges and prospects. Meat Sci 92:297-301.

Quinn LS, Ong LD, Roeder RA. 1990. Paracrine control of myoblast proliferation and differentiation by fibroblasts. Dev Biol 140:8-19.

Rando TA, Blau HM. 1994. Primary mouse myoblast purification, characterization, and

300 transplantation for cell-mediated gene therapy. J Cell Biol 125:1275-1287.
301 Roe JA, Baba AS, Harper JM, Buttery PJ. 1995. Effects of growth factors and gut regulatory
302 peptides on nutrient uptake in ovine muscle cell cultures. Comp Biochem Physiol A
303 Physiol 110:107-114.
304 Syverud BC, Vandusen KW, Larkin LM. 2016. Effects of dexamethasone on satellite cells and
305 tissue engineered skeletal muscle units. Tissue Eng Part A 22:480-489.
306 Troy A, Cadwallader AB, Fedorov Y, Tyner K, Tanaka KK, Olwin BB. 2012. Coordination of
307 satellite cell activation and self-renewal by par-complex-dependent asymmetric
308 activation of p38alpha/beta mapk. Cell Stem Cell 11:541-553.
309 Yin H, Price F, Rudnicki MA. 2013. Satellite cells and the muscle stem cell niche. Physiol Rev
310 93:23-67.
311 Zammit PS, Golding JP, Nagata Y, Hudon V, Partridge TA, Beauchamp JR. 2004. Muscle
312 satellite cells adopt divergent fates: A mechanism for self-renewal? J Cell Biol 166:347-
313 357.
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## Figure legends

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

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

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

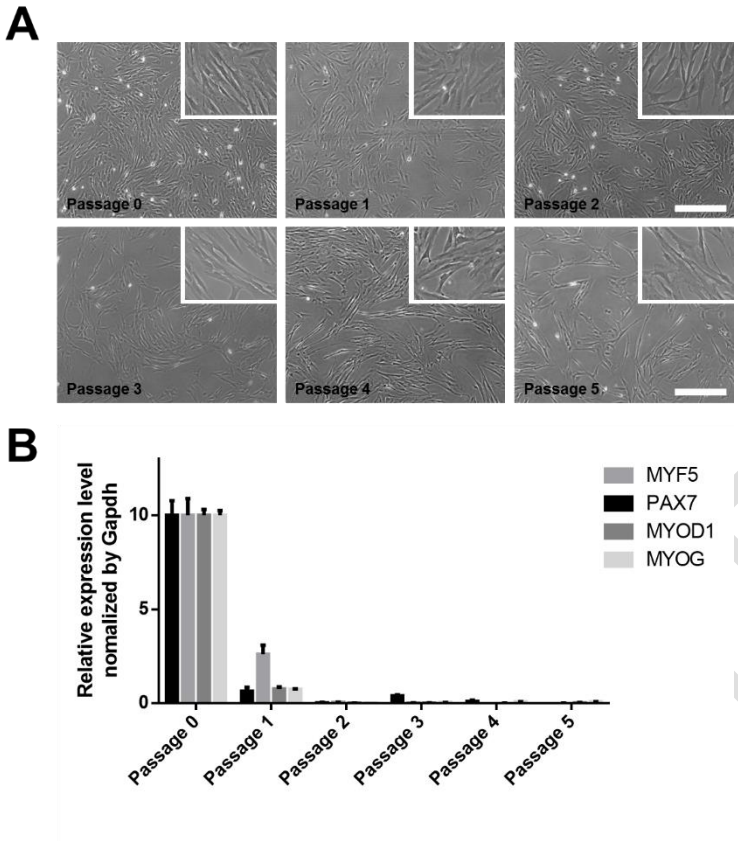
To find suitable *in vitro* culture conditions for pig muscle stem cells, various culture components were tested. (A) Comparative analysis of the proliferation rate of pig muscle stem cells cultured with 10% FBS-supplemented MEM and SkGM-2. (B) The effect of basal media on pig muscle stem cells during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

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

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

was examined and defined using immunostaining of myosin heavy chain (MHC). Red and green fluorescence represent nuclei and myosin heavy chain, respectively. Scale bar = 400 μm.

340 **Fig. 1**

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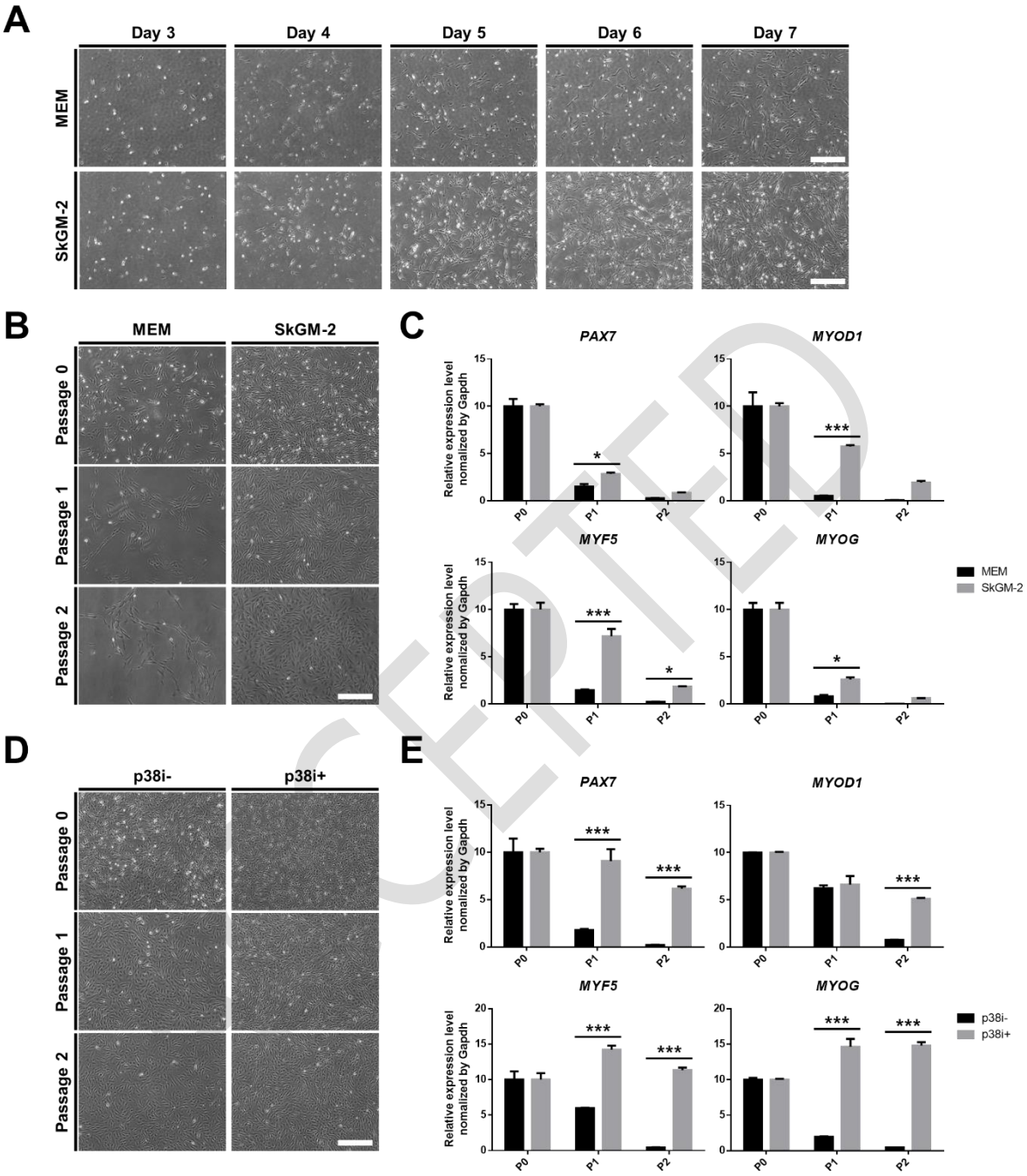
342 **Fig. 2**

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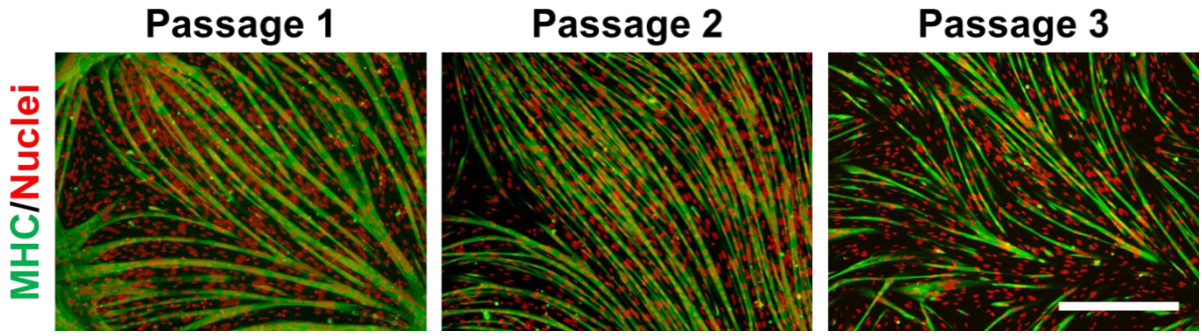
344 **Fig. 3**

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