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Development of a Three-dimensional Hydrogel System for the Maintenance of Porcine Spermatogonial Stem Cell Self-renewal

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ABSTRACT

Porcine spermatogonial stem cells (SSCs) prefer three-dimensional (3D) culture systems to 2D ones for the maintenance of self-renewal. Of the many 3D culture systems, agar-based hydrogels are candidates for supporting porcine SSC self-renewal, and there are various types of agar powder that can be used. In this study, we sought to identify an agar-based 3D hydrogel system that exhibited strong efficacy in the maintenance of porcine SSC self-renewal. First, 3D hydrogels with different mechanics were prepared with various concentrations of Bacto agar, lysogeny broth (LB) agar, and agarose powder, and the 3D hydrogel with the strongest alkaline phosphatase (AP) activity and greatest increase in colony size was identified for the different types of agar powder. Second, among the porcine SSCs cultured in the different 3D hydrogels, we analyzed the colony formation, morphology, and size; AP activity; and transcription and translation of porcine SSC-related genes, and these were compared to determine the optimal 3D hydrogel system for the maintenance of porcine SSC self-renewal. We found that 0.6% (w/v) Bacto agar-, 1% (w/v) LB agar-, and 0.2% (w/v) agarose-based 3D hydrogels showed the strongest maintenance of AP activity and the most pronounced increase in colony size in the culture of porcine SSCs. Moreover, among these hydrogels, the strongest transcription and translation of porcine SSC-related genes and largest colony size were detected in porcine SSCs cultured in the 0.2% (w/v) agarose-based 3D hydrogel, whereas there were no significant differences in colony formation and morphology. These results demonstrate that the 0.2% (w/v) agarose-based 3D hydrogel can be effectively used for the maintenance of porcine SSC self-renewal.

(Key Words : Agar-based hydrogel, Pig, Self-renewal, Spermatogonial stem cells, Three dimension)

INTRODUCTION

Spermatogonial stem cells (SSCs), which play a pivotal role as the foundation of spermatogenesis throughout life (Zohni et al., 2012), undergo infinite self-renewal in the basement membrane of seminiferous tubules and differentiate into spermatozoa via spermatogenesis in the adluminal compartment of seminiferous tubules post-puberty (Waheeb and Hofmann, 2011). Therefore, they can be effectively used for transferring useful genetic information to the next generation (Yeh et al., 2007) and for developing male infertility treatments (Kanatsu-Shinohara et al., 2003). Development of an understanding of the acquisition of the pluripotency of SSCs, which results from the induction of specific external or internal stimulations (Park et al., 2016), will make the development of techniques related to patient-specific cell-based therapies in males possible (Rao and Condic, 2008).

In spite of their usefulness, no two-dimensional (2D) culture systems stimulating proliferation or inhibiting differentiation of SSCs for the long term have been developed, regardless of species. Moreover, self-renewal of SSCs derived from pigs has been difficult to maintain *in vitro* even over the short term (Marret and Durand, 2000). However, a recent report demonstrated that agarose-based 3D hydrogels could maintain porcine SSC self-renewal (Park et al., 2017), indicating that agar-based 3D

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hydrogels could be used to establish a porcine SSC culture system.

Generally, agar-based 3D hydrogels can be constructed using Bacto agar, lysogeny broth (LB) agar, and agarose, which form 3D hydrogels with different mechanical properties according to the type or concentration of agar used. Moreover, the degree of maintenance of porcine SSC self-renewal can vary according to the different conditions of agar-based 3D hydrogels. To elucidate the conditions of an agar-based 3D hydrogel optimized for the maintenance of porcine SSC self-renewal, we measured and compared the undifferentiated state and proliferation of porcine SSCs cultured in each 3D hydrogel prepared using different concentrations of Bacto agar, LB agar, and agarose.

MATERIALS AND METHODS

1. Animals

Crossbred (Landrace×Yorkshire) or purebred (Yorkshire×Yorkshire) male piglets between 1 and 5 days old were kindly donated by Gumbo Inc. (Wonju, Korea), and testes from neonatal piglets was isolated through routine castration surgery performed at a local farm (Gumbo Inc.). The Institutional Animal Care and Use Committee (IACUC) of Kangwon National University (IACUC approval No. KW-131106-1) approved all animal procedures, and all experiments were performed according to the Animal Care and Use Guideline of Kangwon National University.

2. Collection of SSCs from Neonatal Porcine Testes

SSCs were collected from neonatal porcine testes using the petri dish plating post-differential plating (DP) method described previously (Park et al., 2014). Briefly, the seminiferous tubules dispersed mechanically from the testes were digested enzymatically using type IV collagenase (Worthington Biochemical, Lakewood, CA, USA), and hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) and trypsin (Welgene Inc., Daegu, Korea) were used sequentially and separately to disperse the fragmented seminiferous tubules. Subsequently, DP was conducted by treating the dissociated cells with red blood cell (RBC) lysis buffer (Sigma-Aldrich) to remove erythrocytes and then incubating the RBC-free dissociated cells on gelatin-coated petri dishes in high-glucose Dulbecco's modified Eagle's medium (DMEM; Welgene) supplemented with 10% (v/v) fetal bovine serum (FBS; Welgene) and 1% (v/v) antibiotic–antimycotic solution (Welgene); the resulting floating cells were collected following a 16-hour incubation. Moreover, Petri dish plating post-DP was performed by incubating the collected cells on petri dishes in DMEM supplemented with 15% (v/v) FBS, 1% (v/v) nonessential amino acids (NEAA; Invitrogen, Carlsbad, CA, USA), 1% (v/v) antibiotic–antimycotic solution, 2 mM L-glutamine (Invitrogen), 0.1 mM β-mercaptoethanol (Invitrogen), 10^3 units/mL mouse leukemia inhibitory factor (LIF; Chemicon International Inc., Temecula, CA, USA), and 10 ng/mL glial cell-derived neurotrophic factor (GDNF; R&D Systems Inc., Minneapolis, MN, USA). After an overnight incubation, the cells that did not attach to the bottom of the petri dish were collected and porcine SSC populations showing above 95% positivity against porcine SSC-predominant proteins (OCT4, NANOG, PLZF and GFRa1) and below 5% positivity against a leydig cell-specific protein (LHR) and a sertoli cell-specific protein (GATA4) through flow cytometry analysis described previously (Park et al., 2014) were justly allocated for this study. Table 1 shows the detailed information and dilution rate of the primary or secondary antibodies used for flow cytometry.

3. Construction of 3D Hydrogels and Encapsulation of Porcine SSCs into 3D Hydrogel

3D hydrogels were constructed using three types of substrates: Bacto agar (BD Biosciences, Franklin Lakes, NJ, USA), LB agar (BD Biosciences), and agarose (Sigma-Aldrich, Madison, WI, USA). First, stock solutions of each substrate were prepared by dissolving 2% (w/v) Bacto agar, 4% (w/v) LB agar and 1% (w/v) agarose powders in DMEM with heating. Subsequently, to construct Bacto agar-, LB agar-, and agarose-based 3D hydrogels with diverse mechanical properties, the stocks were diluted with DMEM to different concentrations (0.4, 0.6, 0.8 and 1% [w/v] for Bacto agar, 0.8, 1 and 1.2% [w/v] for LB agar, and 0.2 and 0.6% [w/v] for agarose) and heated to 37°C. Encapsulation of porcine SSCs in the 3D hydrogels was achieved by mixing the cells with the different substrate solutions at 37°C and then solidifying at 31°C in a humidified chamber under 95% air and 5% CO_2.

4. Culture of porcine SSCs in 3D Hydrogels

1×10^5 porcine SSCs were inserted into Bacto agar-, LB agar- and agarose-based 3D hydrogels. Subsequently, porcine SSCs exposed to 3D hydrogels were cultured for 6 days in SSC

Table 1. Primary and secondary antibodies.

Antibody	Catalog number	Company	Application	Dilution rate	Reference
Rabbit anti-Human PLZF IgG	Sc-22839	Santa cruz biotechnology	FC[a], FI[b]	1:100	Park et al., 2014
Rabbit anti-Human OCT3/4 IgG	Sc-9081	Santa cruz biotechnology	FC[a], FI[b]	1:100	Park et al., 2014
Rabbit anti-Human SOX2 IgG	AB5603	Chemicon International, Inc.	FI[b]	1:100	Park et al., 2014
Rabbit anti-Human TRA-1-60 IgG	MAB4360	Millipore	FI[b]	1:100	Park et al., 2014
Rabbit anti-Human GFRa1 IgG	Sc-10716	Santa cruz biotechnology	FC[a]	1:100	Park et al., 2014
Rabbit anti-Human NANOG IgG	Sc-33759	Santa cruz biotechnology	FC[a]	1:100	Park et al., 2014
Goat anti-Human LHR IgG	Sc-26341	Santa cruz biotechnology	FC[a]	1:100	Park et al., 2014
Goat anti-Mouse GATA4 IgG	Sc-1237	Santa cruz biotechnology	FC[a]	1:100	Park et al., 2014
Alexa Fluor-488® Chicken anti-Rabbit lgG	A-21441	Molecular probes	FC[a], FI[b]	1:100	Park et al., 2014
Alexa Fluor-568® Donkey anti-Goat lgG	A-11057	Molecular probes	FC[a]	1:100	Park et al., 2014

[a]FC=Flow Cytometry, [b]FI= Fluorescence Immunoassay

culture medium consisting of Stempro-34 medium (Invitrogen) supplemented with insulin-transferrin-selenium (ITS; Invitrogen), 60 μM putrescine dihydrochloride (Sigma-Aldrich), 6 mg/mL D-(+)-glucose (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 0.11 mg/mL sodium pyruvate (Sigma-Aldrich), 1 μl/mL DL-lactic acid (Sigma-Aldrich), 5 mg/mL bovine serum albumin (BSA, Sigma-Aldrich), 2 mM L-glutamine, 0.05 mM β-mercaptoethanol, 1% (v/v) FBS, 1% (v/v) NEAA, MEM vitamin solution (Sigma-Aldrich), 1% (v/v) antibiotic–antimycotic solution, 0.1 mM ascorbic acid (Sigma-Aldrich), 10^3 units/mL mouse LIF, 10 ng/ml GDNF, 30 ng/mL β-estradiol (Sigma-Aldrich), 60 ng/mL progesterone (Sigma-Aldrich), 20 ng/mL human epidermal growth factor (EGF; Peprotech, Inc., Rocky Hill, NJ), and 10 ng/mL human basic fibroblast growth factor (bFGF; Peprotech, Inc.). Medium was changed freshly every second day. The cultured porcine SSC were separated from 3D hydrogels by mechanical dissociation. After trypsinization of the porcine SSCs, the fragmented hydrogels were removed by filtration using 40 μm nylon mesh (SPL Life Sciences CO., Ltd., Pocheon, Korea) and the dissociated porcine SSCs were allocated to the following experiments.

5. Alkaline Phosphatase (AP) Staining

Porcine SSCs cultured in 3D hydrogels were fixed with 4% (v/v) paraformaldehyde (Junsei Chemical Co., Ltd., Chuo-ku, Japan). After washing with DPBS, the fixed cells were stained with AP staining solution consisting of 0.1 M pH 8.2 Tris buffer (Sigma-Aldrich) supplemented with 0.2 mg/mL Naphthol AS-MX phosphate (Sigma-Aldrich), 2% (v/v) N,N-dimethyl formamide (Sigma-Aldrich), and 1 mg/mL Fast Red TR salt (Sigma-Aldrich) for 90 min at room temperature. Next, the stained cells were rinsed two times with DPBS and the proportion of AP-positive cells among above 200 stained cells was measured using a hemocytometer under an inverted microscope (CKX-41; Olympus, Tokyo, Japan).

6. Measurement of Colony Diameter

The diameter of colonies derived from porcine SSCs cultured in each 3D hydrogel was determined by averaging the horizontal and vertical diameters of colonies using i-Solution Lite version 10.0 software (IMT i-Solution Inc., Vancouver, Canada).

7. Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

According to each manufacturer's instruction, total mRNA was extracted using a Dynabeads® mRNA Direct™ Kit (Ambion, Austin, TX), followed by cDNA synthesis using a ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). Subsequently, a THUNDERBIRD™ SYBR® qPCR Mix (Toyobo) under the 7500 Real time PCR system (Applied Biosystem, Foster City, CA) was used for qRT-PCR. PCR specificity was identified by analyzing melting curve data and normalization of the specific gene expression was conducted by comparison to the mRNA level of *GAPDH*. Relative mRNA level was calculated as $2^{-\triangle\triangle Ct}$, where Ct = threshold cycle for target amplification, $\triangle Ct = Ct_{\text{target gene}}$ (specific genes for each sample) - $Ct_{\text{internal reference}}$ (*GAPDH* for each sample), and $\triangle\triangle Ct = \triangle Ct_{\text{sample}}$ (treatment sample in each experiment) - $\triangle Ct_{\text{calibrator}}$ (control sample in each experiment). Table 2 shows general information and sequences of primers designed by Primer3 software (Whitehead Institute/MIT Center

Table 2. Primers and PCR cycling condition.

Genes	GenBank number	Primer sequence		Size (bp)	Annealing temp[a] (ºC)
		Sense	Anti-sense		
GAPDH	NM_001206359.1	5′-GATGGTGATGGCCTTTCCATTG-3′	5′-AGGGCTGCTTTTAACTCTGGCAA-3′	180	60
NANOG	NM_001129971.1	5′-GTTTCCAAGACGGCCTCCAAAT -3′	5′-AACCAAACCTGGAACAGCCAGAC-3′	158	60
EPCAM	NM_214419.1	5′-TGCATCTCGCCCATCTCCTTT-3′	5′-CAATGCAGGGTCTACAGGCTGG-3′	154	60
THY1	NM_001146129.1	5′-TCTTGCTGGAGATGCTGGGC-3′	5′-GTGCTCTTGGGCACTGTGGG-3′	178	60
UCHL1	NM_213763.2	5′-TAGAGGTGGCCATCCACGTTGT-3′	5′-TCCGGAAGACAGAGCAAAATGC-3′	150	60

[a]temp: temperature

for Genome Research) with cDNA sequences derived from GenBank for pig.

8. Fluorescence Immunoassay

The fixation process was conducted by incubating the cultured porcine SSCs for 15 minutes in 4% (v/v) paraformaldehyde. After rinsing twice with DPBS, the fixed cells were stained for 1 hours at 4°C with anti-rabbit OCT3/4, SOX2, and PLZF primary antibodies diluted in DPBS supplemented with 0.01% (v/v) Triton X-100 (Sigma-Aldrich) or with anti-rabbit TRA-1-60 primary antibodies diluted in DPBS. Subsequently, primary antibodies were detected using Alexa Flour 488-conjugated secondary antibody diluted in DPBS. Table 1 shows the detailed information and dilution rate of the used primary or secondary antibodies. After 1 hour, the stained cells were washed twice with DPBS and fluorescence intensity derived from them was measured using SoftMax® Pro 6.2.2. (Molecular Devices Cooperation, Sunnyvale, CA) after adding 100 μl of DPBS to the stained cells.

9. Statistical Analysis

The Statistical Analysis System (SAS) software was used for analyzing statistically all the numerical data shown in each experiment. When a significance of the main effects through varience (ANOVA) analysis in the SAS package was detected, comparison of each treatment was conducted by the least-square or DUNCAN method. Moreover, significant differences among treatments were determined when P value was less than 0.05.

RESULTS

1. Optimization of the mechanical properties of Bacto agar-, LB agar-, or agarose-based 3D hydrogels for self-renewal of porcine SSCs

Bacto agar-, LB agar-, or agarose-based 3D hydrogels have been used as 3D scaffold systems for porcine SSC culture. To determine the mechanical conditions required for the effective maintenance of the self-renewal and growth of porcine SSCs in each 3D hydrogel, we evaluated the alkaline phosphatase (AP) activity and colony size of porcine SSCs cultured in 3D hydrogels prepared using a range of Bacto agar, LB agar, and agarose concentrations. Despite no significant differences among the different preparations, porcine SSCs cultured in the 0.6% (w/v) Bacto agar-based 3D hydrogels showed the highest level of AP activity (Fig. 1A), and the largest colony size was also detected in porcine SSCs cultured in 0.6% (w/v) Bacto agar-based 3D hydrogels compared with the 0.4, 0.8, and 1.0% (w/v) Bacto agar-based 3D hydrogels (Fig. 1B). Moreover, a significantly higher level of AP activity (Fig. 1C) and larger colony size (Fig. 1D) were observed in 1% (w/v) LB agar-based 3D hydrogels compared with porcine SSCs cultured in 0.8 and 1.2% (w/v) LB agar-based 3D hydrogels. In the agarose-based 3D hydrogel, higher AP activity of porcine SSCs was detected with 0.2% (w/v) agarose compared with 0.6% (w/v) agarose, although the differences among the different agarose concentrations were not significant (Fig. 1E). The use of 0.2% (w/v) agarose induced a significant increase in the colony size of porcine SSCs compared with the use of 0.6% (w/v) agarose (Fig. 1F). These results demonstrate that the 3D hydrogels prepared with 0.6% (w/v) Bacto agar, 1% (w/v) LB agar, or 0.2% (w/v) agarose can effectively support the maintenance of undifferentiated porcine SSCs and stimulate proliferation of porcine SSCs, indicating the establishment of mechanical properties optimized for the maintenance of porcine SSC self-renewal for each biomaterial-based 3D scaffold.

2. Determination of the 3D hydrogel demonstrating the best maintenance of porcine SSC self-renewal

Among the Bacto agar-, LB agar-, and agarose-based 3D hydrogels, the 3D hydrogel exhibiting the best performance in the maintenance of porcine SSC self-renewal was determined

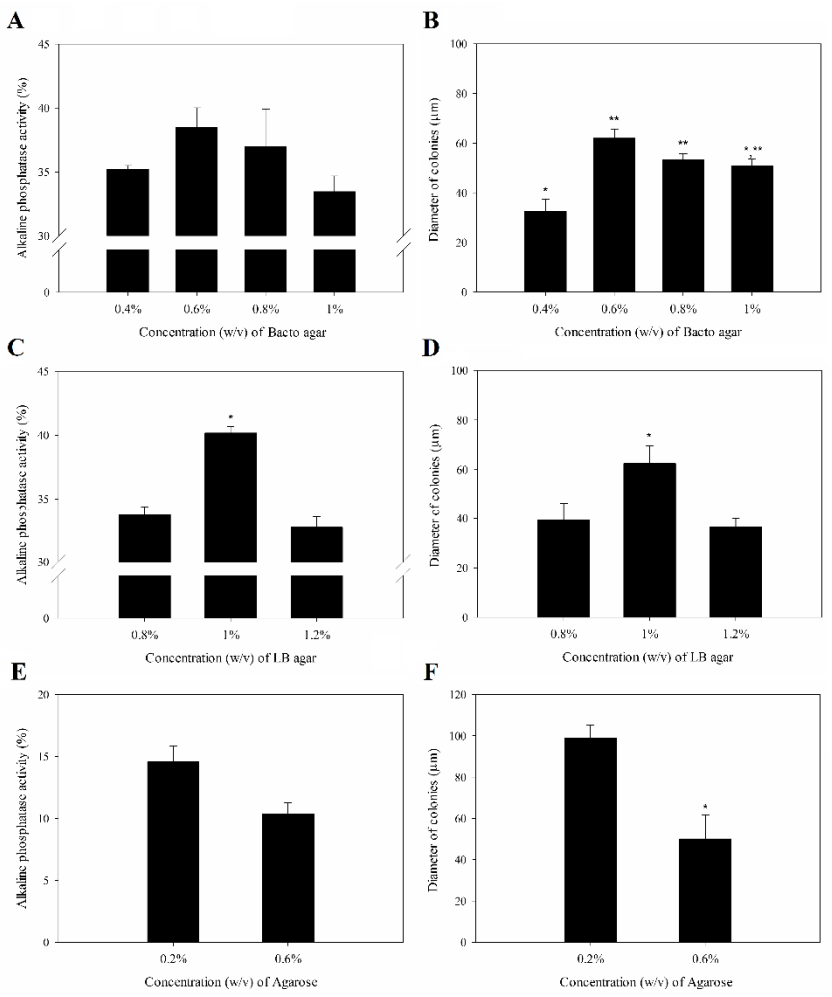

Fig. 1. Optimization of Bacto agar-, LB agar- or agarose-based 3D hydrogel conditions supporting maintenance of AP activity and stimulation of proliferation in porcine SSC cultures. Porcine SSCs were cultured for 6 days in 3D hydrogels constructed using several concentrations (w/v) of Bacto agar, LB agar or agarose. Analysis of AP activity in the cultured porcine SSCs was conducted by calculating the percentage of cells staining positively for AP and proliferation was analyzed by measuring the diameters of colonies derived from porcine SSCs. Although there were no significant differences among the treatments, the highest AP activity was detected in porcine SSCs cultured in 0.6% (w/v) Bacto agar-based 3D hydrogels (A), which showed significantly the largest diameter of porcine SSC-derived colonies (B). Porcine SSCs cultured in 1% (w/v) LB agar-based 3D hydrogels showed significantly higher AP activity (C) and larger colony diameter (D) than those in 0.8 and 1.2% (w/v) LB agar-based 3D hydrogels. In addition, porcine SSCs showed higher AP activity when cultured in 0.2% (w/v) compared with 0.6 (w/v) agarose-based 3D hydrogel (E), although no significant differences were detected among the treatments. Simultaneously, a significant increase in the diameter of porcine SSC-derived colonies was observed in 0.2% (w/v) compared with 0.6% (w/v) agarose-based 3D hydrogels (F). All data are represented as means ± SEM of three independent experiments. $^{*,**}p<0.05$.

by evaluating colony formation, morphology, and size; AP activity; and transcriptional and translational regulation of self-renewal-related genes specific to porcine SSCs. Regardless of the type of hydrogel biomaterial, uniform formation of spherical porcine SSC colonies was observed at 6 days after culture in 3D hydrogels, and the newly formed porcine SSC colonies with spherical morphology stained positively for AP (Fig. 2). However, the 0.2% (w/v) agarose-based 3D hydrogel showed the strongest transcription (Fig. 3) and translation (Fig. 4) of self-renewal-related genes specific to porcine SSCs. Compared with those of 0.6% (w/v) Bacto agar- and 1.0% (w/v) LB agar-based 3D hydrogels, there were significant increases

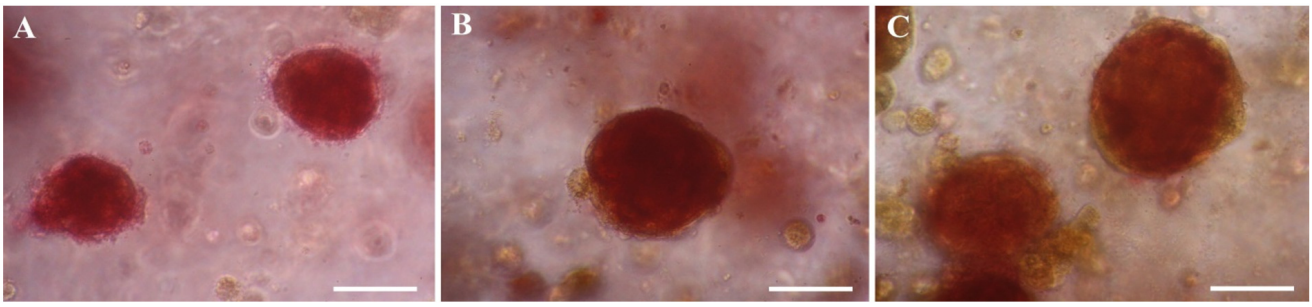

Fig. 2. The morphology and AP activity of colonies derived from porcine SSCs cultured in 0.6% (w/v) Bacto agar-, 1.0% (w/v) LB agar-, and 0.2% (w/v) agarose-based 3D hydrogels. Culture of undifferentiated porcine SSCs were conducted for 6 days in each 3D hydrogel. Subsequently, the cultured porcine SSCs were subjected to AP staining. Regardless of the type of hydrogel biomaterials, strong AP staining (red color) was detected in spherical colonies derived from porcine SSCs cultured in 0.6% (w/v) Bacto agar- (A), 1.0% (w/v) LB agar- (B), and 0.2% (w/v) agarose-based 3D hydrogels (C). *n*=3. Scale bar= 50μm.

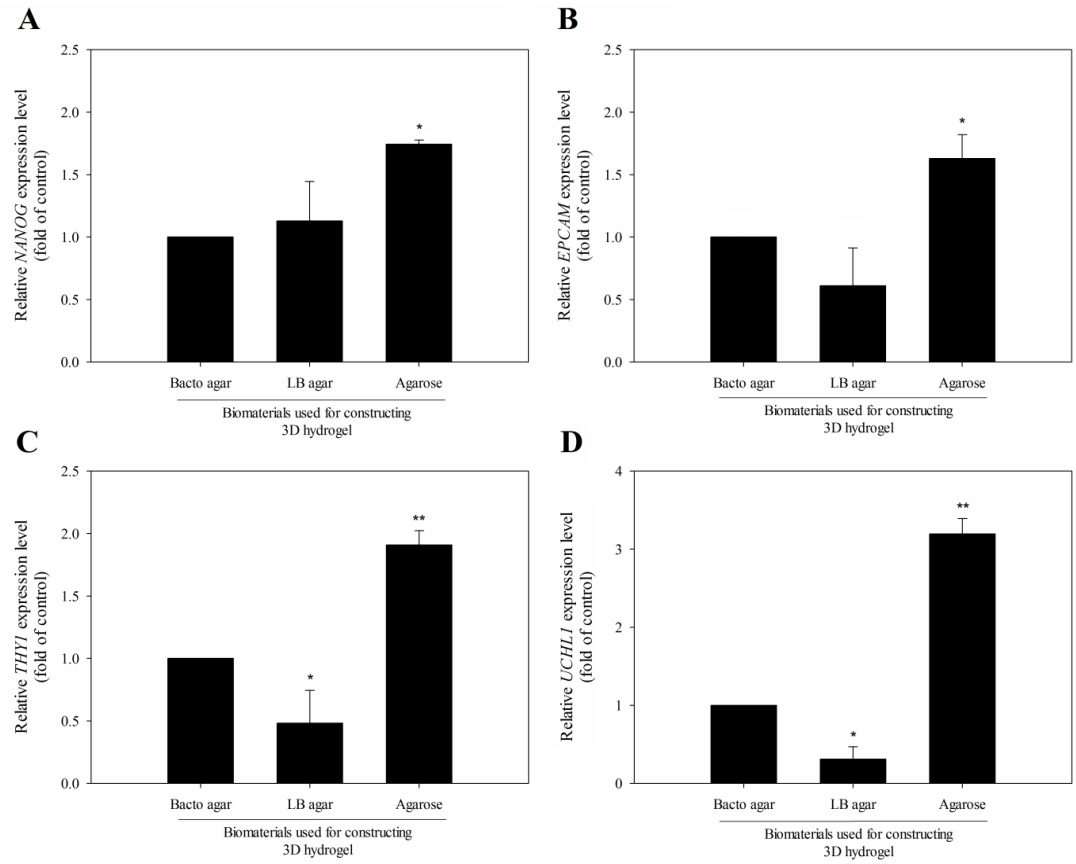

Fig. 3. Transcriptional levels of porcine SSC-predominant genes in porcine SSCs cultured in 0.6% (w/v) Bacto agar-, 1.0% (w/v) LB agar- or 0.2% (w/v) agarose-based 3D hydrogels. Undifferentiated porcine SSCs were cultured for 6 days in each 3D hydrogel. Then, transcript levels of porcine SSC-predominant genes were quantified by real-time PCR. Compared with 0.6% (w/v) Bacto agar- and 1.0% (w/v) LB agar-based 3D hydrogel, the 0.2% (w/v) agarose-based 3D hydrogel induced significant transcriptional up-regulation of *NANOG* (A), *EPCAM* (B), *THY1* (C), and *UCHL1* (D). In addition, porcine SSCs cultured in 0.6% (w/v) Bacto agar-based 3D hydrogel showed significantly higher transcriptional level of *THY1* (C) and *UCHL1* (D) than those in 1.0% (w/v) LB agar-based 3D hydrogel, whereas no significant differences in the transcriptional level of *NANOG* (A) and *EPCAM* (B) were observed in porcine SSCs cultured in 0.6% (w/v) Bacto agar-based 3D hydrogel, compared with those in 1.0% (w/v) LB agar-based 3D hydrogel. All data are represented as means ± SEM of four independent experiments. $^{*,**}p<0.05$.

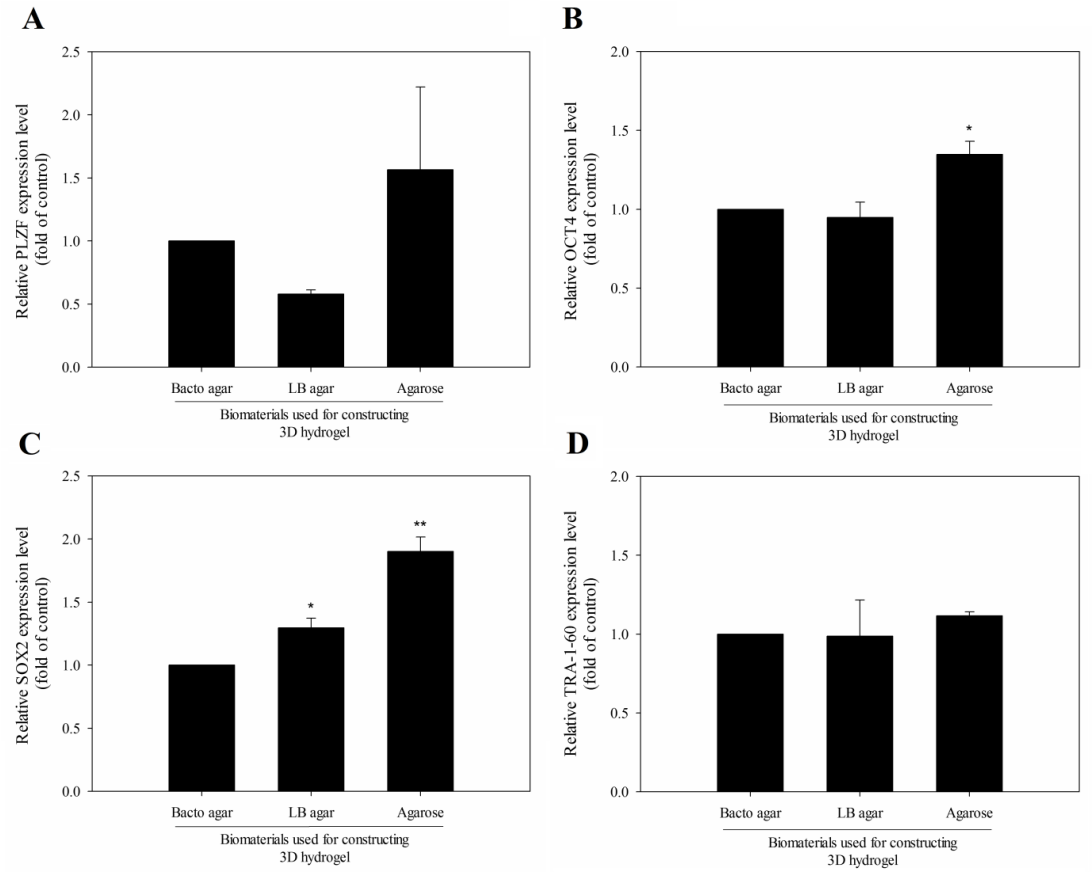

Fig. 4. Translational levels of porcine SSC-predominant genes in porcine SSCs cultured in 0.6% (w/v) Bacto agar-, 1.0% (w/v) LB agar- or 0.2% (w/v) agarose-based 3D hydrogels. Undifferentiated porcine SSCs were cultured for 6 days in each 3D hydrogel. Then, protein levels of porcine SSC-predominant genes were quantified by fluorescence immunoassay. The relative expression level of each porcine SSC-predominant protein was calculated as the ratio of fluorescence intensity of cells cultured in 1.0% (w/v) LB agar- or 1.2% (w/v) agarose-based 3D hydrogel to fluorescence intensity of cells cultured in 0.6% (w/v) Bacto agar-based 3D hydrogel (control). Compared with 0.6% (w/v) Bacto agar- and 1.0% (w/v) LB agar-based 3D hydrogel, the 0.2% (w/v) agar-based 3D hydrogel induced significant increase of OCT4 (B) and SOX2 (C) expression. Simultaneously, levels of PLZF (A) and TRA-1-60 (D) expression were numerically higher in the porcine SSCs cultured in 0.2% (w/v) agarose-based 3D hydrogel than those in 0.6% (w/v) Bacto agar- and 1.0% (w/v) LB agar-based 3D hydrogel. All data are represented as means ± SEM of four independent experiments. $^{*,**}p<0.05$.

in the transcript levels of *NANOG*, *EPCAM*, *THY1*, and *UCHL1* and in the protein levels of OCT4 and SOX2 in porcine SSCs cultured in 0.2% (w/v) agarose-based 3D hydrogels. The protein expression of PLZF and TRA-1-60 was also greater in porcine SSCs cultured in 0.2% (w/v) agarose-based 3D hydrogels compared with those in the 0.6% (w/v) Bacto agar- and 1.0% (w/v) LB agar-based 3D hydrogels, although there were no significant differences among the different hydrogel biomaterials. Additionally, transcription of *THY1* and *UCHL1* was significantly increased in 0.6% (w/v) Bacto agar-based 3D hydrogels compared with porcine SSCs cultured in 1.0% (w/v) LB agar-based 3D hydrogels, whereas the transcription of *NANOG* and *EPCAM* did not differ significantly between 0.6% (w/v) Bacto agar- and 1.0% (w/v) LB agar-based 3D hydrogels. The protein expression level of SOX2 in porcine SSCs was maintained in 1.0% (w/v) LB agar-based 3D hydrogels compared with 0.6% (w/v) Bacto agar-based 3D hydrogels, and no significant differences in the protein levels of OCT4, PLZF, and TRA-1-60 were observed between the 0.6% (w/v) Bacto agar- and the 1.0% (w/v) LB agar-based 3D hydrogels. As shown in Figure 5, compared with the 0.6% (w/v) Bacto agar- and 1.0% (w/v) LB agar-based 3D hydrogels, the 0.2% (w/v) agarose-based 3D hydrogel induced

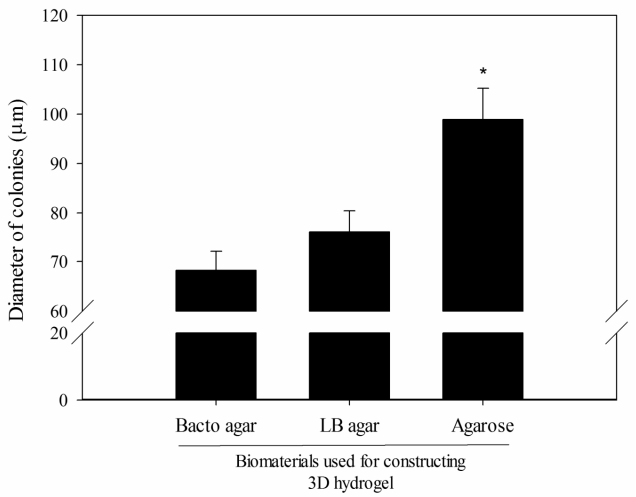

Fig. 5. Diameters of colonies derived from porcine SSCs cultured in 0.6% (w/v) Bacto agar-, 1.0% (w/v) LB agar- or 0.2% (w/v) agarose-based 3D hydrogels. Undifferentiated porcine SSCs were cultured for 6 days in each 3D hydrogel and the diameters of porcine SSC-derived colonies was measured and compared for evaluating their proliferation competence. As the results, porcine SSCs cultured in 0.2% (w/v) agarose-based 3D hydrogel showed significant increase of colony diameter compared with those in 0.6% (w/v) Bacto agar- and 1.0% (w/v) LB agar-based 3D hydrogel. All data are represented as means ± SEM of four independent experiments. $^{*}p<0.05$.

a significant increase in porcine SSC-derived colony diameter, indicating that proliferation of porcine SSCs can be strongly stimulated in 3D hydrogels prepared with 0.2% (w/v) agarose. Accordingly, we suggest that 0.2% (w/v) agarose-based 3D hydrogels can be effectively used for the maintenance of porcine SSC self-renewal.

DISCUSSION

Here, we report that, among 3D hydrogels prepared using a variety of agar powders, 0.2% (w/v) agarose-based 3D hydrogels were optimal for maintaining porcine SSC self-renewal. Compared with 3D hydrogels derived from other biomaterials, the 0.2% (w/v) agarose-based 3D hydrogel exhibited the greatest degree of maintenance of the undifferentiated state and the highest level of stimulation of proliferation in porcine SSC culture. However, porcine SSCs cultured in 3D hydrogels induced the formation of spherical colonies, regardless of the biomaterial used and the hydrogel mechanics. These results suggest that a 3D culture microenvironment is essential for the formation of uniform colonies derived from porcine SSCs, and the internal mechanics derived from the 0.2% (w/v) agarose-derived 3D hydrogel play an important role in maintaining the undifferentiated state of porcine SSCs and in stimulating the proliferation of porcine SSCs, indicating that a 0.2% (w/v) agarose-based 3D hydrogel is adequate for the *in vitro* culture of porcine SSCs.

Generally, among the commercialized agar powders, Bacto and LB agar include agaropectin (Bao et al., 2010), whereas agarose does not (Lam et al., 2012). Agaropectin is a heterogeneous mixture containing two anionic groups, sulfate and pyruvate (Albuquerque et al., 2016). Previous reports have demonstrated that sulfation of heparin sulfate and the non-saccharide GAG mimetic inhibits stem cell self-renewal (Hirano et al., 2012; Pate et al., 2014), indicating that the presence of a sulfate group on a scaffold in stem cell culture negatively affects the maintenance of stem cell self-renewal. Therefore, we suggest that the improved maintenance of porcine SSC self-renewal in agarose-based 3D hydrogels may be a result of the absence of sulfate groups in agaropectin-free agarose powder.

Overall, the non-cellular niche derived from 0.2% (w/v) agarose-based 3D hydrogels without agaropectin was effective in maintaining the self-renewal of porcine SSCs. Although the manipulation of agarose is challenging because of its high melting temperature and untimely coagulation, and the retrieval of cells from－and incorporation of adhesion molecules into－agarose-based 3D hydrogels is difficult, information on the mechanics of 0.2% (w/v) agarose-based 3D hydrogels will contribute to the development of an advanced non-cellular niche customized for the maintenance of porcine SSC self-renewal using a new biodegradable 3D scaffold system. Therefore, precise measurement of the mechanical properties, including stiffness, elastic modulus, and swelling ratio, should be performed in future studies.

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