



Effects of Culture Dimensions on Maintenance of Porcine Inner Cell Mass-Derived Cell Self-Renewal

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Despite the fact that porcine embryonic stem cells (ESCs) are a practical study tool, *in vitro* long-term maintenance of these cells is difficult in a two-dimensional (2D) microenvironment using cellular niche or extracellular matrix proteins. However, a three-dimensional (3D) microenvironment, similar to that enclosing the inner cell mass of the blastocyst, may improve *in vitro* maintenance of self-renewal. Accordingly, as a first step toward constructing a 3D microenvironment optimized to maintain porcine ESC self-renewal, we investigated different culture dimensions for porcine ICM-derived cells to enhance the maintenance of self-renewal. Porcine ICM-derived cells were cultured in agarose-based 3D hydrogel with self-renewal-friendly mechanics and in 2D culture plates with or without feeder cells. Subsequently, the effects of the 3D microenvironment on maintenance of self-renewal were identified by analyzing colony formation and morphology, alkaline phosphatase (AP) activity, and transcriptional and translational regulation of self-renewal-related genes. The 3D microenvironment using a 1.5% (w/v) agarose-based 3D hydrogel resulted in significantly more colonies with stereoscopic morphology, significantly improved AP activity, and increased protein expression of self-renewal-related genes compared to those in the 2D microenvironment. These results demonstrate that self-renewal of porcine ICM-derived cells can be maintained more effectively in a 3D microenvironment than in a 2D microenvironment. These results will help develop novel

culture systems for ICM-derived cells derived from diverse species, which will contribute to stimulating basic and applicable studies related to ESCs.

Keywords: agarose, culture dimension, embryonic stem cells, pig, self-renewal

INTRODUCTION

Among domestic animals, pigs have overwhelming anatomical, physiological, immunological, and genomic similarities to humans (Lunney, 2007; Meurens et al., 2012; Walters et al., 2001). As a laboratory animal model suitable for human medical research (Lunney, 2007), clinical translational studies (Hughes et al., 2003), drug efficacy evaluation (Donato et al., 1999), protein-based drug production (Van Cott and Velandier, 1998), and xenotransplantation studies (Valdés-González et al., 2005) have been conducted extensively in porcine species. However, trials at the individual, organ, or tissue level have major limitations in effectiveness, efficiency, and accuracy, resulting in the need for more delicate manipulations at the cellular level. Accordingly, porcine embryonic stem cells (ESCs), which self-renew indefinitely and differentiate into the three germ layers that form the body (Vassiliev et al., 2010), offer a practical tool to address these needs.

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Unfortunately, research concentrating on *in vitro* culture microenvironments to optimize physicochemical and physiological niches maintaining self-renewal has failed to identify effective culture technologies for long-term maintenance of undifferentiated porcine ESCs. Interest has been focused on the extracellular matrix (ECM) niche of culture systems. However, two-dimensional (2D) culturing of porcine ESCs on plates coated with ECM proteins, which contributes to self-renewal, has also failed to effectively maintain porcine ESCs in an undifferentiated state long term (Son et al., 2009).

In vivo, cells are surrounded by three-dimensional (3D) microenvironments that contain multiple ECM components and mixed cell populations (Baker and Chen, 2012), and 3D communication networks are formed through cell-to-cell and cell-to-ECM interactions that maintain the specificity of cells (Pampaloni et al., 2007). The provision of *in vivo*-like 3D microenvironments is very important for maintaining such specificity *in vitro*. Providing porcine ESCs with a 3D microenvironment similar to that enclosing the inner cell mass of blastocysts may improve the *in vitro* maintenance of ESC self-renewal.

Accordingly, as a first step toward constructing synthetic 3D microenvironments optimized to maintain porcine ESC self-renewal, the conditions needed to construct agarose-based 3D scaffolds were determined, and we sought to identify the culture dimension preferences of these cells. Porcine inner cell mass(ICM)-derived cells were cultured on 2D plates with or without feeder cells or on optimized agarose-based 3D scaffolds, and alkaline phosphatase (AP) activity and transcriptional and translational expression of genes specific to the undifferentiated state were analyzed.

MATERIALS AND METHODS

Cells and animals

Porcine ICM-derived cells with characteristics of ESCs derived from inner cell mass of porcine *in vivo* blastocysts (Supplementary Fig. S1) were used in all experiments. Fetuses were derived from 13.5-day-old pregnant female ICR mice purchased from DBL (Korea) and used as embryonic fibroblast donors. All animal housing, handling, and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Kangwon National University (IACUC approval no. KW-140904-1) and conducted in accordance with the Animal Care and Use Guidelines of Kangwon National University.

Preparation of agarose-based 3D hydrogels and encapsulation of porcine ICM-derived cells

To construct agarose-based 3D hydrogels with different mechanical characteristics, 0.5, 1.0, or 1.5% (w/v) agarose powder (Sigma-Aldrich, USA) was dissolved in 1:1 low-glucose Dulbecco's modified Eagle's medium (DMEM; Welgene, Korea):Ham's F-10 (Invitrogen, USA) with heating. Encapsulation of porcine ICM-derived cells into agarose-based 3D hydrogels was conducted by mixing cell clumps with each of the agarose solutions at 37°C and allowing them to solidify on glass slides coated with Sigmacote® (Sigma-Aldrich) at 31°C in a humidified chamber under

95% air and 5% CO₂.

Culture of porcine ICM-derived cells

For 2D cultures, clumps derived from porcine ICM-derived cells dissociated mechanically were seeded in culture plates coated with or without mouse embryonic fibroblasts (MEFs) inactivated mitotically by 10 µg/ml mitomycin C (Sigma-Aldrich). For 3D cultures, porcine ICM-derived cell-derived clumps were incorporated into agarose-based 3D hydrogels as described above. Subsequently, porcine ICM-derived cells exposed to 2D or 3D microenvironments were cultured for 7 days in 1:1 low-glucose DMEM:Ham's F-10 supplemented with 15% (v/v) heat-inactivated ES cell-screened fetal bovine serum (Hyclone, USA), 0.2 mM β-mercaptoethanol (Invitrogen), 1% (v/v) nonessential amino acids (Invitrogen), 1% (v/v) antibiotic-antimycotic solution (Welgene), and 2 ng/ml basic fibroblast growth factor (PeproTech, Inc., USA). The medium was replaced every second day. The characterized porcine ICM-derived cells (Supplementary Fig. S1) were maintained over Passage 24, and porcine ICM-derived cells at Passage between 25 and 29 were allocated to each experiment.

Alkaline phosphatase (AP) staining

Cultured porcine ICM-derived cells were fixed in 4% (v/v) paraformaldehyde (Junsei Chemical Co., Japan). After two washes with Dulbecco's phosphate-buffered saline (DPBS; Welgene), the fixed cells were stained with a solution containing 0.2 mg/ml naphthol AS-MX phosphate (Sigma-Aldrich), 2% (v/v) N,N-dimethylformamide (Sigma-Aldrich), and 1 mg/ml Fast Red TR salt (Sigma-Aldrich) in 0.1 M Tris buffer (pH 8.2) for 90 min at room temperature. Subsequently, the stained cells were rinsed twice with DPBS, and the proportion of AP-positive cells was measured using a hemocytometer and an inverted microscope (CKX-41; Olympus, Japan).

Quantitative real-time polymerase chain reaction (PCR)

According to the manufacturer's instructions, total mRNA was extracted from porcine ICM-derived cells harvested mechanically from agarose-based 3D hydrogels using the Dynabeads® mRNA Direct™ Kit (Ambion, USA), and cDNA synthesis was performed using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Japan). Subsequently, the expression levels of the specific genes were quantified using THUNDERBIRD™ SYBR® qPCR Mix (Toyobo) on a 7500 Real time PCR system (Applied Biosystem, USA), and melting curve data were analyzed to determine PCR specificity. mRNA levels are presented as 2^{-ΔCt} values, where Ct = the threshold cycle for target amplification, ΔCt = Ct_{target gene} (specific gene Ct in a sample) - Ct_{internal reference} (GAPDH Ct in same sample). Primer sequences were designed using Primer3 software (Whitehead Institute/MIT Center for Genome Research) based on porcine cDNA sequences obtained from GenBank. See Supplementary Table S1. for additional PCR information and primer sequences.

Immunocytochemistry

Porcine ICM-derived cells harvested mechanically from aga-

Table 1. Effects of different agarose concentrations on the maintenance of AP activity in the culture of porcine ICM-derived cells inside agarose-based 3D hydrogels.

Concentration (% w/v) of agarose	No. of porcine ICM-derived cell colonies inserted into agarose-based 3D hydrogels	No. (%) ^a of porcine ICM-derived cell colonies ^b stained positively by AP staining
0.5	28	24 (85.7)
1.0	27	24 (88.9)
1.5	29	28 (96.6)

Model effect of treatments in the number of colonies stained positively by AP staining is 0.3662 (p value).

^aPercentage of the number of porcine ICM-derived cell colonies inserted into agarose-based 3D hydrogels.

^bColonies were derived from porcine ICM-derived cells cultured for 7 days in the 3D hydrogels based on agarose with the specific concentration.

Table 2. Effects of different agarose concentrations on the proliferation of porcine ICM-derived cells cultured in agarose-based 3D hydrogels.

Concentration (% w/v) of agarose	No. of porcine ICM-derived cell colonies inserted into agarose-based 3D hydrogels	No. (%) ^a of porcine ICM-derived cell colonies ^b experiencing proliferation ^c
0.5	34	2 (5.9)
1.0	34	9 (26.5)
1.5	40	12 (30.0)

Model effect of treatments in the number of colonies stained positively by AP staining is 0.5068 (p value).

^aPercentage of the number of porcine ICM-derived cell colonies inserted into agarose-based 3D hydrogels.

^bColonies were derived from porcine ICM-derived cells cultured for 7 days in the 3D hydrogels based on agarose with the specific concentration.

^cColony experiencing increase of size post-*in vitro* culture was regarded as the proliferated colony.

rose-based 3D hydrogels at 7 day of culture were fixed using 4% (v/v) paraformaldehyde for 20 min, washed twice with DPBS, and incubated for 5 min with REAL peroxidase blocking solution (Dako, Denmark). Cells were then incubated for 30 minutes at room temperature with a primary antibody detecting either OCT4, SOX2, NANOG, TRA-1-60, TRA-1-81, neurofilament, α -smooth muscle actin or cytokeratin 18 and rinsed twice with DPBS. Primary antibodies were localized by incubating for 30 min at room temperature using the REAL EnVision/HRP detection system (Dako), followed by two washes with DPBS and a 90-minute incubation with REAL DAB+ chromogen (Dako). Details and antibody dilutions used are provided in [Supplementary Table S2](#). After staining, IMT iSolution Lite software (ver. 10.0, IMT iSolution Inc., Canada) was used to measure the colony areas and the percentage of porcine ICM-derived cells within a colony expressing self-renewal-related proteins; the latter was presented as the area ratio \times 100, where area ratio = the area of a colony stained positively with the specific antibody/total area of each colony.

Statistical analysis

All numerical data were analyzed using the Statistical Analysis System (SAS). Porcine ICM-derived cell colonies that proliferated and stained positively for AP were compared among treatment groups using a generalized linear model (PROC-GLM) in the SAS package. Protein and mRNA levels were compared by the least-squares or DUNCAN method when significant differences were detected by analysis of variance using SAS. p values less than 0.05 were regarded as

indicative of significant differences.

RESULTS

Determination of optimal agarose-based 3D hydrogel conditions for maintenance of porcine ICM-derived cell self-renewal

To establish a formula for developing agarose-based 3D hydrogels that effectively support the maintenance of porcine ICM-derived cell self-renewal, agarose-based 3D hydrogels with different mechanical characteristics were constructed using varying agarose concentrations and evaluated by counting the number of porcine ICM-derived cell colonies showing evidence of AP activity and proliferation. As shown in [Table 1](#), although there were no significant differences among experimental groups, the highest percentage of AP-positive colonies (96.6%) was seen in the 1.5% (w/v) agarose-based 3D hydrogels, whereas the 0.5% (w/v) (85.7% AP positivity) and 1.0% (w/v) (88.9% AP positivity) agarose-based 3D hydrogels showed less than 90% AP positivity. Moreover, although the differences among experimental groups were not significant, substantial evidence of proliferation was observed in the porcine ICM-derived cell colonies in 1.0% (w/v) (26.5% of colonies) and 1.5% (w/v) agarose-based 3D hydrogels (30.0% of colonies); 0.5% (w/v) agarose-based 3D hydrogels showed the lowest percentage of colonies undergoing proliferation (5.9%) ([Table 2](#)). These results indicate that the 1.5% (w/v) agarose-based 3D hydrogels were the most competent in maintaining AP activity and stimulating proliferation in a 3D culture microenviron-

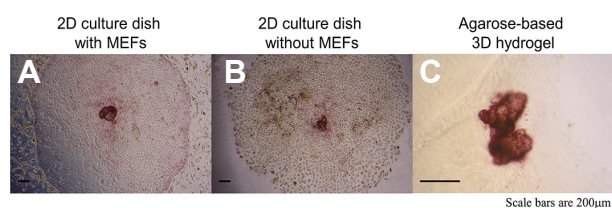


Fig. 1. AP activity and colony morphology of porcine ICM-derived cells cultured in 2D and 3D microenvironments. Undifferentiated porcine ICM-derived cells were maintained on 2D culture plates coated with (A) or without (B) MEFs, or in 3D microenvironments in 1.5% (w/v) agarose-based 3D hydrogels (C). After 7 days of incubation, AP activity and colony morphology were characterized by histochemical staining and stereomicroscopy. Porcine ICM-derived in MEF-free 2D cultures showed no AP positivity (B), whereas those in 2D cultures with MEFs or in 1.5% (w/v) agarose-based 3D hydrogels showed strong positivity (A and C, red color). Colonies with well-defined boundaries were primarily observed in the porcine ESCs cultured in 2D with MEFs, while MEF-free 2D cultures induced formation of colonies with discontinuous boundaries. Colonies in the 3D microenvironment showed stereoscopic morphology. Scale bars are 200 μ m.

ment customized for the maintenance of porcine ICM-derived cell self-renewal.

Effects of the 3D culture microenvironment on the maintenance of porcine ICM-derived cell self-renewal

Colony formation and morphology, AP activity, and the transcript and protein levels of self-renewal-related genes were compared between porcine ICM-derived cells cultured in 2D versus 3D microenvironments. As shown in Fig. 1, while successful formation of colonies was observed in all cultures, colony morphology and AP activity differed between the culture microenvironments. MEF-free 2D cultures induced the formation of colonies with discontinuous boundaries, extremely weak AP activity in the center and no AP activity in the rest except the center (Fig. 1B), whereas porcine ICM-derived cells cultured with MEFs in a typical 2D culture microenvironment formed colonies with well-defined boundaries, strong AP activity in the center and boundary and less intense AP activity in the rest except the center and boundary (Fig. 1A). Colonies with 3D morphology and overall strong AP activity were detected in the 3D culture microenvironment (Fig. 1C). In evaluating the potential to maintain AP activity during *in vitro* culture (Table 3), no colonies with AP activity were observed in MEF-free 2D cultures. However, in both MEF-coated 2D and MEF-free 3D cultures, all colonies exhibited AP activity, indicating that the 3D culture microenvironment prevents the decrease in AP activity characteristic of undifferentiated porcine ICM-derived cells. Regarding the transcriptional regulation of self-renewal-related genes (Fig. 2), the 3D culture microenvironment induced significantly stronger up-regulation of *OCT4*, *SOX2*, and *TERT* transcription than did the 2D culture microenvironments, with or without MEFs. However, compared with the expression levels seen in MEF-coated 2D cultures, *NANOG* transcription

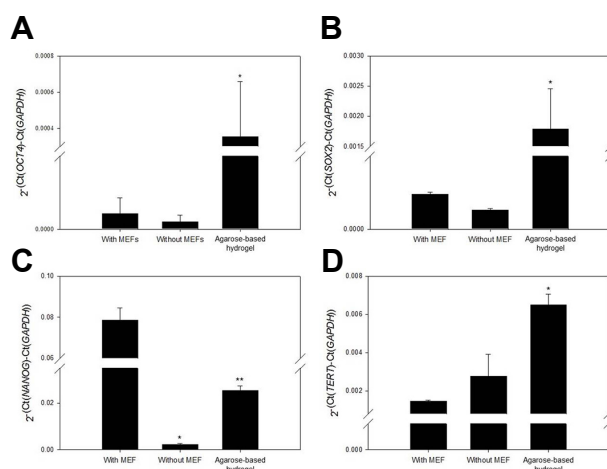


Fig. 2. Effects of the culture dimension *in vitro* on the transcript levels of self-renewal-related genes in porcine ICM-derived cells.

Porcine ICM-derived cells were cultured for 7 days on 2D culture plates coated with or without MEFs or in 1.5% (w/v) agarose-based 3D hydrogels. Subsequently, transcript levels of self-renewal-related genes were estimated by real-time PCR. Porcine ICM-derived cells in agarose-based 3D hydrogels showed significantly higher transcript levels of *OCT4* (A), *SOX2* (B), *NANOG* (C) and *TERT* (D) relative to those in 2D cultures without MEFs. The MEF-free agarose-based 3D hydrogel itself induced significant transcriptional up-regulation of *OCT4* (A), *SOX2* (B) and *TERT* (D) and down-regulation of *NANOG* (C) in porcine ICM-derived cells, compared with MEF-coated 2D culture plates. Error bars represent S.D. $n = 3$. *, ** $p < 0.05$.

was significantly down-regulated in 3D cultures, albeit still higher than in MEF-free 2D cultures. Subsequently, strong expression of OCT4, SOX2, NANOG, TRA-1-60, and TRA-1-81 proteins was detected in porcine ICM-derived cells cultured in both 2D cultures with MEFs and 3D cultures, with weaker expression in MEF-free 2D cultures (Supplementary Fig. S2). Furthermore, quantification of the expressed self-renewal-related proteins (Fig. 3) showed no significant differences in the protein expression of OCT4, NANOG and TRA-1-60 between porcine ICM-derived cells cultured in 2D with MEFs and porcine ICM-derived cells cultured in 3D. Rather, significant increases in SOX2 and TRA-1-81 proteins were observed in porcine ICM-derived cells cultured in agarose-based 3D hydrogels, relative to 2D cultures with MEFs. Porcine ICM-derived cells in MEF-free 2D culture microenvironments showed significantly lower protein expression of all self-renewal-related genes except *SOX2*. Thus, these results demonstrate that self-renewal of porcine ICM-derived cells can be maintained effectively, not only in 2D cultures with MEFs but also in 3D culture microenvironments, which appear to replace the need for MEFs in 2D cultures.

DISCUSSION

Here, we report the strong efficacy of 3D culture microenvironments for *in vitro* maintenance of porcine ICM-derived

Table 3. Effects of dimensional difference in *in vitro* culture on the maintenance of AP activity in porcine ICM-derived cells.

Dimension	Culture condition		No. of porcine ICM-derived cell colonies seeded	No. (%) ^a of porcine ICM-derived cell colonies ^b stained positively by AP staining
	Without (-) or With (+) MEFs			
2D	+		15	15 (100.0) ^c
	-		14	0 (0.0) ^d
3D	-		16	15 (93.8) ^c

Model effect of treatments in the number of colonies stained positively by AP staining was less than 0.0001 (p value).

^aPercentage of the number of porcine ICM-derived cell colonies seeded.

^bColonies were derived from porcine ICM-derived cells cultured for 7 days in the 3D hydrogels based on agarose with the specific concentration.

^{c,d}Different superscripts within a column are significantly different, $p < 0.0001$.

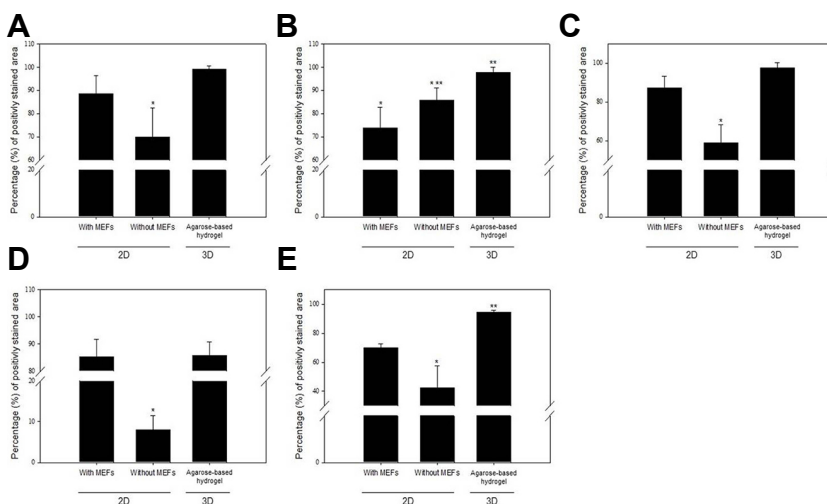


Fig. 3. The percentage of porcine ICM-derived cells expressing self-renewal-related proteins in 2D and 3D cultures. Porcine ICM-derived cells were cultured for 7 days on 2D plates with or without MEFs or in 1.5% (w/v) agarose-based 3D hydrogels and then immunostained for self-renewal-related proteins expressed in undifferentiated porcine ICM-derived cells. The percentage of porcine ICM-derived cells expressing self-renewal-related proteins within a colony was calculated by dividing the positively stained area of each colony by the total area of that colony. Porcine ICM-derived cells cultured in MEF-free 2D culture plates showed significantly lower percentages of cells positive for all self-

renewal-related proteins evaluated except SOX2 (B). There were no significant differences in the percentages of porcine ICM-derived cells expressing OCT4 (A), NANOG (C) and TRA-1-60 (D) between 2D cultures with MEFs and agarose-based 3D hydrogels; significantly more porcine ICM-derived cells cultured in agarose-based 3D hydrogels were positive for SOX2 (B) and TRA-1-81 (E) than in 2D cultures with MEFs. Error bars represent S.D. $n = 3$. *, ** $p < 0.05$.

cell self-renewal. Porcine ICM-derived cells cultured in 1.5% (w/v) agarose-based 3D hydrogels showed significantly greater maintenance of AP activity and of self-renewal-related mRNA and protein expression than did those in 2D MEF-free cultures. Moreover, compared with 2D cultures coated with MEFs, no significant decrease in AP activity or in self-renewal-related protein expression was seen in 1.5% (w/v) agarose-based 3D hydrogels; in fact, porcine ICM-derived cells in these gels showed significant transcriptional and translational up-regulation of such genes. Accordingly, we suggest that the culture dimension should be considered when establishing *in vitro* culture systems for specific cells. The 3D scaffold itself, without added ECM analogs providing extracellular signals, can replace a variety of such signals derived from feeder cells that support the maintenance of self-renewal in typical 2D culture systems.

The mechanical properties of agarose-based 3D hydrogels can be regulated by altering the agarose concentration (Ulrich et al., 2011). Low concentrations of agarose result in softer hydrogels, whereas high concentrations result in mechanically stronger hydrogels. In the present study, porcine

ICM-derived cells cultured in 1.5% (w/v) agarose-based hydrogels (the stiffest gels tested) showed the best AP activity and proliferation, indicating that porcine ICM-derived cells prefer a strong 3D scaffold to maintain their self-renewal. The effects of 3D hydrogel mechanics on stem cell self-renewal appear to vary among species and genetic backgrounds. Human (Dixon et al., 2014; Gerecht et al., 2007; Jang et al., 2013; Musah et al. 2012) and mouse (Lee et al., 2010; 2012) ESC self-renewal is maintained effectively using very strong (8-arm) and somewhat weak (4-arm) polyethylene glycol-based 3D hydrogels, respectively, where strength increases with the arm number. Therefore, we suggest that the optimal mechanical strength of scaffolds used in the construction of 3D microenvironments must be one of the factors considered in determining specific cell needs.

In previous studies, naive mouse ESCs formed colonies from single cells with spherical morphology and clear boundaries in 3D culture (Lee et al., 2012). However, irregular colony morphologies, dependent upon the shapes of cell clumps incorporated into 3D hydrogels, were observed in the culture of primed porcine ESC colonies from mechanical-

ly dissociated clumps (Supplementary Fig. S1) (Yuguo and David, 2013). This phenomenon was also observed in the culture of primed human ESCs, with characteristics similar to those of primed porcine ESCs in 3D culture (Jang et al., 2013; Lou et al., 2015). Therefore, we propose that the 3D microenvironment itself may be valuable in identifying pluripotent states (naïve or primed) of uncharacterized stem cells.

In this study, we have confirmed that self-renewal of porcine ICM-derived cells can be maintained more effectively in 3D microenvironments consisting of 1.5% (w/v) agarose-based 3D hydrogels than in 2D microenvironments, in the absence of any extracellular signals derived from ECM proteins. In addition, maintenance of porcine ICM-derived cell self-renewal *in vitro* should be possible by developing synthetic 3D microenvironments incorporating integrin signaling with ECM analogs to regulate cellular characteristics. These findings emphasize the importance of 3D microenvironments in the *in vitro* culture of various stem cells.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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