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Effects of Knockout Serum Replacement in the Culture Medium on the Proliferation of Porcine Fetal Fibroblasts In Vitro

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Human fibroblasts that maintain the structural integrity of connective tissues by secreting precursors of the extracellular matrix are typically cultured with serum. However, there are potential disadvantages of the use of serum including unnatural interactions between the cells and the potential for exposure to animal pathogens. To prevent the possible influence of serum on fibroblast cultures, we devised a serum-free growth method and present in vitro data that demonstrate its suitability for growing porcine fetal fibroblasts. These cells were grown under four different culture conditions: no serum (negative control), 10% fetal bovine serum (FBS, positive control), 10% knockout serum replacement (KSR) and 20% KSR in the medium. The proliferation rates and viabilities of the cells were investigated by counting the number of cells and trypan blue staining, respectively. The 10% FBS group showed the largest increase in the total number of cells (1.09×10^5 cells/ml). In terms of the rate of viable cells, the results from the KSR supplementation groups (20% KSR: 64.7%; 10% KSR: 80.6%) were similar to those from the 10% FBS group (68.5%). Moreover, supplementation with either 10% (3.0×10^4 cells/ml) or 20% KSR (4.8×10^4 cells/ml) produced similar cell growth rates. In conclusion, although KSR supplementation produces a lower cell proliferation rate than FBS, this growth condition is more effective for obtaining an appropriate number of viable porcine fetal fibroblasts in culture. Using KSR in fibroblast culture medium is thus a viable alternative to FBS.

Key words: fibroblast, serum replacement, serum-free culture

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Introduction

The mammalian cell culture has been applied to various fields. Viral vectors used for gene therapy and recombinant proteins such as erythropoietin are produced by mammalian cell culture to maintain the bioactivity required in producing such factors. Mammalian cell culture has been used in the large scale production of initially viral vaccines and more recently complex protein pharmaceuticals (Quek *et. al.*, 2009). Mammalian cells are the preferred production host for many biopharmaceuticals because complex post-translational modifications of the proteins (such as glycosylation) are generally not properly performed by microbial systems, although some progress has been made recently with 'humanized' yeasts (Wildt and Gerngross, 2005; Li *et. al.*, 2006). Furthermore, *ex vivo* culture of various cells, such as chondrocytes and immunocytes, has been performed for cell therapy (Masahiro *et. al.*, 2005).

Fibroblasts are the most common cells of connective tissue in animals and play a critical role in wound healing. Fibroblasts and its related cells are primarily responsible for creating an extracellular matrix that fulfills the need of a specific tissue. Connective tissue components are synthesized by fibroblasts and secreted into the environment where they polymerize into various supramolecular structures (Paul *et. al.*, 1992). Fibrillar collagens (type I, II, III and V) are major constituents of various organs such as skin, tendon, bone and cartilage (Paul *et. al.*, 1992). Human fibroblasts that maintain the structural integrity of connective

tissues by secreting precursors of the extracellular matrix have been often cultured with serum. However, potential disadvantages from the use of serum have been noted for several reasons. First, it is unnatural for fibroblasts to have contact with serum since somatic cells are not exposed to serum within a living organism. Under normal physiological conditions, only blood cells are surrounded by serum, and the use of serum for cultivation of other cell types may thus be questioned (Figenschau *et. al.*, 1997). Serum is an undefined substance containing multiple potential factors that might influence cell function in unknown ways. Serum may affect the function of the fibroblast because of a variety of factors included, such as polypeptides, hormones, growth factors, and cytokines. The content of them and soluble receptors for these is undefined (Figenschau *et. al.*, 1997). If animal serum is used in cell culture for human therapy, there is possible exposure to animal pathogens such as bacteria and viruses. The field of regenerative medicine offers the possibility of delivering new stem cell-based therapies for the treatment of diseases including Type II diabetes, Parkinson's and a variety of other neuro-degenerative conditions and injuries (Notara *et. al.*, 2007). There is, therefore, a need for safe, scalable and reproducible technologies to expand populations of adult and embryonic stem cells to meet potential clinical need. With growing concerns regarding the potential transmission of adventitious agents such as prions and animal viruses, it would be preferable to culture cells for human transplantation under xenobiotic-free conditions that can maintain stem cells (Notara *et. al.*, 2007). Medium formulations utilizing animal-derived serum may face regulatory barriers for any human clinical application. Related works in cartilage tissue engineering have demonstrated the additional benefit of serum-free medium, as these formulations typically resulted in improved construct maturation in comparison to serum-containing medium (Kisiday *et. al.*, 2005; Byers *et. al.*, 2008). Thus serum-free culture is implemented through medium including serum replacement and growth factors to prevent the possible influence of serum on the culture system (Amit *et. al.*, 2004).

In this study, the author presented *in vitro* data demonstrating the suitability of serum-free culture by comparing the differences between the growth of porcine fetal fibroblast after culturing with/without serum or serum replacement. This study was undertaken to find that serum-free culture could provide appropriate environment for the growth of porcine fibroblasts.

Materials and Methods

Chemicals

All inorganic and organic compounds other than sera and liquid solutions were purchased from Sigma-Aldrich Korea Ltd. (Yongin, Korea) and all liquid solutions and sera were purchased from Invitrogen-Gibco Korea (Seoul, Korea).

Fibroblast culture

Fibroblasts were obtained from porcine conceptuses that were collected between embryonic Days 28 and 39. The head, extremities, and internal organs were removed, and the remaining tissues were cut into small pieces. Cells were dispersed by exposure to 0.25% (w/v) trypsin solution and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS; F-DMEM). For each passage, cells were cultured until confluent, disaggregated by incubation in a 0.25% trypsin solution for 1 min at 37°C, and fractionated into three new dishes. Incubation was carried out at 37°C in humidified air with 5% CO_2. The primarily cultured cells after 6 passages, named P_6, were continued to culture to monitor its growing behavior before splitting to pass (Experiment 1). Total 10×10^4 cells were cultured in 35 mm dishes with 2 ml FDMEM (5×10^4 cells/ml), then the number of cells were counted from 3 different dishes everyday until Day 4 (starting day of culture = Day 0) for Experiment 1 and until Day 3 for Experiment 2. The cells were counted using hemocytometer. Based on the first series of experiment, Day 3 was set to be the point of time when cell counting began for Experiment 2. In the second series of experiment, the fibroblasts were cultured in four different groups of media for 3 days, and 5 culture dishes were prepared for each group. Four experimental groups were as follows: 1) Medium A: Dulbecco's modified Eagle's medium (DMEM) + 10,000 IU/l penicillin + 1 mg/l streptomycin; 2) Medium B: Medium A + 10% FBS (v/v); 3) Medium C: Medium A + 10% KSR (v/v); 4) Medium D: Medium A + 20% KSR (v/v). In the second series of experiment, only the cells attached on the bottom of the dish were harvested and counted to monitor cell proliferation and viability precisely. To fix the condition and number of attached cells, the cells were initially cultured in FBS supplemented medium for 24 h and moved to four different culture conditions on Day 1. The results were expressed by treatment as mean ± SD.

Cell viability test by Trypan blue staining

Trypan blue staining method was applied to test cell viability. Viable or dead cells on the hemocytometer were assessed under the inverted microscope (Olympus Korea, Seoul, Korea). The cells were dissociated from the dishes with the use of trypsin and neutralized with PBS. After centrifugation, the pellet was diluted in 1 ml PBS. The dispersed cell pellets of 200 μl were mixed with same volume of the trypan blue solution. As trypan blue was passed through cell membrane by diffusion, only viable cells were able to excrete this solution by exocytosis using ATP, and the non-stained viable cells were distinguished from stained dead ones.

Table 1. Proliferation of porcine fetal fibroblasts in culture*

Day of cell count	Cell population ($\times 10^4$ cells/ml ± SD)
Day 0	5 ± 0
Day 1	8 ± 3
Day 2	23 ± 1
Day 3	25 ± 0
Day 4	25 ± 1

*Five replicates.

Table 2. The number of cells cultured in FBS or KSR supplemented media

Medium	Cell population on Day 1* ($\times 10^3$ cells/ml ± SD)	Cell population on Day 3* ($\times 10^3$ cells/ml ± SD)
A (Control)	14 ± 0	9 ± 7
B (10% FBS)	14 ± 0	109 ± 30
C (10% KSR)	14 ± 0	30 ± 12
D (20% KSR)	14 ± 0	48 ± 13

*The cells only attached on the bottom of the dish were harvested and counted.

Results

Primary cell culture and passing

In the first series of experiment, counting of cultured cells was implemented everyday for 4 days. After Day 3, the number of attached cells became confluent and no longer grew well (Table 1) and Day 3 was chosen for the timing of cell counting in the next series of experiment.

Effects of KSR on the growth and viability of porcine fibroblasts

The results of total cell numbers and the proportion of viable cells are shown in Table 2 and Table 3, respectively.

Table 3. The viability of porcine fibroblasts cultured in FBS or KSR supplemented media*

Medium	Total**	Viable	Dead	Viability
	($\times 10^3$ cells/ml ± SD)			
A (Control)	13 ± 5	3 ± 2	10 ± 2	23.0%
B (10% FBS)	89 ± 21	61 ± 14	28 ± 8	68.5%
C (10% KSR)	36 ± 2	29 ± 2	7 ± 3	80.6%
D (20% KSR)	34 ± 9	22 ± 7	12 ± 3	64.7%

*Total 5×10^4 cells/ml were seeded on 35 mm dish in each group.
**The cell viability was checked on Day 3.

The highest total cell number was from Medium B supplemented with 10% FBS, and Medium D and C were followed (Table 2). Culture without serum or with serum replacement (Medium A) did not support to grow cells *in vitro*. The highest proportion of viable cells was shown in Medium C (10% KSR). Medium B and medium D showed similar cell viabilities. Medium A did not support the viability of the cells (Table 3, Fig. 1).

Discussion

In general, human fibroblasts are cultured in FBS supplemented medium which may expose them to animal pathogens. In this study, a defined serum-free culture system based on the use of KSR was demonstrated. There were four kinds of cultural systems that were implemented; no serum/serum replacement, 10% FBS, 10% KSR and 20% KSR. The 10% FBS group showed the highest increase in total cell number. Although KSR did not support cell proliferation when compared to FBS counterpart, the viabilities of the cells in KSR supplementation groups was similar to those in FBS supplementation group.

The FBS has been commonly used in cell culture, which

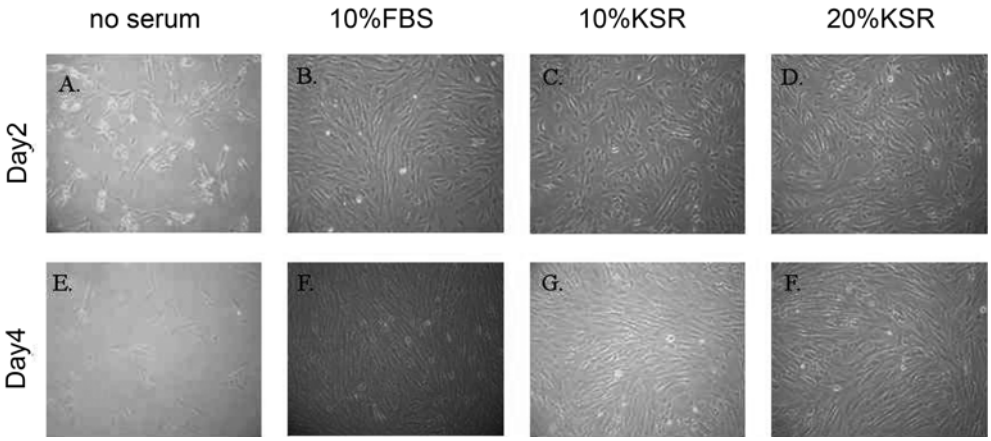

Fig. 1. *In vitro* growth of porcine fetal fibroblast cells cultured in serum, serum-free and knockout serum replacement (KSR) environments from Day 2 to Day 4. Representative photomicrographs of attached cells cultured in the media supplemented with: no serum (A, E), 10% FBS (B, F), 10% KSR (C, G), and 20% KSR (D, F) for 2 and 4 days, respectively. Medium without serum did not support the viability of the cells.

plays important role (s) in nutrition for cultured cells *in vitro*. However, it contains many undefined factors, which promote growth and differentiation of embryonic stem (ES) cells (Bettiol *et. al.*, 2007). It has been also reported that the uptake and the expression of FBS-derived materials on the cell surface may induce immune responses upon transplantation (Martin *et. al.*, 2005). In addition, overall variation in the lots of FBS is significant, and each lot must be screened prior to use. Hemoglobin and endotoxin levels in FBS are also noted for the similar response as well. A currently growing concern throughout European countries is that animal-derived adventitious agents such as bovine spongiform encephalitis, called BSE, cannot be detected effectively using an *in vitro* diagnostic method. For that reason, a defined, serum-free alternative would be a preferred approach for therapeutic applications (Notara *et. al.*, 2007). All of the drawbacks of serum-supplemented ES cell culture, including heat-inactivation of FBS, can be alleviated with the use of KSR. The KSR is a medium supplement widely used for the serum-free culture of ES cells. Its defined formulation provides consistent growth conditions for ES cell cultures (Goldsborough *et. al.*, 1998; Cheng *et. al.*, 2004). This culture system is an advanced step taken to derive completely defined culture conditions for human cells, and will promote further development of a xeno-free culture system for human cells. Because KSR for replacement of serum in this study resulted in lower cell proliferation rates than FBS supplementation, addition of growth factors or other substances in KSR will be considered for the clinical application. Growth factor supplementation can affect maturation of tissue engineered constructs (Byers *et. al.*, 2008). Although the KSR used in the present study is not entirely animal-free because it contains Albumax^{TM}, a lipid-enriched bovine serum albumin, more defined cell culture condition should give more information to make a completely defined animal- and serum-free human cell culture system which is important for the use of the cells in regenerative medicine or cell therapy.

In conclusion, although KSR supplementation showed lower cell proliferation rates than FBS counterpart, it was the effective in obtaining appropriate number of viable porcine fetal fibroblasts in culture. Therefore, replacing serum with KSR may allow avoiding potential/unknown risking factors caused by serum in culture.

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References

Amit M, Shariki C, Margulets V, Itskovitz-Eldor J. Feeder layer- and serum-free culture of human embryonic stem cells. Biol of Reprod. 2004;70:837-45.

Bettiol E, Sartiani L, Chicha L, Krause KH, Cerbai E, Jaconi ME. Fetal bovine serum enables cardiac differentiation of human embryonic stem cells. Differentiation. 2007;75: 669-81.

Byers BA, Mauck RL, Chiang IE, Tuan RS. Transient exposure to transforming growth factor beta 3 under serum-free conditions enhances the biomechanical and biochemical maturation of tissue-engineered cartilage. Tissue Eng Part A. 2008;14:1821-34.

Contard P, Jacobs L 2nd, Perlish JS, Fleischmajer R. Collagen fibrillogenesis in a three-dimensional fibroblast cell culture system. Cell Tissue Res. 1993;273:571-5.

Cheng J, Dutra A, Takesono A, Garrett-Beal L, Schwartzberg PL. Improved generation of C57BL/6J mouse embryonic stem cells in a defined serum-free media. Genesis. 2004;39: 100-4.

Figenschau Y, Sundsfjord JA, Yousef MI, Fuskevag OM, Sveinbjornsson B, Bertheussen K. A simplified serum-free method for preparation and cultivation of human granulosa-luteal cells. Hum Reprod. 1997;12:523-31.

Goldsborough MD, Tilkins ML, Price PJ Lobo-Alfonso J, Morrison JR, Stevens ME, Meneses J, Pedersen R, Koller B, Latour A. Serum-free culture of murine embryonic stem (ES) cells. Focus. 1998;20:8-12.

Kishi Y, Inoue M, Tanaka Y, Shibata H, Masuda S, Ikeda T, Hasegawa M, Hanazono Y. Knockout serum replacement (KSR) has a suppressive effect on sendai virus-mediated transduction of cynomolgus ES cells. Cloning Stem Cells. 2008;10:307-12.

Kisiday JD, Kurz B, DiMicco MA, Grodzinsky AJ. Evaluation of medium supplemented with insulin-transferrin-selenium for culture of primary bovine calf chondrocytes in three-dimensional hydrogel scaffolds. Tissue Eng. 2005;11:141- 51.

Koch R, Goode RL, Simpson GT. Serum-free keloid fibroblast cell culture: an *in vitro* model for the study of aberrant wound healing. Plastic Reconstr Surg. 1997;99:1094-8.

Lima EG, Bian L, Ng KW, Mauck RL, Byers BA, Tuan RS, Ateshian GA, Hung CT. The beneficial effect of delayed compressive loading on tissue-engineered cartilage constructs cultured with TGF-beta3. Osteo Arthritis and Cartilage. 2007;15:1025-33.

Li HJ, Sethuraman N, Stadheim TA, Zha D, Prinz B, Ballew N, Bobrowicz P, Chio BK, Cook WJ, Cukan M, Houston-Cummings NR, Davidson R, Gong B, Hamilton SR, Hoopes JP, Jiang Y, Kim N, Mansfield R, Nett JG, Rios S, Strawbridge R, Wildt S, Gerngross TU. Optimization of humanized IgGs in glycoengineered Pichia pastoris. Nat Biotechnol. 2006; 24:210-5.

Martin MJ, Muotri A, Gage F, Varki A. Human embryonic stem cells express an immunogenic nonhuman sialic acid. Nat Med. 2005;11:228-32.

Notara M, Haddow DB, MacNeil S, Dniels JT. A xenobiotic-free culture system for human limbal epithelial stem cells.

Regen Med. 2007;2:919-27.
Quek LE, Dietmair S, Kromer JO, Nielsen LK. Metabolic flux analysis in mammalian cell culture. Metab Eng. 2009;10:1-10.
Risbud MV, Di Martino A, Guttapalli A, Seghatoleslami R, Denaro V, Vaccaro AR, Albert TJ, Shapiro IM. Toward an optimum system for intervertebral disc organ culture: TGF-beta 3 enhances nucleus pulposus and anulus fibrosus survival and function through modulation of TGF-beta-R expression and ERK signaling. Spine. 2006;31:884-90.
Sasaki M, Kato Y, Yamada H, Terada S. Development of a novel serum-free freezing medium for mammalian cells using the silk protein sericin. Biotechnol Appl Biochem. 2005;42:183-8.
Wildt S, Gerngross TU. The humanization of N-glycosylation pathways in yeast. Nat Rev Microbiol. 2005;3:119-28.