Growth Factors Supplementation in Culture Medium Leads to Active Proliferation of Porcine Fibroblasts

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ABSTRACT

Fibroblasts of large animals are easy to isolate and to maintain *in vitro* culture. Thus, these cells are extensively applied to donor cell for somatic cell nuclear transfer, and to substrate cells to generate induced pluripotent stem cells after transfection of required genes to be essentially required for direct reprogramming. However, limited mitotic activity of fibroblasts to differentiate along a terminal lineage becomes restrictive for their versatile application. Recently, commercial culture medium and systems developed for primary cells are provided by manufactures. In this study, we examined whether one of the systems developed for primary fibroblasts of human are effective on porcine ear skin fibroblasts. To this end, we performed proliferation assay after five days culture *in vitro* of porcine fibroblasts in medium DMEM, which is generally used for fibroblasts culture, and medium M106 for human dermal fibroblasts, supplemented with various concentrations of FBS and LSGS contained mainly growth factors, respectively. Consequence was that presence of 15% FBS and 0.1 X concentrations of LSGS in DMEM showed most active proliferation of porcine fibroblasts.

(Key words : Fibroblasts, Culture medium, Proliferation)

INTRODUCTION

Fibroblasts, an essential component of skin are connective tissue cells and synthesize extracellular matrix. Different subpopulations of fibroblasts exists in skin, of which the most are derived from two distinct dermal layers, papillary and reticular dermis (Sorrell and Caplan, 2004). *In vitro* culture separately of fibroblasts from each of these layers shows different growth potential (Harper and Grove, 1979). Skin also harbors multilineage differentiation potential stem- and/or -like cells (Alt *et al.*, 2011; Dyce *et al.*, 2004; Huang *et al.*, 2010; Lermen *et al.*, 2010; Lorenz *et al.*, 2008).

Pig skin has been considered as biomedical animal model for human therapeutic applications because of its similarity of histological and physiological properties to human (Dick and Scott, 1992; Jacobi *et al.*, 2007; Simon and Maibach, 2000). Furthermore, generation of fetal and skin fibroblast derived induced pluripotent stem cells by direct reprogramming from mouse (Takahashi and Yamanaka, 2006; Wernig *et al.*, 2007), human (Takahashi *et al.*, 2007; Yu *et al.*, 2007) and pig (West *et al.*, 2010) might potentially permit pig skin fibroblasts to be provided as accessible sources not only to study on stem cell therapies for human diseases but also to investigate cell differentiation and embryonic development, cell fate and pathways involved in cell differentiation and embryonic development.

Since Campbell *et al.*(1996) reported initially generation of cloned sheep by nuclear transfer of primary somatic cells, the approach to generating knock-out large animal has been become available (McCreath *et al.*, 2000). Eventually, an important advance has been made by nuclear transfer technology to generate of α1,3-galactosyltransferase knock-out pigs, a highly relevant step for xenotransplantation (Ahn *et al.*, 2011; Dai *et al.*, 2002; Harrison *et al.*, 2004; Lai *et al.*, 2002; Phelps *et al.*, 2003; Ramsoondar *et al.*, 2003; Takahagi *et al.*, 2005). Skin fibroblasts of pig have been most widely applied for generation of genetically modified donor cells.

Primary fibroblasts isolated from porcine skin consist of heterogeneous cells, which have different growth potentials and longevities, consequently resulting in different colony size when their individual cells are cultured *in vitro* (Smith *et al.*, 1978; Smith and Whitney, 1980) and finally, progressing differentiation along a terminal lineage when cultured *in vitro* for a long peri-

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od (Bayreuther *et al.*, 1988). Given that introduction of a foreign gene including a gene targeting vector into fibroblasts and then selection of clones a foreign gene-introduced are prerequisite steps prior to choosing donor cell clones for nuclear transfer, suitable *in vitro* culture conditions and microenvironment should be provided for porcine fibroblasts to prolong their growth potentials. In this study, we examined influence of serum and growth factors contained low serum growth supplement (LSGS) on fibroblast proliferation under basal medium DMEM and M106. Consequence was that presence of 15% FBS and 0.1 X concentrations of LSGS in DMEM showed most active proliferation of porcine fibroblasts.

MATERIALS AND METHODS

Isolation of Porcine Fibroblasts

Fibroblasts were isolated from ear skin of 10-day old NIH miniature pig. Biopsied skin tissues were washed with HBSS containing 1% antibiotic-antimycotic (Invitrogen, CA, USA), and then removed crudely the epidermis and cartilage. Subsequently, the tissues cut into small pieces were transferred to 6cm dishes and maintained in culture medium. The proliferating fibroblasts, which were observed after 4~5 days of tissue culture, were maintained on culture vessels up to 80~90% confluence, and were frozen for further experiments. The skin tissue culture medium was Dulbecco's modified Eagle's medium (DMEM; Invitrogen, CA, USA), supplemented with 15% fetal bovine serum (Invitrogen), 1% non-essential amino acids (Invitrogen), 0.1 mM β-mercaptoethanol (Invitrogen), and 1% antibiotic/antimycotic (Invitrogen).

Porcine Fibroblasts Culture for Proliferation Assay

Frozen fibroblasts were cultured in tissue culture medium for 2~3 days to make the cells restored to normal growth phase. Subsequently the cells were trypsinized and seeded into 96-well culture vessel at density of 2×10^3 cells of a well. The cells were maintained for 5 days on different culture mediums, are supplemented with various concentrations of FBS and LSGS into DMEM and M106 (Invitrogen), respectively, as shown in Table 1. Proliferation assays were performed daily up to 5 day in triplicate.

WST-1 Cell Proliferation Assay

Cell proliferation assay was performed using WST-1 cell proliferation assay system (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. Briefly, 10 μl Premix WAT-1 solution was added into a well of 96 culture vessels keeping the cells seeded with 100 μl culture medium. Subsequently, the cells were incubated for 1 hour at 37℃. The quantity of formazan was measured using a Benchmark Plus microplate spectrophotometer (Bio-Rad, CA, USA) at a wavelength of 450 nm.

Coomassie Blue Staining of Fibroblast

Fibroblasts were seeded into 24-well culture vessel coated with gelatin for 24 hours and cultured up to 5 days. For staining, the cells were washed with 1X phosphate buffered saline (PBS) three times and fixed by 4% paraformaldehyde (Sigma, MO, USA) for 30 minutes. The fixed cells were washed with PBS three times and stained by 1X coomassie blue solution (Bio-Rad). The stained images were photographed using a distal camera (Canon G12; Canon Inc, Japan) coupled to a microscope (Axiovert 40 CFL; Carl Zeiss, Göttingen, Germany).

RESULTS AND DISCUSSION

It is well known that serum in culture medium provides a complex mixture of hormones, nutrients, growth factors which is required to support cell proliferation of common cells, including primary cells (Orly and Sato, 1979; Savion *et al.*, 1981). Indeed, (Ramirez *et al.*, 2001) demonstrated that serum addition into culture medium were able to lead to rescue skin fibroblasts, which are arrested by *in vitro* culture in chemically defined medium with 0.25% serum, to become actively proliferative. Although 10% serum is widely used for common cell culture, yet suboptimal concentration of

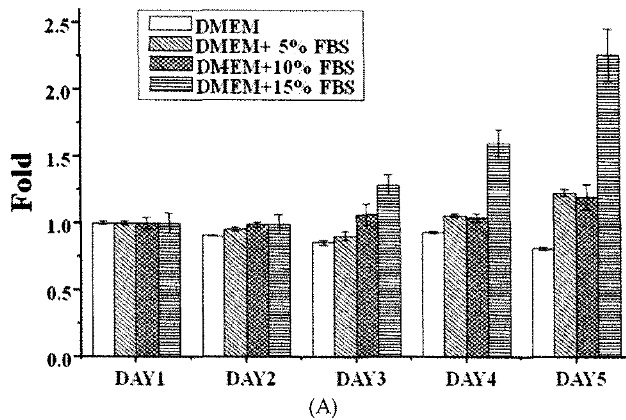
Table 1. Composition of culture medium used in this study

Basal medium	DMEM				M106			
Concentration of FBS (%)	0	5	10	15	0	5	10	15
Concentration of LSGS	0	0	0	0	0	1X	1X	1X
	0.1X*	0.1X	0.1X	0.1X				
	0.5X	0.5X	0.5X	0.5X				
	1X	1X	1X	1X	1X			

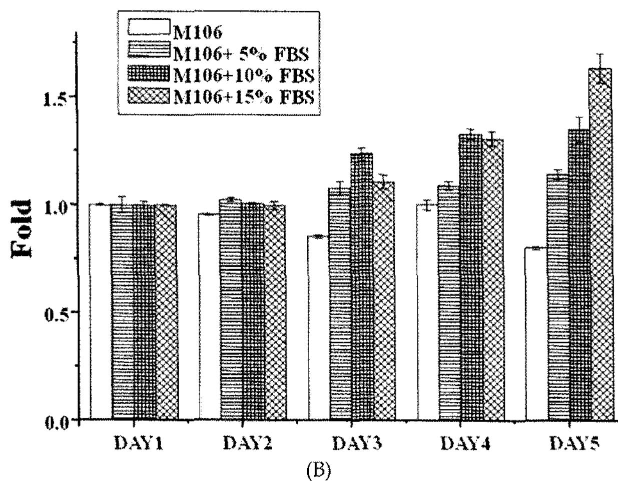
* LSGS was provided with 50X concentrated solution

serum for the porcine ear skin fibroblasts has not been exactly determined.

The basal medium DMEM is commonly used for *in vitro* culture of primary fibroblasts. Alternatively, basal medium M106 is provided for culture of human dermal fibroblasts by a manufacture. To find suboptimal concentration of serum, we performed cell culture up to five days under DMEM medium supplemented with different concentrations of fetal bovine serum (FBS) 5%, 10% and 15%, respectively. Absence of FBS into DMEM showed no proliferation of porcine fibroblasts through 5-days culture. Ultimately, addition of 15% FBS into DMEM allowed dramatic increase of cell numbers at day 5 culture compared to those from 5% and 10%, showing 2.26±0.19, 1.22±0.02, and 1.19±0.09 proliferation rates relative to those at day 1 control culture, respectively (Fig. 1A), indicating that presence of serum in DMEM is essentially required for porcine fibroblasts.



(A)



(B)

Fig. 1. Influence of different concentrations of serum on proliferation of porcine fibroblasts. Porcine fibroblasts were cultured in medium DMEM (A) and M106 (B) supplemented with 5%, 10%, and 25% FBS, respectively. Proliferation assay was performed daily up to 5 days in triplicate. The value was indicated as relative fold compared to that of day 1. Standard deviation was represented on the bar.

Next, we investigated whether M106 medium is suitable to and presence of FBS in that medium affects proliferation of porcine fibroblasts. Results indicated that only 15% FBS influenced slightly porcine fibroblast proliferation through 5-days culture, showing 1.63±0.06 proliferation rate at day 5 (Fig. 1B).

According to manufacturer's instruction, M106 for the culture of human dermal fibroblast requires addition of LSGS, which is concentrated at 50X and includes the components of 2% FBS, 1 μg/ml hydrocortisone, 10 ng/ml epidermal growth factor (EGF), 3 ng/ml basic fibroblast growth factor (bFGF), and 10 μg/ml heparin at 1X final concentrations. The proliferation rates by addition of 1X LSGS solution into M106 were 1.07±0.06, 1.13±0.12, 1.48±0.07, and 1.79±0.03 at day 2, day 3, day 4, and day 5, respectively (Fig. 2). Importantly, presence of FBS in LSGS supplemented M106 led to acceleration of cell proliferation (2.74±0.31 proliferation rate at day 5 by additions of 15% FBS and LSGS into M-106; Fig. 2), likely that either LSGS or FBS is not sufficient for active proliferation of porcine fibroblasts, but both of LSGS or FBS are synergistically influenced on fibroblasts to be more actively proliferated.

To determine whether presence of LSGS and FBS in DMEM lead to synergistic influence on fibroblast proliferation, we cultured porcine fibroblasts for 5 days in DMEM supplemented with different concentrations of LSGS and FBS. As shown in Fig. 3A, single addition of 1X concentration of LSGS showed as many increased proliferation rate (2.62±0.14 at day 5) as single addition of 15% FBS did (2.26±0.19 at day 5, see Fig. 1A). Inter-

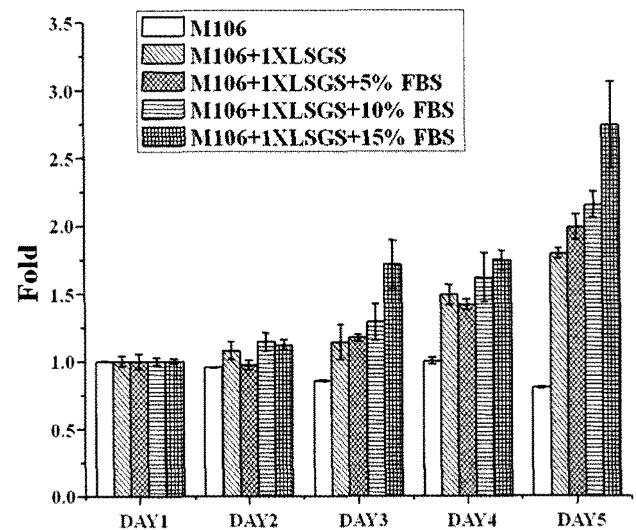


Fig. 2. Influence of different concentrations of serum supplemented into 1X LSGS contained M106 medium on proliferation of porcine fibroblasts. Concentrations of supplemented FBS were indicated above graph. Proliferation assay was performed daily up to 5 days in triplicate. The value was indicated as relative fold compared to that of day 1. Standard deviation was represented.

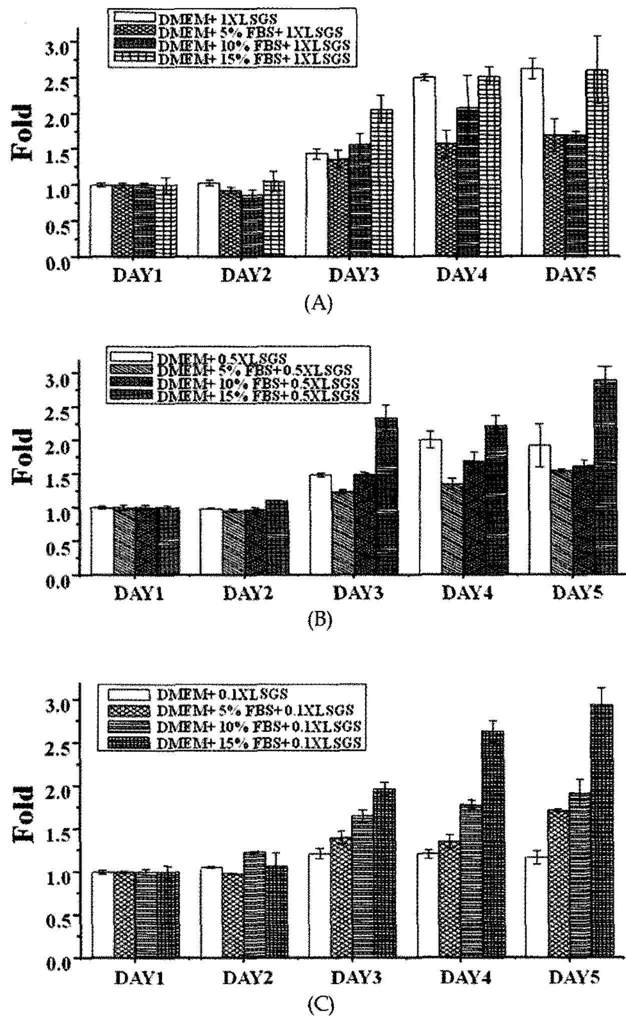
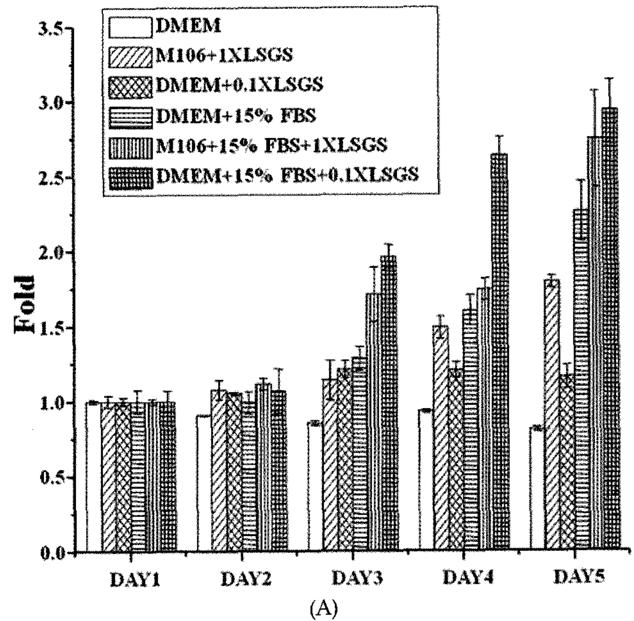


Fig. 3. Influence of combinations of different serum concentrations on proliferation of porcine fibroblasts. Porcine fibroblasts were cultured in 5%, 10%, and 15% FBS supplemented into 1X LSGS (A), 0.5X LSGS (B), and 0.1X LSGS contained DMEM, respectively. Proliferation assay was performed daily up to 5 days in triplicate. The value was indicated as relative fold compared to that of day 1. Standard deviation was represented.

estingly, presence of 5% and 10% FBS into 0.5X and 1X LSGS in DMEM, respectively, did not showed synergistic influence on proliferation of the cells, suggesting that combination at suboptimal concentrations of LSGS and FBS are important for active proliferation of fibroblasts. The combination of 0.1X LSGS and 15% FBS in DMEM led to highest proliferation rates of porcine fibroblast (2.93±0.2 at day 5; Fig. 3C).

Yu *et al.*(2011) reported presence of EGF and bFGF in culture medium for 7 days resulted in 2-times greater proliferation than in control cultures of human dermal fibroblasts. They got results by applying 2, 10 or 50 μg/ml of EGF and 2 and 10 μg/ml of bFGF into 10 % FBS contained culture medium. In addition, Zhu *et al.*(2004) demonstrated that population doubling time cul-



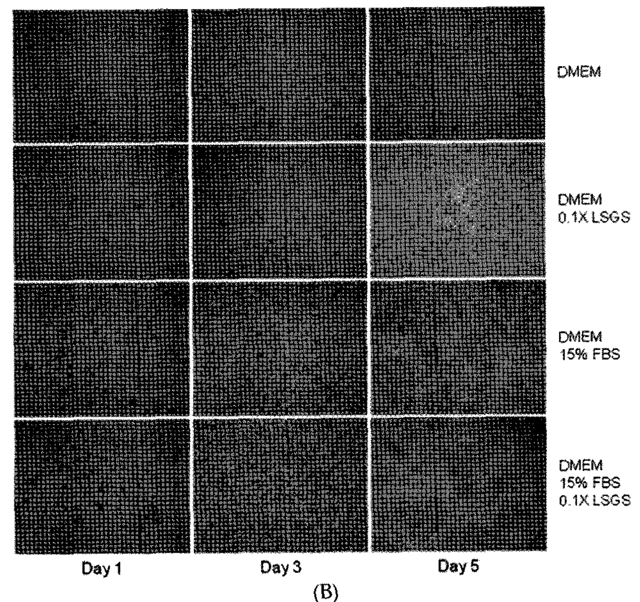


Fig. 4. (A) Summary of overall data shown in Fig. 1, 2, and 3. Importantly, culture conditions of 0.1X LSGS and 15% FBS in DMEM, and 1X LSGS and 15% FBS in M106 showed higher proliferation rates. (B) Proliferating fibroblasts were visualized by coomassie blue staining.

tured in 15% FBS of porcine fetal fibroblasts was shorter than that in 10% FBS, whereas presence of 20% FBS did not further increase fibroblast growth rate. In this study, we used LSGS additive contained EGF and b-FGF at concentrations of 10 ng/ml, and 3 ng/ml, respectively, although other components are included. As shown to Fig. 4A, which showed overall data in this study, supplementation of 0.1X and 1X LSGS into 15% FBS contained culture medium DMEM and M106 led

to maximum proliferation rates of porcine fibroblasts as 2.74±0.31 and 2.93±0.2, respectively. Consequently, these results implied that additional supplementation of growth factors at low concentrations into 15% FBS contained culture medium could further facilitate proliferation of porcine fibroblasts. With regard to costs required for cell culture, we suggest that 0.1X LSGS and 15% FBS in DMEM culture medium (Fig. 4B) is more valuable than 1X LSGS and 15% FBS in M106.

Prolonged growth potential during *in vitro* culture for long period is essential to extend versatile applications of fibroblasts. In this study, we examined proliferation rates of porcine fibroblasts by short period culturing up to 5 days at different culture conditions. Instead, whether 0.1X LSGS and 15% FBS in DMEM culture condition is able to lead to proliferative state of long period cultured porcine fibroblasts is still remain elusive.

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