Influence of tissue origins and external microenvironment on porcine foetal fibroblast growth, proliferative life span and genome stability

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Abstract. One of the challenges of manipulating genes in primary cells is that the cells have a finite proliferation capacity. This, combined with the lower gene targeting efficiency of somatic cells, makes identification of targeted clones very difficult. The objective of this study was to establish a system that allows porcine foetal fibroblasts to reach their maximal proliferation capacity *in vitro*. The influence of fibroblast origin, stage of foetal development, cell seeding densities and concentration of foetal bovine serum (FBS) on the population doublings, the percentage of β -galactosidase-activity-positive cells and the genome stability of foetal fibroblasts during *in vitro* culture was investigated. It was found that porcine foetal fibroblasts could be cultured for over 80 population doublings in the appropriate culture system. Fibroblasts from earlier stages of foetal development were better candidate cells than those from the later stages. Cells from the heart were more actively proliferative and more resistant to replicative senescence than those from the liver. Compared to 10% FBS content, 15% FBS provided better homeostatic support, not only to proliferative performance, but also in maintaining a normal karyotype. In addition, the proliferative life span of porcine foetal fibroblasts is also dependent on seeding density of the culture.

INTRODUCTION

Since the first report of somatic cell nuclear transfer in sheep (cloning), using a differentiated cell line (Campbell *et al.* 1996), this approach in farm animals has developed and advanced. The establishment of the technology has provided new opportunities for the production of transgenic animals using site-specific genetic modifications. An ideal system for producing transgenic livestock is 'gene targeting' in combination with nuclear transfer. The system is advantageous over DNA microinjection in that insertion of exogenous DNA sequences into a predetermined site may confer high levels of gene expression, preferably without affecting the general viability and productivity of the animal.

Gene targeting involves transfection then selection of the targeted donor cells, which necessitates the cells being grown in culture during the period of selection. In contrast to embryonic stem cells, somatic cells have a limited proliferative life span when cultured *in vitro* (Hayflick

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& Moorhead 1961; Nicole *et al.* 2001). In addition, it has been generally accepted that both culture environmental conditions and genetic factors appear to influence the proliferative life span of somatic cells (Cristofalo *et al.* 1989; Cristofalo & Pignolo 1993). Several recent studies have suggested that this limit to life span is perhaps more an artifact of culture than an inherent cellular property (Sherr & DePinho 2000; Nicole *et al.* 2001; Tang *et al.* 2001). Interestingly, it has been shown that normal rodent Schwann cells do not undergo replicative senescence when cultured in certain settings (Nicole *et al.* 2001), raising the possibility that somatic cells may proliferate indefinitely if the optimal culture conditions were identified. It is therefore important to establish an appropriate culture system that allows somatic cells to reach their maximal proliferative capacity so that transfected and targeted cells could be maximized during the selection period prior to nuclear transfer.

Our hypothesis was that somatic cells with different internal microenvironments have different proliferative capabilities and that their life spans would be influenced by external microenvironment *in vitro*.

MATERIALS AND METHODS

Cell isolation and culture

Yorkshire pig foetal fibroblasts were isolated as previously described (Boquest *et al.* 1999) with modifications. For organ-dependent experiments, fibroblasts were obtained from fetuses of 55 days' gestation, and fibroblasts used in further experiments in the current study were obtained from fetuses of 22 days' gestation. Foetal skin, lung, heart, brain, liver and uterus or whole foetal carcasses were washed twice with phosphate-buffered saline (PBS) and finely chopped (1–2 mm³) using scissors. Tissue pieces were then suspended in 6 ml of Dulbecco's modified Eagle's medium (DMEM; Gibco Burlington, Canada) supplemented with penicillin (100 IU/ml; Gibco) and streptomycin (100 µg/ml; Gibco) and 10% foetal bovine serum (FBS; Gibco), in tissue culture dishes (100 mm × 15 mm). Explants from each fetus were cultured individually under 5% CO₂ in air at 38.5 °C. Cells migrated from the tissue pieces onto the surface of tissue culture dishes. The floating pieces of tissues were removed by aspiration on day 5–6 of culture and monolayers of fibroblast-like cells were harvested with 0.05% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA; Gibco) and washed with DMEM. Each aliquot of these cells was resuspended in a freezing medium [10% dimethyl sulfoxide (Sigma, St. Louis, MO, USA), 10% FBS in DMEM], and stored in liquid nitrogen as passage 0 until use. Subsequently, aliquots of the cells were cultured and passaged in the medium described above.

Cell subculture and passaging

Cells were cultured then trypsinized (0.05% trypsin, 0.02% EDTA) and washed when they reached subconfluency (about 80%). To identify the initial plating density that facilitates cell proliferation, plating density experiments were performed with low density (5×10^4 and 1×10^5) and high density (8×10^4) (culture dish growth area 56 cm²). Cells were counted using a haemocytometer. In FBS concentration effect experiments, cells from two groups were subcultured at the same time interval (from 48 to 96 h).

The number of population doublings (PD) was calculated using the following formula (Romanov *et al.* 2001):

$$PD = \log[A/(BC)]/\log 2$$

where A is the number of collected cells, B is the number of plated cells, and C is the attachment efficiency (efficiency of cell attachment 12 h postplating). Accumulated population doubling was the sum of all the previous population doublings.

$$\text{Doubling time} = \text{Cultural period (hours)}/\text{PD}$$

β -galactosidase activity test

β -galactosidase (β -gal) activity was detected as previously described (Serrano *et al.* 1997; Wei & Sedivy 1999) with minor modifications. When cultured cells reached subconfluency, culture medium was removed and cells were washed with PBS and fixed (2% formaldehyde, 0.2% glutaraldehyde in PBS) for 15 min at room temperature. After fixing, cells were washed twice with PBS, and incubated overnight in staining solution [40 mM citric acid/sodium phosphate (pH 6), 0.15 M NaCl, 2 mM MgCl_2 , 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 1 mg/ml of 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside] at 38.5 °C. Cells staining blue were scored as β -gal-positive cells.

Chromosome analysis

Chromosomes were analysed by standard karyotypic analysis. Briefly, thymidine was added to the culture medium to a final concentration of 1.2 mg/ml when cultured cells were ~60% confluent, after overnight incubation at 5% CO_2 and 38.5 °C. Culture medium was removed and the cells were washed with PBS. Cells were cultured in fresh medium for 6 h then trypsinized. The harvested cells were incubated in 6 ml hypotonic solution (1 : 6 dilution of serum in distilled H_2O) for 20 min at 38.5 °C, then fixed in 1 ml solution (methanol : acetic acid, 3 : 1) and centrifuged immediately at 500 *g* for 7 min. Cells were resuspended in fixative solution (4 °C) and were then dropped on cold (4 °C), dry, methanol-cleaned slides for smear preparation. Slides were air-dried and stained with 1.5% Giemsa solution (EM Science Darmstadt, Germany) for 5 min. The morphology of chromosomes was examined using a digital image system (Openlab 2.25, Improvion Ltd, Guelph, Canada) in conjunction with light microscopy. Chromosome number was determined from a minimum of 100 separate spreads from each sample.

Statistics

Cells from one to three fetuses were used in each experiment. Experiments were repeated three or four times with cells isolated from fetuses after different gestation periods. The data were analysed using SAS (version 8), as a randomized complete block design in which blocks were independent experiments. Differences among different groups were determined using the Tukey–Kramer Multiple Comparisons test. Statistical significance was inferred at $P < 0.05$.

RESULTS

Influence of cell origin on proliferation

Fibroblasts and granulosa cells are the most commonly used donor cells in nuclear transfer. In our preliminary experiments, granulosa cells divided only three times before entering senescence *in vitro*. They are therefore not suitable candidates for gene-manipulation. Fibroblasts were then chosen in our subsequent experiments.

To study whether fibroblasts of varying origin have different proliferating performances *in vitro*, foetal fibroblasts from skin, lung, heart, brain, liver, uterus and carcass were isolated and

cultured *in vitro*. As seen in Fig. 1(a), the cumulative population doubling of fibroblasts from foetal skin, lung, carcass, heart, brain and liver were 13.2 ± 1 , 12.7 ± 1 , 11.3 ± 0.7 , 15.0 ± 0.5 , 10.9 ± 0.1 and 8.4 ± 0.2 , by between 30 and 35 days of culture. Growth curves were measured using a linear regression of cumulative population doubling on the appropriate days. Statistical analysis revealed that slopes of growth curves were significantly different between fibroblasts from heart and those from liver ($P < 0.05$). The time required for the fibroblasts to double their number in culture (population doubling time) was longest in fibroblasts from liver and shortest in those from heart. (Fig. 1b; $P < 0.05$)

Influence of foetal development stage on fibroblast proliferation

Fibroblasts isolated from foetuses at 22 and 55 days of gestation, respectively, were cultured for 10 passages and population doubling times were determined. The average population doubling time of fibroblasts from the 22-day foetuses was much shorter than that from the 55-day foetuses (27.7 ± 0.5 h vs. 65.6 ± 3.4 h; $P < 0.01$). These data suggested that fibroblasts from an earlier stage of foetal development were proliferatively more active compared to those from a later stage.

Concentration of FBS has a impact on the proliferation of foetal fibroblast

Data from our study also showed that FBS concentration influenced fibroblast proliferation. In the early stages (passage 1–20), the population doubling times for fibroblasts cultured in the presence of 10% and 15% FBS were 36.6 ± 1.0 and 31.5 ± 0.9 h, respectively ($P = 0.2$). The difference in doubling time between cells in 10% and 15% FBS cultures became significant when they were at their mid- (passage 21–40; $P < 0.05$) and late- (passage 41–70; $P < 0.05$) stages of culture (Fig. 2a), suggesting that there may be a greater proportion of cells staying in the proliferative stage when cultured in 15% serum. There were also significant differences between the slopes of their growth curves ($P < 0.01$; Fig. 2b). A higher concentration of FBS (20%), however, did not further increase fibroblast growth rate (data not shown).

The *in vitro* proliferation life span of porcine foetal fibroblasts is dependent on seeding density

Cell–cell interaction plays an important role in the control of cell growth and responses of cells to extracellular signals (Lallemand *et al.* 1998; Richardson *et al.* 1999; Cheng *et al.* 2001). To identify the initial plating density that facilitates cell proliferation, plating density experiments were performed. Cells seeded at low density (5×10^4 and 1×10^5) underwent fewer than 40 population doublings before entering a growth plateau, when cells were larger in size, flat in shape and became vacuolated. The proliferation capability of fibroblasts was plating-density-dependent (Fig. 3), they reached growth plateau at 18.0 ± 0.7 (5×10^4) and 37.3 ± 2.3 (1×10^5) population doublings, respectively. However, when cells were seeded at 8×10^5 , fibroblasts could divide up to almost 100 times (Fig. 3). These experiments suggest that a minimum starting density is required to maintain the proliferation capability of fibroblasts *in vitro*. Of the three cell lines used in the 8×10^5 seeding density study, one became senescence at 161 population doublings and the other two became senescent at 92 and 86 population doublings, respectively.

Figure 1. Influence of tissue-origin on foetal fibroblast proliferation. Fibroblasts from different origins were plated at a seeding density of at 8×10^5 on a culture dish with growth area of 56 cm². Cells were counted and subcultured when they reached 80% confluence in culture. The parameters evaluated included cumulative population doubling (a) and average population doubling time (b) Data expressed as mean $\pm$ SEM of three experiments. Letter subscripts indicate statistical significant differences ($P < 0.05$). H, Heart; S, Skin; Lu, Lung; Ca, Carcass; B, Brain; L, Liver.

Figure 2. The influence of FBS concentration on the proliferation of foetal fibroblasts. Fibroblasts were cultured in DMEM with 10% or 15% FBS. Cells were counted and passaged at 48–72-h intervals. (a) Population doubling time: average population doubling time of early passages (E; passage 1–20), mid-passages (M; passage 21–40) and late passages (L; passage 41–70). Data expressed as mean $\pm$ SEM of three experiments. * $P < 0.05$; compared to respective control (10% FBS). (b) Growth curve of foetal fibroblasts in the presence of different concentrations of FBS. Data expressed as mean $\pm$ SEM of three experiments.

Influence of culture microenvironment and cell origin on β -gal activity

It has been suggested that pH-sensitive β -gal (present at pH 6) activity is associated with replicative senescence in fibroblasts (Dimri *et al.* 1995; Thomas *et al.* 1997). In our current study, the percentage of β -gal-positive cells was similar (3–5%) in fibroblasts from different organs during passage 2–3. However, at passage 11, the proportion of β -gal-positive cells was greatest in fibroblasts from liver compared to cells from the other organs (Fig. 4a). As shown in Fig. 4(b), consistent with the proliferation study results, the increase of β -gal-positive cells during passaging in 10% FBS culture was greater than that in 15% FBS culture ($P < 0.05$ when comparing the slopes of the two plots).

Influence of FBS concentration and cell origin on karyotype of fibroblasts

Karyotype analysis using conventional staining of metaphase-arrested chromosome spreads showed that abnormal karyotypes were present in fibroblasts from different organs. The abnormalities included aneuploidy (monosomy and trisomy), polyploidy (triploidy and tetraploidy; Fig. 5) and chromatin breaks. The percentage of fibroblasts with abnormal karyotypes was similar

Figure 3. Dependence of proliferative life span of porcine foetal fibroblasts on seeding density. Foetal fibroblasts were initially from the same subpopulation and plated at the seeding density indicated, on a culture dish with a growth area of 56 cm^2 . The cell number was determined and used to calculate population doubling when cells reached 80% confluence. They were then subcultured at the respective density until they reached growth plateaux, except for the 8×10^5 density group cells, which were still proliferating after 250 days of culture. Data expressed as mean $\pm$ SEM of three to five experiments.

Figure 4. Influence of culture microenvironment and cell origin on β -galactosidase activity. (a) Proportion of β -gal-positive cells: fibroblasts from different origins (passage 11). Letter subscripts indicate statistically significant differences ($P < 0.05$). (b) Proportion of β -gal-positive cells: fibroblasts from different concentrations of FBS. Data are mean $\pm$ SEM of three experiments. The slopes of the two plots are significantly different ($P < 0.05$).

(~3%) irrespective of their origin, and no significant increase was observed when they were cultured up to passage 11 (data not shown). Figure 6 shows that the proportion of cells with abnormal karyotypes increased as population doubling increased, and that cells in the 10% FBS culture group increased more rapid ($P < 0.05$). Significant increases in abnormal karyotypes were first observed at 21 and 47 population doublings ($P < 0.01$ for both) in the 10% and 15% FBS groups, respectively. Moreover, the percentage of cells with abnormal karyotype was greater in the 10% FBS group at late passages (22.3 ± 1.3 at population doubling 58) compared to that from the 15% FBS group (11.8 ± 1 at population doubling 66; $P < 0.01$).

DISCUSSION

The foetal fibroblast is usually the donor cell of choice for livestock cloning because of its ease of isolation and potential for multiple cell divisions *in vitro* before senescence. One of the

Figure 5. Representative chromosome spreads from fibroblasts. (a) Normal ($n = 38$), (b) polyploid (triploid; $n = 57$) karyotype.

Figure 6. Kinetics of accumulation of karyotypic abnormalities in fibroblasts cultured in different FBS concentrations. Data are mean $\pm$ SEM of three experiments.

challenges of manipulating genes in primary cells is that they have a finite proliferation capacity. The transfection, selection, colony growth, and genetic testing preceding nuclear transfer have to be accomplished before the cells enter the senescent stage (Betthausen *et al.* 2000).

It has been suggested in gene targeting studies in sheep and pigs, that there is considerable heterogeneity of life span of the cells in culture, with many of the selected colonies having a life span considerably shorter than others (Denning *et al.* 2001a,b). This heterogeneity may be the result of fibroblasts from diverse organs having different proliferative performances *in vitro*. Our finding that such fibroblasts indeed demonstrated different population doubling curves is consistent with this notion. It is unclear why foetal fibroblasts from the heart were proliferatively more active compared to those from the liver. Interestingly, the percentage of β -gal-positive cells in the liver-originated fibroblasts was significantly higher than those from other organs, suggesting a greater proportion of cells underwent replicative senescence in this group during the experiment. Difference in population doubling time between cells from the heart and those from the liver may have been because the latter not only have a lower proliferation rate, but also have a larger proportion of cells prematurely entering senescence during the *in vitro* culture period.

Our findings, that cells from earlier stages of foetal development had shorter population doubling times and thus more population doublings for the same period of culture suggested that they are better candidate cells for gene targeting and selection. Shorter population doubling times mean that the targeted recombinants can be expanded into clonal cultures faster and that they require shorter exposure time to selection drugs. Thus, cells with the desired genetic modifications will have a better chance to be reprogrammed appropriately to develop into transgenic pigs. Developmental stage differences in cell proliferation may reflect the cells' capability to respond to the microenvironment *in vitro*. It has been shown that foetal and adult cells responded to growth factors and matrix composition differently (Wharton 1984; Schor *et al.* 1988; Furth *et al.* 1997). Whether similar differences also exist in cells from different stages of foetal development is to be investigated.

Although serum-free culture can offer a controlled and standardized environment for cell growth, the importance of serum instead of defined medium in maintenance of long-term proliferation, has been indicated recently (Ramirez *et al.* 2001). Most cultured cells grow in a highly specialized microenvironment that may be mimicked *in vitro* by diluting serum into an appropriate concentration. Serum provides a complex mixture of hormones, nutrients, growth and attachment factors which are necessary to support cell proliferation (Orly & Sato 1979; Savion *et al.* 1981). Although growth factors, hormones, binding proteins and nutrients in serum have been identified, FBS still contains many undefined components that are necessary to support the maximal growth of cells. To date, 10% FBS is a common concentration that is widely used in cell culture media, yet the optimal concentration for long-term foetal fibroblast growth has to be determined. In our study, no significant difference was observed in population doubling times (during early passages) between 10% and 15% FBS culture groups. However, foetal fibroblasts cultured in 15% FBS showed much better growth performance compared to those of the 10% FBS group during mid- and late-passages. Cell populations doubled more frequently and doubling times were shorter in the 15% FBS group. It is possible that higher levels of growth factors and thus higher concentrations of FBS were required to stimulate proliferation as cells aged *in vitro*. Alternatively, although it did not reflect the cells' proliferative performance, a certain level of FBS concentration perhaps played a role in maintaining their intrinsic machinery for long-term proliferation during early and mid-passages.

Cell-cell interactions play an important role in the control of cell growth and the responses of cells to extracellular signals. It has been shown recently that cell density affects the expression of transcription factor Sp1 and stress-activated protein kinase as well as the binding and activity of basic fibroblast growth factor (Lallemant *et al.* 1998; Richardson *et al.* 1999; Cheng *et al.* 2001). Consistent with findings in human fibroblasts (Balin *et al.* 2002), the proliferative life span of porcine primary cells was directly related to seeding density in cell culture in our current study. Proliferative life spans (total population doubling) increased as initial plating density increased. Progressive loss in proliferative capacity in low density cultures may result from cumulative handling stress imposed or by inadequate cell-cell interactions (Sherr & Depinho 2000).

Senescence of somatic cells is associated with the induction of a particular isoform of β -gal (SA- β -Gal), whose activity is detectable at pH 6 (Dimri *et al.* 1995; Nicole *et al.* 2001). Here, β -gal-positive cells increased in number during passaging in both 10% and 15% FBS culture groups. The increase was, however, greater in the 10% FBS group. These data suggest that FBS concentration also plays a role in the control of replicative senescence. The increase in β -gal-positive cells was consistent with passage-dependent and time-dependent increases in population doubling time (Figs 2a and 4b), suggesting that the decline in cell proliferative performance might be partly the result of the proportion of cells entering cellular senescence.

In somatic cell nuclear transfer, a normal karyotype is essential for donor cells if they are to be reprogrammed appropriately and develop to term. It has been shown that prolonged culture increases the proportion of cells with abnormal karyotypes (Romanov *et al.* 2001). A system that supports maintenance of the normal karyotype during the growth of cells will thus be critical to the improvement of nuclear transfer efficiency. In a typical gene targeting experiment, approximately 50 population doublings are required for transfection, selection of targeted cells and preparation of the cells for nuclear transfer to be accomplished. In our study, the percentage of cells with abnormal karyotype was significantly lower in the 15% FBS group compared to those in the 10% FBS group (9.1% vs. 19.7%; Fig. 6) when cells were approaching their 50th population doubling. This suggested that 15% FBS provided better support for the cells to maintain normal karyotype. The percentage of cells with abnormal karyotype at 20–50 population doublings, in the current study, was $14.6 \pm 2.1\%$ and $6.3 \pm 0.8\%$ for the 10% and 15% FBS group, respectively (Fig. 6). These percentages were lower compared to findings in a recent report on porcine fibroblasts at similar stages of culture (20–51 population doublings; 25% of cells with abnormal karyotype). It is possible that genome stability of cells from different breeds of pig is different *in vitro*, fibroblasts from 'Large White' pig foetuses were used in that study (Denning *et al.* 2001b).

In summary, porcine foetal fibroblasts can be cultured *in vitro* for over 80 population doublings in the appropriate culture system. This number of population doublings exceeds the minimum number that is required for genetic engineering, including transfection, selection of targeted cells and preparation of the clonal culture for nuclear transfer, to produce transgenic pigs. Fibroblasts from earlier stages of foetal development were better candidate cells than those from later stages of development. Cells from the heart were proliferatively more active, and more resistant to replicative senescence than those from the liver. The 15% FBS provided better support, not only to proliferative performance, but also to maintain normal cell karyotype. In addition, when expanding targeted recombinants into clonal culture after selection, it is important to passage them at a higher level of seeding density to prevent premature senescence.

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