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Establishment of transformed swine fibroblast cell lines using SV40 large T antigen

C. S. Choi[1], **M. P. Murtaugh**[2], and **T. W. Molitor**[1]

Departments of [1] Large Animal Clinical Sciences and [2] Veterinary Pathobiology, College of Veterinary Medicine, University of Minnesota, St. Paul, Minnesota, U.S.A.

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Summary. Swine testicle cell lines were established by transformation of primary swine testicle (PST) cells with an SV40 plasmid (pSV3-neo), which contains genes conferring resistance to neomycin and expressing SV40 large T antigen. Plasmid DNA was transfected into PST cells using a lipofection system. Two related plasmids, pSV2-neo and pSV5-neo, failed to induce transformed cells. Cells transformed with pSV3-neo formed single colonies that were resistant to the antibiotic, G418, and expressed large T antigen. Upon two cycles of cloning by endpoint dilution method, three transformed clones, designated transformed swine testicle (tST)-3, tST-14 and tST-18, were selected and characterized in regards to cell replication and susceptibility to swine viruses. The resultant clones were compared with a counterpart non-transformed ST cell line (ATCC-ST). The three tST cell lines showed longer or the same doubling times and higher saturation densities compared to ATCC-ST cells. These cells were free from a range of adventitious agents and supported the replication of porcine parvovirus (PPV), pseudorabies virus (PRV) and transmissible gastroenteritis virus (TGEV), comparable to ATCC-ST cells. All three cell lines have been maintained in continuous cultures for over 60 passages with no changes in growth characteristics. These findings indicate that lipofection with pSV3-neo is an efficient means for the introduction of exogenous DNA into porcine cells and for establishment of transformed immortalized cell lines.

Introduction

Normal cells proliferate in an unaltered form for a number of cell generations after which proliferation declines due to a limited life span in vitro [16]. Changes in mammalian cells which give rise to immortality and continuous growth are called "in vitro transformation," and may occur spontaneously, or be induced physically [2], chemically [18] or virally [19].

Transformation of cells in vitro has been well established with viruses including vaccinia virus [20], Shope fibroma virus [27], retroviruses [1], lymphotropic papovavirus [21] and simian virus 40 (SV40) [19, 25, 34]. The most common method for transformation has been the immortalization of cells by SV40. SV40 is tumorigenic in vivo and can induce in vitro transformation of a variety of mammalian cells [8]. The early region of SV40 encodes the large and small T antigen and is responsible for its tumorigenic and transforming properties. Whereas SV40 small T antigen appears to play a role in the transformation of certain kinds of cells, SV40 large T antigen alone can be used to induce transformation of a wide variety of cell types [8].

A variety of methods have been developed for the introduction of foreign DNA into living mammalian cells in a form allowing expression in the recipient cells. Collectively, these methods are referred to as "transfection". Under appropriate conditions, eukaryotic cells can take up foreign DNA, and a portion of this DNA becomes localized in the nucleus. Due to the size and charge of DNA and to the multitude of enzymatic and membrane barriers imposed by the cells, the spontaneous entry of intact DNA into cells is a very inefficient process [10].

A number of techniques have been introduced to facilitate the entry of DNA into cells. These methods include precipitation with calcium phosphate [15, 29], microinjection [14], electroporation [30], liposome fusion [32], centrifugation loading [3] and scrape or sonication loading [9]. While various cell lines have been transformed, the outlook has been much less encouraging for the transformation of primary cells.

Recently, a synthetic cationic lipid (DOTMA), N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethyl ammonium chloride, that forms liposomes has been applied in the transfection of foreign DNA into cells [10]. Liposomes containing DOTMA interact with DNA, spontaneously fuse with cells and facilitate the delivery of foreign DNA into cells. Transfection using liposomes has been designated "lipofection." Lipofection with DOTMA is convenient, reproducible, efficient and can be used to transfect various cells with different classes of polynucleotides including DNA, mRNA and ds-RNA [11]. One striking advantage of lipofection is that it offers the promise of a gentle and simple method for transfection useful for primary cultures [24].

In this study, primary swine testicle (PST) cells were transfected with various SV40 plasmids to develop a straightforward, reproducible system for the establishment of transformed swine testicle cells. The resultant transformed cell lines were characterized for their growth kinetics and sensitivity to selected porcine viruses.

Materials and methods

Cells

Primary swine testicle (PST) cells were obtained from a male, 40-day old, crossbred pig. Primary cells were prepared as described by Kumagai et al. [22]. The isolated cells were propagated in 25 cm^2 tissue culture flasks containing Autopow MEM (Sigma) with 10%

heat inactivated fetal calf serum and antibiotics. PST cells can be passaged only 7 to 10 times. A non-transformed swine testicle cell line (ATCC CRL 1746) was obtained from American Type Tissue Culture Collection (ATCC) and designated ATCC-ST. ATCC-ST cells have been successfully passaged over 400 times in our laboratory.

Plasmids and reagents for transfection

Hybrid pSV-neo plasmids, pSV2-neo, pSV3-neo, and pSV5-neo were used for transformation of PST cells [33]. Plasmid pSV2-neo was derived from pSV2-β globin (BG) by excising the β-globin cDNA segment from pSV2-BG and substituting the neo fragment from pBR-neo. The plasmids pSV3-neo and pSV5-neo had been constructed from pSV2-neo by insertion of either an intact SV40 early region or an intact polyoma early region, respectively. All plasmids replicated efficiently in *E. coli* strain HB101 and conferred resistance to ampicillin and neomycin.

Lipofectin (BRL, Gaitherburg, NY), a cationic lipid, was employed for cationic liposome-mediated transfection (lipofection). Geneticin (G418, Gibco, Grand Island, NY), structurally similar to neomycin and an aminoglycoside antibiotic, was used for selection of transfected cells. G418 interferes with the function of 80S ribosomes and blocks protein synthesis in eukaryotic cells [7]. These antibiotics can be inactivated by the bacterial enzyme, phosphotransferase. Antibiotic resistance results from the acquisition, maintenance and continued expression of the phosphotransferase. Stock solutions of G418 were prepared at 40 mg/ml in 100 mM HEPES (pH 7.3) and stored at –20 °C. Appropriate concentrations of G418, 400 μg/ml for initial selection and 200 μg/ml for subsequent maintenance, were added to cell culture medium.

Transfection procedures

PST cells (three times passaged) were transfected with one of three pSV-neo plasmids using the lipofection system, following methods described by Felgner and Holm [11] with some modifications. Confluent PST cells in six well plates were washed three times with serum free Opti-MEM (Gibco) immediately prior to transfection to remove transfection inhibitory factors present in serum. Lipofection reagent (20 μg) and plasmids (5 μg of pSV2-neo, pSV3-neo, or pSV5-neo) were mixed in 0.75 ml of serum-free Opti-MEM, and the mixture was added to the washed PST cells. Treated cells were further incubated at 37 °C. Medium was removed from 2 wells each at 2, 3, and 5 h post-transfection and replaced with 3 ml of Opti-MEM containing 10% fetal calf serum. 72 h post-transfection, the media was removed and the cells were incubated in Opti-MEM media containing 10% FCS and G418 (400 μg/ml) to select transformed cells. Clones of G418 resistant cells became visible at 12 days after transfection, and transfection efficiency was determined by scoring these clones.

Cloning

Transformed ST (tST) cells were cloned twice by limiting dilution as described by Helmke et al. [17] with some modifications. Cultures in Opti-MEM containing 10% FCS were diluted and plated at 10, 3, and 0.5 cells per 200 μl in 96 well plates (30 wells per each dilution). Each well was replenished with 100 μl of Opti-MEM with 10% FCS every other day. The cultures were observed microscopically for identification of wells containing single cells, and subsequently for their proliferation. Fourteen days later, proliferating cultures were expanded by transferring to sequentially larger vessels, i.e., 24 well plates, 6 well plates, 25 cm^2 flasks and 75 cm^2 flasks. Three clones were selected based on their cell morphology and designated tST-3, tST-14 and tST-18. These clones were characterized for large T antigen expression, growth kinetics, presence of adventitious agents and sensitivity to porcine viruses.

Detection of SV40 large T antigen

At 24 h post-transfection, the cells were trypsinized and replated in 12 well slide glasses (Cell Line Assoc., Newfield, NJ). Expression of large T antigen was evaluated by indirect fluorescence antibody (IFA) staining using a monoclonal antibody (50 µl of 5 µg/ml) to SV40 large T antigen (Oncogene Science Inc., Manhasset, NY).

Growth kinetics

Transformed cells (tST-3, tST-14, and tST-18) were evaluated for their growth kinetics by comparing with those of a conventional ST cell line (ATCC-ST). Cells growing in Opti-MEM containing 10% FCS were trypsinized and replated at 2×10^4 cells per well in 24 well plates. At two days intervals for eight days, the total number of cells was removed from wells by trypsinization and viable cells/well were counted by trypan blue staining [31].

Detection of adventitious agents

Transformed ST cells were tested for the presence of common contaminants in cultured cells, such as porcine parvovirus (PPV), bovine viral diarrhea virus (BVDV) and mycoplasma. The presence of PPV in cell cultures was evaluated using a non-isotopic hybridization protocol [28]. Immunoprecipitation of ^{35}S-labelled proteins was used to detect BVDV as described earlier [6]. Mycoplasma contamination was tested using the fluorescent dye DAPI (Boehringer Mannheim Biochemicals) according to the manufacturer's specifications.

Susceptibility of transformed ST cells to selected porcine viruses

The susceptibility of transformed ST (tST) cells to three porcine viruses, PPV, pseudorabies virus (PRV) and transmissible gastroenteritis virus (TGEV), was examined. Ten-fold dilutions of each virus were inoculated onto tST cells and ATCC-ST cells grown on 12 well slide glasses. At 48 h post-infection (PI), titers of each virus were measured by indirect fluorescent antibody (IFA) staining using rabbit antisera against each virus. Titers were expressed as fluorescent focus units (FFU)/ml.

Results

Establishment of transformed swine testicle cell lines

Primary swine testicle (PST) cells from the testicles of young male swine were transfected with pSV2-neo, pSV3-neo, or pSV5-neo using lipofection to establish transformed ST cell lines. Attempts to obtain transformants with pSV5-neo were unsuccessful. Although some transformed colonies were initiated from pSV2-neo, transfected cells died after 2 to 3 passages. Successful long-term cultures of transformed ST cells were routinely obtained from PST cells transfected with pSV3-neo. Transformants formed single colonies in the presence of G418 at 12 days post-transfection showing single proliferating colonies and exhibited T antigen specific nuclear fluorescence (Fig. 1). The transformation frequency did not vary appreciably depending on the transfection time, i.e., 8, 12, and 8 colonies per 10^4 cells were obtained after 2, 3, and 5 h transfection, respectively (Table 1). Transformation frequencies ranged from 1×10^{-3} to 8×10^{-4}. When the monolayers were further subcultured at ratios of 1:2, transfected cells showed a total recovery in the presence of G418. Transformed

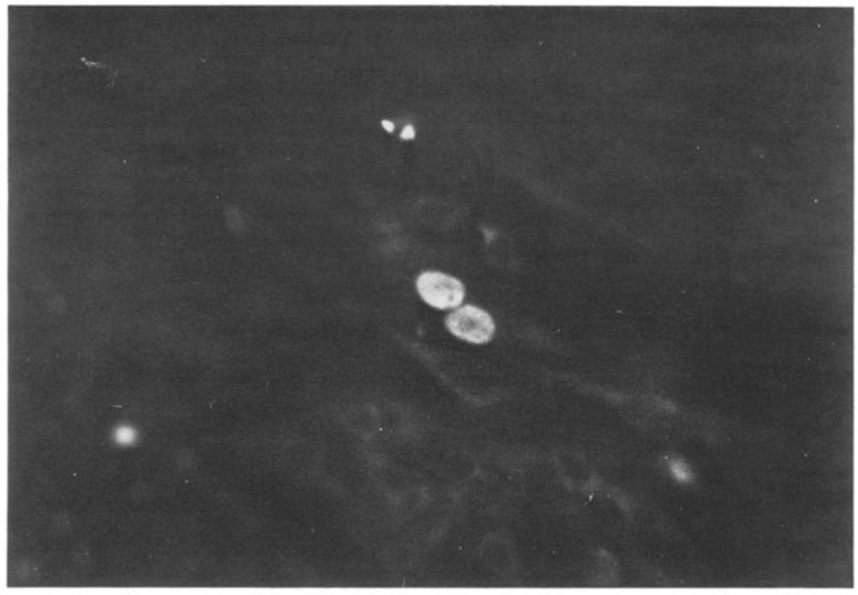

Fig. 1. Immunofluorescence of SV40 large T antigen. Cells growing in 12 well slides were fixed in methanol/acetone and incubated with monoclonal antibody against SV40 T antigen followed by staining with FITC-conjugated goat anti-mouse immunoglobulin. SV40 T antigen positive fluorescences were shown in nuclei 24 h post transfection

Table 1. Transformation frequency of pSV3-neo transfected primary swine testicle (PST) cells

Transfection time (h)	Transfection frequencies (colonies per 10^4 cells plated)
0	0 (0)
2	8×10^{-4} (8)
3	1×10^{-3} (12)
5	8×10^{-4} (8)

cells formed large, dense colonies with a piled-up morphology. These cells showed resistance to G418 at a concentration of 400 μg/ml and showed T antigen specific fluorescence in the nucleus indicating the evidence of successful transformation with pSV3-neo.

Cloning and expansion of cultures

At the fourth passage, selected transformed cells were subjected to cloning by endpoint dilution. From the wells seeded with the equivalent of 0.5 cell, more than 70% contained single colonies at 10 days after culture. Cells in these colonies had the morphological appearance of fibroblasts, epithelial cells and

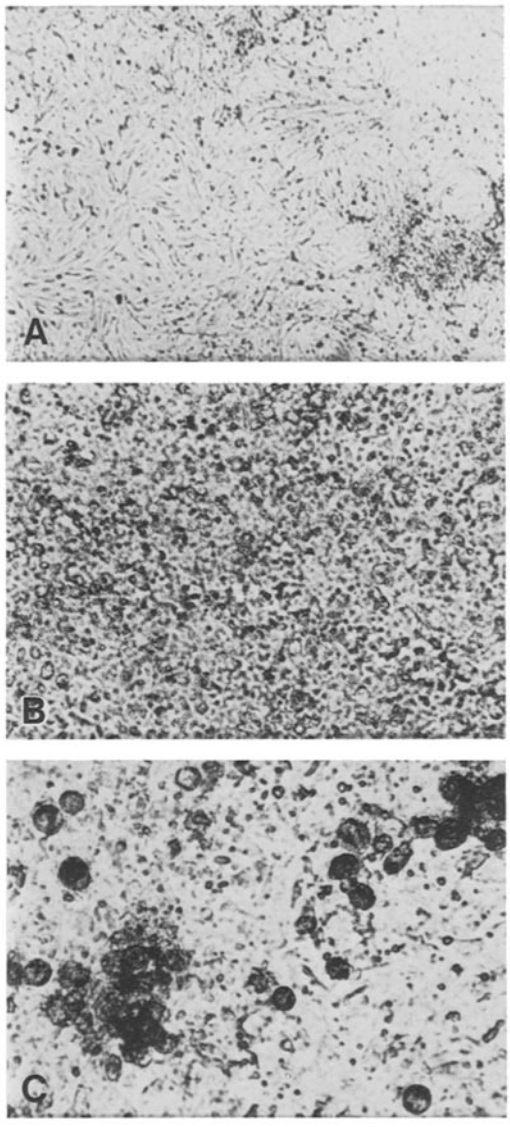

Fig. 2. Morphology of tST cells. Single colonies were continuously cultured by transferring cells from 96 well plates to 24 and 6 well plates and finally in 75 cm^2 flasks. Upon subcloning, three clones were selected from different cell types: tST-3 **(A)**, tST-14 **(B)**, and tST-18 **(C)**

round cells. Five clones from each morphological type were subcloned. Single colonies were visible at 10 to 12 days after cultures in wells cloned at 0.5 cell/well. Four clones showing the original morphology of each type were further expanded. All clones were capable of continuous growth in the presence of G418. One clone from each morphology, designated tST-3 (Fig. 2A), tST-14 (Fig. 2B), and tST-18 (Fig. 2C), respectively, was selected for further characterization.

Growth kinetics

Cloned tST cells were examined for growth kinetics relative to ATCC-ST. The doubling times of tST-3 and tST-14 were approximately 48 h compared to 24 h for tST-18 and ATCC-ST (Fig. 3). Saturation densities of tSTs were between

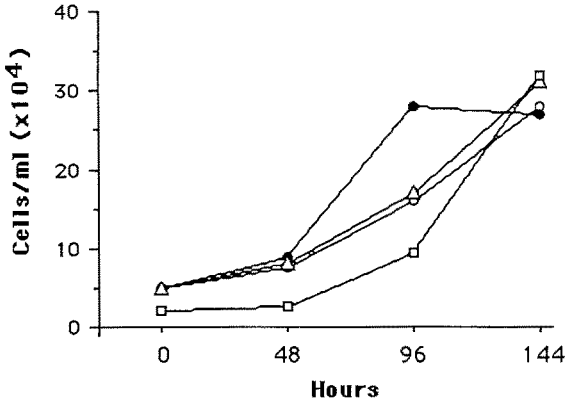

Fig. 3. Growth kinetics of tST cells. Two to five $\times 10^4$ cells/ml of each clone, tST-3 (○), tST-14 (△), and tST-18 (□), as well as ATCC-ST (●) were seeded into 24 well plates per clones and cultured for six days. At 48 h intervals, cells from 4 wells were trypsinized, pooled and counted using trypan blue. Cell numbers were expressed as total cells per well (total cells from 4 wells/4)

3 to 3.5 $\times 10^5$ per well which was higher than that of ATCC-ST (2.7×10^5). However, ATCC-ST cells reached saturation density approximately 24 h faster than tST cell lines. All tST cells were capable of growth to confluent monolayers at a reduced concentration (3 to 5%) of fetal calf serum, but not at concentrations lower than 3%. In addition, these cells proliferated as rapidly in Autopow MEM or DMEM with 5% fetal calf serum as in Opti-MEM with 10% fetal calf serum. Since their initial cloning, all three cell lines have been continuously passaged more than 60 times.

Detection of adventitious agents

The cloned tST cells were tested for the presence of contaminating adventitious agents such as BVDV, PPV, and mycoplasma. These agents often contaminate cell lines inadvertently from fetal calf serum, trypsin, and handling, respectively. At passage number 20, all transformed cells were examined for possible BVDV, PPV, or mycoplasma contamination. No BVDV polypeptides were recognized by BVDV hyperimmune sera. By non-isotopic hybridization, no specific PPV DNA was detected from DNA extracts of transformed cells. Mycoplasma also were not detected in tST cells by the DAPI method. Similar results were obtained from ATCC-ST cells.

Susceptibility of tST cells to various porcine viruses

Transformed ST cells and ATCC-ST cells were compared for susceptibility to porcine viruses, PPV, TGEV, and PRV (Fig. 4). PPV exhibited similar growth patterns in tST-3 and ATCC-ST cells, giving rise to titers of $10^{8.2}$ and $10^{8.0}$ FFU/ml, respectively. In contrast, a 1.5 log lower titer of PPV ($10^{6.5}$ FFU/ml) was detected in tST-14 cells. Interestingly, all 3 tST cells showed more cell destruction upon PPV infection compared to ATCC-ST cells. Infectious titers of PRV and TGEV were similar in the transformed cells and ATCC-ST cells.

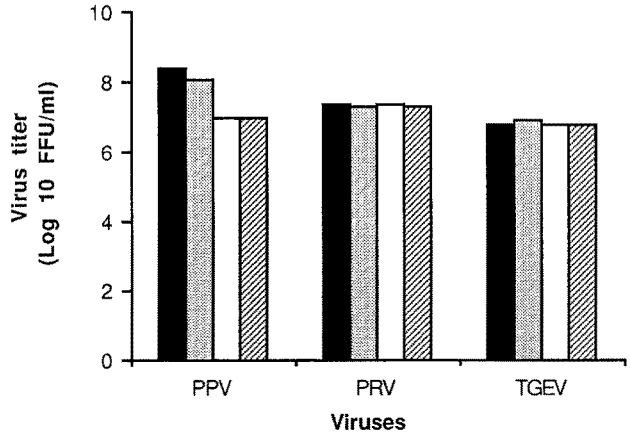

Fig. 4. Replication of selected porcine viruses in transformed ST cells. Ten-fold dilutions of porcine parvovirus (*PPV*), pseudorabies virus (*PRV*), or transmissible gastroenteritis virus (*TGEV*) were inoculated onto tST cells, tST-3 (▨), tST-14 (□) and tST-18 (▨), or ATCC-ST (■) cells grown in 96 well plates. At 48 h post-infection (PI), virus specific fluorescence was examined for each virus employing virus specific antisera. Endpoint titers are expressed as focus forming unit (FFU)/ml

Discussion

DNA transfection systems are often a critical component of studies for exploring eukaryotic gene expression. Previous attempts at immortalizing primary baby rat kidney cells using standard transfection methods have produced cell lines at low frequency and with minimal reproducibility [5]. Nonimmortalized primary cells appear to be less competent recipients for DNA-mediated gene transfer under conditions of calcium phosphate precipitation [12]. Recently, a synthetic lipid, Lipofectin Reagent, has been described as a method to transfect rat pituitary gland cells, a primary cell [24]. The purpose of this study was to develop a straightforward, reproducible system for the establishment of immortalized porcine cell lines. Using a recombinant plasmid (pSV3-neo) carrying SV40 T antigen and neomycin resistance genes and transfected by the lipofection method, transformed swine testicle (tST) cells were established. A 4:1 ratio of Lipofectin (20 μg) and DNA (5 μg) resulted in satisfactory transfection efficiencies. A more thorough investigation of optimizing the ratio may further increase the transformation frequencies. The optimal incubation time with DNA/Lipofectin complexes is different depending on cell types, 3 h for JZ.1 cell line, 5 h for CV-1 and Cos-7 [10], and 6 h for primary rat pituitary gland cells [24]. Incubation periods can range from 3 to 24 h depending on the ability of cells to tolerate serum-free cultures in the presence of the liposome/DNA complexes [11]. In this study, the incubation periods of 2, 3, and 5 h for primary ST cell resulted in satisfactory transformation frequencies (8×10^{-4} to 1×10^{-3}). These frequencies were relatively higher than those obtained from other transfection methods, such as calcium phosphate precipitation. Felgner

et al. [10] reported that lipofection is 5 to 100 times more efficient than either calcium phosphate or the DEAE-dextran transfection technique. Transformation frequencies obtained in this study were comparable to those reported by Felgner et al. [10].

Transformed colonies in the presence of selective media containing G418 were first detected at 12 days post transfection. Other studies detected colonies from 7 days [4] to 2–3 weeks [23]. The selection strategy of permitting cell growth prior to the addition of G418 was adopted due to a significant reduction in transformation frequency if G418 was added before 48 h [33]. Consequently, if cells become transformed early after transfection, cell division prior to selection may result in overestimation of the transformation frequency. Considering these circumstances, G418 was added 72 h after transfection in this study.

Immunofluorescent staining revealed that transfected cells contained the SV40 specific T antigen, whereas non-transfected control cells did not. The number of T antigen fluorescent foci increased to virtually 100% of cells after cloning, indicating that transformed colonies resulted from transfection with pSV3-neo. Additional support for this phenomenon was that all cells exhibited G418 resistance.

Compared to ATCC-ST cells (24 h), tST cells showed similar (24 h for tST-18) or longer (48 h for tST-3 and -14) doubling times. Saturation densities of tST cells were higher than that of ATCC-ST cells while tST cells reached saturation densities more slowly than ATCC-ST cells. Cloned tST cells were capable of replicating in low percent fetal calf serum (3% compared to 10% in initial stage of selection). In addition, these cells showed no contact inhibition and were capable of growing in semisolid agar (data not shown) indicating anchorage independent growth. These cells have been successfully passaged more than 60 times with no changes in growth properties. Such characteristics of cloned cells meet with the criteria for transformed cell lines [13], such as loss of contact inhibition, low serum requirement and anchorage independence. The three characterized cloned tST cells were found to be free of adventitious agents known to contaminate cultured cells. Trypsin is the major source of porcine parvovirus contamination because trypsin is prepared from the pig pancreas. Fetal calf serum is known to be the major source of BVDV contamination.

The susceptibility of tST cells to selected porcine viruses was examined and compared to ATCC-ST cells. Only slight differences were found in PPV infected cells. PPV caused more thorough cell destruction compared to PPV infected ATCC-ST cells. A recent report has shown that minute virus of mouse (MVM) induced more severe cell destruction in transformed cells compared to non-transformed parents cells, suggesting that transformation might trigger cellular functions required for the completion of the parvoviral lytic cycle [26]. Malignant cells may be a preferential target for the viral cytotoxic action and enhanced susceptibility of transformed cells to autonomous parvoviruses.

This study demonstrates that porcine primary cells can be immortalized by lipofection-mediated transformation with pSV3-neo. Further studies will determine if other primary cells can be transformed with similar frequencies. In addition, these cells will provide useful models for in vitro cellular and molecular studies, i.e., cytokine expression.

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Authors' address: T. Molitor, Department of Large Animal Clinical Sciences, College of Veterinary Medicine, University of Minnesota, St. Paul, MN 55108, U.S.A.

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