Development of a clonal equine myoblast cell line capable of terminal differentiation into mature myotubes in vitro

Richard J. Piercy VetMB, PhD

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From the Comparative Neuromuscular Diseases Laboratory, Department of Clinical Sciences and Services, Royal Veterinary College, London, NW1 0TU, England.

Address correspondence to Dr. Piercy (rpiercy@rvc.ac.uk).

OBJECTIVE
To produce a clonal equine myoblast cell line that retains the ability to divide for multiple passages and differentiate into multinucleated myotubes during specific conditions.

SAMPLE
Cultured primary equine skeletal muscle–derived cells from a healthy Thoroughbred.

PROCEDURES
Cell cultures were transfected by electroporation with a plasmid (pNIT) that expresses the temperature-sensitive simian vacuolating virus 40 large T antigen (TAg), which can be controlled by a doxycycline-responsive promoter. Cells that stably integrated the TAg were selected and expanded to passage 25. For each passage, differentiation and fusion properties of the cells were determined and immunocytochemical analyses were performed to evaluate expression of TAg and other muscle-specific proteins. Optimum conditions that led to cell differentiation into myotubes were also determined.

RESULTS
Compared with nontransfected control cells, myogenic, desmin-positive cells expressed the TAg when incubated at 33°C and could be maintained in culture for numerous passages. Reduced expression of TAg was identified in cells incubated at 37°C or when incubated with doxycycline at 33°C. Expression of TAg was not detected when cells were incubated with doxycycline at 37°C, and when serum was withdrawn from the culture medium, those clones differentiated into a pure population of multinucleated myotubes.

CONCLUSIONS AND CLINICAL RELEVANCE
Results indicated that production of an immortalized clonal equine skeletal muscle cell line was possible. A clonal equine skeletal muscle cell line will be a valuable in vitro tool for use in equine physiology and disease research. (Am J Vet Res 2015;76:608–614)

Cell-culture models are attractive tools for the performance of in vitro studies of muscle physiology and disease. Compared with in vivo experiments, in vitro experiments are less expensive, help reduce the number of animals required for research, and allow for the application of treatments not possible in vivo. Several established and widely available murine myoblast cell lines are used extensively in muscle research; however, the physiologic behavior of muscle cells differs among species. Isolation of clonal equine satellite cell lines capable of fusing to form multinucleated cells that express muscle-specific proteins has been described, but isolation of additional cell lines that are capable of long-term expansion in culture would be a valuable resource for investigation of specific responses of equine muscle cells.

Given the high prevalence of primary myopathies in horses, equine muscle physiology is an important area of research. Recurrent exertional rhabdomyolysis is estimated to affect 6.7% of Thoroughbred racehorses, and types 1 and 2 polysaccharide storage myopathy affect > 50% of the horses of some draft breeds. Also, skeletal muscle is secondarily affected during endotoxemia, chronic obstructive pulmonary disease, hyperadrenocorticism, diabetes mellitus, and disuse atrophy, and loss of muscle mass is positively associated with the patient mortality rate in other species. Additionally, further elucidation of equine muscle physiology might provide useful insight into processes that lead to optimal athletic performance.

The use of primary muscle cell cultures for in vitro experiments has several challenges. Cells must be obtained by an invasive muscle biopsy, and the production of viable cell cultures is time-consuming. Pri-
ary muscle cell cultures typically contain a mixed population of fibroblasts and myoblasts. Because the cell cycle of fibroblasts is generally quicker than that of myoblasts, the percentage of fibroblasts within a culture tends to increase with each passage. Furthermore, primary muscle cells often proliferate for only a limited number of divisions before entering senescence, such that the proliferative capacity of the culture decreases as the number of passages increases. Consequently, muscle biopsy must be repeated to acquire fresh source cells, and primary muscle cell cultures are suboptimal for extensive or repeated in vitro experiments.

Many of the disadvantages associated with primary muscle cell cultures can be avoided by the use of clonal cell line cultures for in vitro studies of skeletal muscle. Cells of cell line cultures can evade senescence and continue to divide by the acquisition of genetic mutations that occur naturally or by genetic manipulation. Another advantage of clonal cell lines is that genetically identical cells can be produced for use in repeated biological experiments. A number of muscle cell lines with extensive mitotic capacity such as the mouse C2C12 subclone line, which consists of spontaneously transformed immortalized murine myoblasts derived from 1 C3H mouse, have been established and widely used in in vitro muscle research.

Cultured myoblasts can be induced to transform into more mature, multinucleated myotubes, which enable researchers to investigate differentiated cells that more closely resemble mature muscle and its properties. Myotube formation is associated with multiple myoblasts exiting the cell cycle and fusing together. Consequently, the immortalizing property needs to be nullified to enable immortalized myoblasts to exit the cell cycle and differentiate into myotubes. Differentiation of myoblasts into myotubes occurs when the cells approach confluence and is typically induced by the reduction of the serum content in the culture medium. The extent of differentiation is affected by the type of culture medium and serum used, cell density, and the substrate on which the cells are grown in a cell line-specific manner.

The stable integration of a proliferation-promoting oncogene such as the temperature-sensitive SV40 large TAg into cells can be used to maintain cellular proliferation, and expression of the transgene can be controlled by environmental conditions. The NIT-TAg plasmid is a packaging-deficient retroviral vector derived from a lentiviral plasmid NIT that contains the SV40 large TAg, which can be controlled by a doxycycline-responsive promoter and a mammalian expression cassette that confers G418 resistance. The large TAg overcomes cell-cycle arrest by inhibition of the P53 proliferation suppressor gene, which results in the upregulation of many genes associated with cell division and progression to the S phase of the cell cycle. During permissive conditions (incubation at 35°C without doxycycline), the TAg is expressed and promotes cell division. When the ambient temperature is increased to 37°C or doxycycline is added to the culture medium, TAg expression is reduced and cell division is inhibited. When the ambient temperature is increased to 37°C and doxycycline is added to the culture medium, TAg expression is inactivated and the cells exit the cell cycle and undergo terminal differentiation.

The purpose of the study reported here was to produce a clonal equine myoblast cell line that retains the ability to divide for multiple passages and differentiate into multinucleated myotubes during specific conditions. We hypothesized that stable integration of a temperature-sensitive SV40 large TAg transgene that could be controlled by an inducible promoter into cells of a mixed skeletal muscle cell culture would result in the selection of a myoblast clone capable of differentiation during specific conditions.

Materials and Methods

Primary equine skeletal muscle cell culture

With owner consent, a skeletal muscle specimen (0.5 cm³) was obtained from a superficial gluteal muscle of a healthy 5-year-old Thoroughbred gelding immediately following euthanasia with IV administration of secobarbital sodium and cinchocaine hydrochloride for reasons unrelated to the study. The specimen was placed in DMEM and stored at 4°C overnight (approx 14 hours). The following morning, the tissue specimen was minced with a sterile scalpel. The tissue pieces were suspended in DMEM that contained 1% penicillin-streptomycin, 1% l-glutamine, and 20% FCS and then evenly divided among the wells of a 6-well culture plate. The plate was incubated at 37°C with 5% CO₂, and the medium was replaced with fresh medium every 48 to 72 hours. After 8 days of incubation, spindle-shaped cells growing away from the tissue pieces were visible.

Primary control cell culture

Primary control cell cultures were established by the use of murine myogenic (C2C12) and nonmyogenic simian (COS7) cell lines grown in DMEM that contained 1% penicillin-streptomycin, 1% l-glutamine, and 10% FCS. The cells were incubated at 37°C with 5% CO₂. The cells were passaged when they approached 90% confluence following incubation with a 1% trypsin-EDTA solution.

Clonal selection from primary muscle cultures

Each of 2 aliquots of 1 X 10⁶ primary skeletal muscle-derived cells (passage 7 [P7]) were resuspended in 100 µL of electroporation solution. Then, NIT-TAg plasmid DNA (3.5 pmol; 16 µg in 37 µL) was added to one aliquot but not the other, which served as a negative control. The cell solutions were transferred to cuvettes and electroporation (program P-022) was performed at room temperature (approx 22°C) by use
of an electroporator in accordance with the manufacturer’s instructions. Each cell solution was transferred to a T75 flask to which additional culture medium (10 mL of DMEM with 1% penicillin-streptomycin, 1% L-glutamine, and 20% FCS) was added. The cells were incubated at 33°C for 48 hours, then the culture medium was supplemented with G418 (400 µg/mL), and the cells were incubated for 14 days. Punctate-cellular clones that survived the G418 treatment were selected and expanded; however, the flask with the control cells had no surviving cells. Expression of the muscle-specific protein desmin as determined by an immunocytochemical procedure was used to distinguish myoblasts from fibroblasts; C2C12 cells were used as the positive control and COS7 cells were used as the negative control during the staining procedure.

Characterization of the myoblast clone

To evaluate terminal differentiation, 2 X 10^4 clonal equine myoblast cells (P15) were plated in each 0.8-cm² well of a chamber slide that was precoated with extracellular matrix substrate diluted in DMEM. Cells were incubated at 33°C until they achieved 95% confluency. Then, the medium was changed to 2% FCS with doxycycline (1 µg/mL), and the cells were incubated at 37°C. Subjective visual evaluation of myotube morphology was used to identify the equine myoblast clone with optimum differentiation, and that clone was selected for further characterization.

An immunocytochemical procedure was used to determine the expression of sarcomeric myosin, actin, desmin, and skeletal muscle RyR1 in cells following 1, 6, and 10 days of incubation at 37°C (ie, after differentiation). An immunocytochemical procedure was also used to evaluate TAg expression in clonal cells (P15) incubated at 33° and 37°C with and without doxycycline and to evaluate selected protein and TAg expression in P25 clonal cells. Cellular morphology of P25 clonal cells was visually evaluated.

Optimization of clonal myoblast differentiation

To determine the optimum conditions for equine myoblast differentiation, 4 concentrations (4 X 10^4 cells/well, 6 X 10^4 cells/well, 8 X 10^4 cells/well, and 10 X 10^4 cells/well) of clonal equine myoblast cells (P10 to P14) were plated in each 0.8-cm² well of a chamber slide that was or was not precoated with fibronectin (40 µg/mL), laminin (20 µg/mL), 10% extracellular matrix substrate, 0.01% poly-L-lysine solution, and 0.05% gelatin. Cells were incubated at 37°C and allowed to differentiate for 14 days in DMEM that contained 2% serum (FCS or equine serum), 1% L-glutamine, and doxycycline (1 µg/mL) with or without 7 X 10⁻⁵% β-mercaptoethanol, 1% nonesterified fatty acids, and 1% insulin, or in enriched medium supplemented with 1% L-glutamine and doxycycline (1 µg/mL). The cells were then fixed, and desmin expression was determined by an immunocytochemical staining procedure. The extent of cell differentiation was determined by an immunocytochemical staining procedure. The extent of cell differentiation was determined by an immunocytochemical staining procedure. The extent of cell differentiation was determined by an immunocytochemical staining procedure. The extent of cell differentiation was determined by an immunocytochemical staining procedure. The extent of cell differentiation was determined by an immunocytochemical staining procedure. The extent of cell differentiation was determined by an immunocytochemical staining procedure.
termined by subjective visual evaluation of myotube morphology.

**Immunocytochemical procedure**

Cells were rinsed in PBS solution prior to being fixed in PBS solution with 4% paraformaldehyde at 4°C for 20 minutes, before permeabilization in PBS solution with 0.1% Triton for 30 minutes at room temperature. Primary mouse monoclonal antibodies against desmin\(^b\) (1:100), TAg\(^c\) (1:100), sarcomeric myosin\(^d\) (1:100), and RyR1\(^e\) (1:100) were diluted in PBS solution and incubated with the cells at room temperature for 1 hour. The cells were rinsed with PBS solution, incubated with a fluorescent (568- or 488-nm conjugate) donkey anti-mouse antibody\(^f\) (1:5,000) at room temperature for 1 hour, and then rinsed 3 times with PBS solution. Fluorescently conjugated phaloidin\(^g\) (1:40) was applied to the cells to evaluate filamentous actin localization. Cells were counterstained with Hoechst\(^h\) stain (1:10,000) for 5 minutes to allow identification of cell nuclei and then mounted in an aqueous mountant\(^i\) on a glass slide. A fluorescent microscope\(^j\) fitted with imaging software\(^k\) was used to evaluate the slides.

**Results**

**Clone selection**

Following electroporation with pNIT-TAg and treatment with G418, 8 separate clones (clones 1 through 8) were obtained. Each clone was individually expanded and immunocytochemical analysis revealed that only 4 clones expressed desmin (Figure 1). From those 4 clones, clone 1 was judged to have optimal differentiation and was selected for further characterization.

**Clonal cell line characteristics**

Expression of TAg was confirmed in the nuclei of the transfected clonal (clone 1) cells at both early (P10) and late (P25) passages, but not in the nuclei of the nontransfected control cells (Figure 2). Maximal expression of TAg occurred when cells were incubated at 33°C, but was reduced when the cells were exposed to doxycycline or the incubation temperature was increased from 33°C to 37°C (Figure 3). Expression of TAg was completely blocked when the clonal cells were incubated with doxycycline at 37°C.

Clonal equine myoblasts differentiated into multinucleated myotubes following incubation in differentiating medium with doxycycline for 10 days at 37°C. The differentiating myotubes expressed sarcomeric myosin and actin following 1, 6, and 10 days of incubation, whereas RYR1 expression became evident only after 10 days of incubation (Figure 4). Optimal cell differentiation was observed when cells were plated at a density of 6 X 10^4 cells/0.8-cm² well and incubated in DMEM supplemented with 2% horse serum, 1% L-glutamine, nonessential amino acids, β-mercaptoethanol, and insulin. Although myotube differentiation was subjectively determined to be suboptimal when cells were cultured on poly-L-lysine, the subjective morphological characteristics of the differentiated myotubes did not differ among the cells cultured on the other substrates. Myoblasts that were frozen at -80°C, stored...
in liquid nitrogen for > 1 year, and then defrosted continued to divide, differentiate, and express myogenic proteins associated with differentiation. Those myoblasts maintained the ability to express TAg and differentiate for 25 passages (Figure 5), which was the highest number of passages assessed in this study.

Discussion

In the present study, a primary mixed equine skeletal muscle cell culture was transduced with a SV40 large TAg transgene that could be controlled by a doxycycline-responsive promoter to isolate a clonal myoblast cell line. That cell line was then characterized, and the optimum conditions required for its culture and differentiation into multinucleated myotubes were determined. The clonal myoblast cell line described in this study was capable of dividing during permissive conditions (incubation without doxycycline at 33°C) for up to 25 passages, which suggested that it could be a valuable tool for in vitro studies of equine muscle physiology and disease.

Primary skeletal muscle cell cultures have been used to investigate myoblast differentiation and muscle regeneration, calcium regulation, and muscle contraction in in vitro models of equine recurrent exertional rhabdomyolysis and the production of reactive oxygen species subsequent to anoxia. However, the difficulties associated with maintaining viable primary muscle cell cultures has restricted their use. To overcome some of those difficulties and because fibroblasts proliferate more readily than do myoblasts, our laboratory group developed a technique for generating equine myoblasts by transfecting equine skin fibroblast cultures with either a lentivirus or adenovirus that forces expression of equine myogenic differentiation factor 1, a muscle-specific transcription factor. That technique yields a heterogeneous cell population, which is useful for certain experiments, especially when variation within and between animals is being investigated. Conversely, the technique described in the present study was used to successfully isolate and produce a presumably genetically identical clonal myoblast cell line. This clonal cell line could have a multitude of diverse applications, and its use should allow for greater standardization between experiments and reduce the need for the acquisition of multiple invasive muscle biopsy specimens.

To produce a cell line that retains the ability to proliferate requires the insertion of an oncogene into the host genome. Several transgenes have been successfully inserted into recipient cell lines, including the large TAg used in this study and other studies, PyMT, Notch, and telomerase. The resulting effect from transgene insertion into a genome appears to be cell-type specific. Transfection of equine myoblasts with a plasmid containing the SV40 large TAg inhibited the P53 proliferation suppressor gene, which resulted in the upregulation of many of the genes associated with cell division and progression to the S phase of the cell cycle. Many other researchers have
used plasmids containing the SV40 large TAg to immortalize in vitro cell lines such as fibroblasts, epithelial cells, and rat and human myoblasts. Results of 1 study indicate that, unlike other immortalized muscle cell lines, those immortalized with SV40 large TAg retained the ability to optimally differentiate into myotubes capable of contractile activity. However, we did not observe spontaneous contraction in any of the equine myotube cultures of the present study. Because muscle physiology researchers generally prefer to perform in vitro experiments with myotubes that possess contractile proteins and closely resemble mature muscle cells, it is important that clonal muscle cell lines contain an oncogene that can be controlled to allow for cell proliferation or differentiation. In the present study, we used a temperature-sensitive mutant of the SV40 large TAg that could be controlled by a doxycycline-responsive promoter. Results indicated that the addition of doxycycline to the culture medium and increasing the incubation temperature from 33°C to 37°C prevented the clonal cells from expressing TAg and allowed them to differentiate into myotubes.

The present report described a technique for producing a clonal equine myoblast cell line that will likely become a valuable research tool with a variety of possible applications. Use of this cell line will allow for investigation of various interventions on equine muscle metabolism and growth and minimize the need for the acquisition of invasive muscle biopsy specimens from live horses. Furthermore, use of a clonal equine muscle cell line culture model is an attractive alternative for evaluating the effects of drugs on muscle or investigating myopathies associated with the ingestion of toxins such as hypoglycin A, which results in the often-fatal atypical myopathy of horses, a condition for which in vivo experiments are likely encumbered by ethical challenges.

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The authors declare that they have no conflicts of interest to report regarding the study reported here.

Footnotes
g. Megacell, Sigma-Aldrich, Gillingham, England.
i. Cellbiochem, Merk Millipore, Darmstadt, Germany.
k. Alexafluor, Life Technologies Ltd, Paisley, Scotland.
l. Hoechst 33342, Life Technologies Ltd, Paisley, Scotland.
m. Hydromount, National Diagnostics, Atlanta, Ga.

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