Journal of Cellular Biochemistry 55:530–544 (1994)

Space Shuttle Flight (STS-45) of L8 Myoblast Cells Results in the Isolation of a Nonfusing Cell Line Variant

David A. Kulesh, Loraine H. Anderson, Bernard Wilson, Ericka J. Otis, Diane M. Elgin, Michael J. Barker, William J. Mehm, and George P. Kearney

Division of Altitude and Hyperbaric Physiology at the Armed Forces Institute of Pathology, Washington, District of Columbia 20306 (D.A.K., L.H.A., B.W., E.J.O., W.J.M.); Division of Surgery at the Walter Reed Army Institute of Research, Washington, District of Columbia 20307 (G.P.K.); Tulane University School of Medicine, New Orleans, Louisiana 70118 (D.M.E.); United States Naval Academy, Annapolis, Maryland 21402 (M.J.B.)

Abstract Myoblast cell cultures have been widely employed in conventional (1*g*) studies of biological processes because characteristics of intact muscle can be readily observed in these cultured cells. We decided to investigate the effects of spaceflight on muscle by utilizing a well characterized myoblast cell line (L8 rat myoblasts) as cultured in the recently designed Space Tissue Loss Flight Module "A" (STL-A). The STL-A is a "state of the art," compact, fully contained, automated cell culture apparatus which replaces a single mid-deck locker on the Space Shuttle. The L8 cells were successfully flown in the STL-A on the Space Shuttle STS-45 mission. Upon return to earth, reculturing of these spaceflown L8 cells (L8SF) resulted in their unexpected failure to fuse and differentiate into myotubes. This inability of the L8SF cells to fuse was found to be a permanent phenotypic alteration. Scanning electron microscopic examination of L8SF cells growing at 1*g* on fibronectin-coated polypropylene fibers exhibited a strikingly different morphology as compared to control cells. In addition to their failure to fuse into myotubes, L8SF cells also piled up on top of each other. When assayed in fusion-promoting soft agar, L8SF cells gave rise to substantially more and larger colonies than did either preflight (L8AT) or ground control (L8GC) cells. All data to this point indicate that flying L8 rat myoblasts on the Space Shuttle for a duration of 7–10 d at subconfluent densities results in several permanent phenotypic alterations in these cells.

Key words: muscle, myogenesis, Space Shuttle, cell culture, microgravity, neoplastic transformation, cartridge

All biological organisms are compelled to physiologically adapt to the unique stresses (vibration and noise of launch, zero gravity, cosmic radiation, and the hypergravity of reentry) imposed by spaceflight. Not surprisingly, the health and safety of astronauts and cosmonauts during extended periods in space has long been a major concern of the United States and Russian space programs. During recent long-term space missions, it has been possible to perform extensive biological and medical research in space. One area receiving considerable attention has been the effects of long duration spaceflight on the musculoskeletal system. The most critical factor affecting the human musculoskeleton system during spaceflight is microgravity, which causes a "loss in the level of the daily external loading history [primarily body weight and the GRF" (ground reaction force)] [Whalen, 1993]. The GRF is the inertia which accelerates and decelerates the center of mass of the body during activity. Spaceflight muscle tissue effects include a general weakening of contractile performance (up to 20%), an occurrence of scattered necrotic foci along myofibers, a loss of detectable sarcosomal (mitochondrial) functionality, an erosion of myofibrils from sarcomeres, and a decreased myosin light chain content concurrent with a decrease in myosin ATPase activity. Also seen are shifts in predominant metabolic pathways for provision of energy to support contraction, and a change in the ultrastructure of the motor end plates [D'Amelio and Daunton, 1992; Ilyina-Kakueva et al., 1976b; Riley et al., 1987]. How-

George P. Kearney's present address is *in*Vitronics, 4201 University Drive, Suite 102, Durham, NC 27707.

Received December 27, 1993; accepted March 10, 1994.

Address reprint requests to David A. Kulesh, Ph.D., Capt, USAF, BSC, Division of Altitude and Hyperbaric Physiology (CPW-P), Armed Forces Institute of Pathology, Washington, DC 20306-6000.

ever, while it has been well established that the skeletal and muscle tissues of astronauts and animals are adversely affected by long-term spaceflight [Loughna et al., 1987; Nicogossian and Parker, 1982; Steffen and Musacchia, 1987], specific alterations at the cellular and molecular levels have yet to be established. The focus of spaceflight investigations should now be extended to the effects of spaceflight, and specifically microgravity, at the macromolecular level as suggested by Allaerts [1991] and Spooner [1992]. These studies should include investigations of cellular ultrastructure along with detailed examinations of cellular and molecular functions including spaceflight-regulated gene expression and regulation. Only when the morphologic, biochemical, and molecular effects of spaceflight are detailed can the information be used to define cellular microgravity-regulated mechanisms. Scientists might then better overcome the deleterious effects of space travel and thus lengthen space flights, expanding our explorative capacity.

Within the 1*g* gravitational field of earth, genes, which code for the basic form and function of an organism, execute their genetic programs "expecting" gravity to be present. Therefore, while the outcome of evolution in gravity may, in fact, be a permanent imprinting of gravity-controlled mechanisms onto the genomes of living organisms [Mesland, 1992], environmental influences may also contribute to the ultimate detailed form of an organism during ontogenesis and morphogenesis. Before the advent of spaceflight, few people questioned whether gravity could influence tissue at the cellular level. A number of biological studies have suggested that spaceflight has a profound effect on the normal pattern of cell proliferation and differentiation in both prokaryotic and eukaryotic cells [for a review see Cogoli and Gmünder, 1991]. However, a methodical and comprehensive program of investigations did not begin until 1983 with the first *Spacelab* mission and was continued by the German Spacelab D-1 and D-2 missions in 1985 and 1993, respectively. Recent advances in cell culture technology have allowed scientists to ask questions regarding the effects of spaceflight and microgravity at the cellular and molecular levels.

The specific goal of any spaceflight cell culture experiment should be to augment whole animal model studies [Ilyina-Kakueva et al., 1976a,b; Loughna et al., 1987; Nicogossian and Parker, 1982; Riley et al., 1985, 1987; Steffen and Musacchia, 1987] so as to simplify the molecular and cellular analysis of microgravity effects on cells. Therefore, in this study, we decided to utilize cultured skeletal muscle cells (myoblasts) which have been widely employed in studies of conventional earthbound muscle function. In addition, a substantial number of the characteristics of intact muscle can be observed in these cells. The very nature of muscle differentiation in vitro makes it a very appealing system for the study of differentiation in microgravity. Myoblast cells exhibit normal proliferation when incubated under appropriate growth conditions. However, following a change of medium to one that favors differentiation rather than proliferation, myoblasts undergo a process called "commitment to myogenic differentiation" [Nadal-Ginard, 1990]. Concurrently, there is a coordinated expression of muscle-specific genes [Benoff and Nadal-Ginard, 1980; Caravatti et al., 1982], closely followed by the fusion of numerous individual myoblasts to form extensive myotubes and the formation of the contractile apparatus which exhibits spontaneous contractions [Emerson, 1990; Funk et al., 1991]. Therefore, these skeletal muscle cells provide an attractive model system for exploring the relationship between muscle cell proliferation and differentiation because activation of muscle-specific genes during myogenesis is coupled to withdrawal of proliferating myoblasts from the cell cycle. These observations, combined with recent physiological, biochemical, and molecular biological studies, provide assurance that the results obtained with cultured myoblast cells are representative of many processes that occur in muscle in vivo [Florini et al., 1989]. The considerable advantages of working with cloned, reproducible cell cultures have prompted many laboratories to use the well characterized rat skeletal myoblast cell lines L6 [Yaffe, 1968] or L8 [Richler and Yaffe, 1970; Yaffe and Saxel, 1977] for the cellular and molecular evaluation of myogenesis.

Our laboratory has decided to investigate the effects of spaceflight on muscle by culturing L8 myoblasts in the Space Tissue Loss Flight Module "A" (STL-A) [Kearney and Arnold, in preparation]. Because cells in culture exhibit homeostatic responses to adverse changes in their local environment, the STL-A was specifically designed to rigorously control the microenvironment surrounding the mass of cultured cells while in space. The meticulously controlled envi-

ronment assures that any changes in cell function during Space Shuttle flight are due solely to the spaceflight and not to local anomalous microgravity-induced cell culture conditions. We wish to report our initial findings as to the growth characteristics of L8 myoblasts cultured in the STL-A module following their flight on Space Shuttle mission STS-45. We hope these studies will contribute to our overall understanding of cell proliferation, cell differentiation, and development in the microgravity environment.

METHODS

The Space Tissue Loss Flight Module "A" (STL-A)

The STL-A is a compact, fully contained, automated cell culture apparatus which replaces a single mid-deck locker on the Space Shuttle (Fig. 1A) [Graeber and Kearney, 1987; Kearney and Arnold, in preparation]. The STL-A module was designed and built at the Walter Reed Army Institute of Research, Washington, DC. Briefly, the design philosophy of the STL-A which flew on the Space Shuttle mission STS-45 adopted a modular approach with significant system redundancy in order to maximize experimental return (Fig. 2). The STL-A system was physically and functionally divided into four experimental rail assemblies, each of which was composed of a media reservoir, routing valves, pumps, oxygenators, four hollow fiber cartridges (based on the Knazek/NIH design), a controlled temperature stage, and embedded temperature and pH sensors. A 5% CO_2, 20% O_2, 75% N_2 certified gas mix was continuously filtered and conditioned as it was applied to the circulating media via membrane exchangers. Solid-state Thermal Environment-Peltier heat pump chips were used to maintain 37°C during the STS-45 flight. The real-time temperature was recorded every 15 min with a full-flight thermal profile used to run the subsequent identically configured ground controls. A detailed characterization of the STL-A module is to be presented elsewhere [Kearney and Arnold, in preparation].

Culture Media

All myoblasts were cultured in the following growth media [Florini et al., 1989]: a 4:1 ratio of Dulbecco's modified Eagle's medium and Medium 199, 89%; chicken embryo extract, 1%; heat-inactivated horse serum, 10%; L-glutamine, 2 mM. Fusion/differentiation media [Florini et al., 1989] consisted of Dulbecco's modified Eagle's medium; L-glutamine, 2 mM; and heat-inactivated horse serum, 1%. All media was purchased from GibcoBRL (Gaithersburg, MD). Antibiotics (200 units/ml of penicillin, 200 μg/ml streptomycin; 0.025 μg/ml fungizone; and 20 μg/ml of gentamicin) were added only to media used specifically for culturing cells in the STL-A modules. Early passage numbers (#19–#35) were used for all experiments and were routinely checked for mycoplasm contamination using the GibcoBRL MycoTect Kit.

Cells

Three L8 myoblast cell lines were employed in all of the described experiments.

L8 stock myoblasts (L8AT). These are the L8 myoblasts purchased from ATCC (CRL 1769) [Richler and Yaffe, 1970; Yaffe and Saxel, 1977] which were maintained exclusively in tissue culture flasks for use as the preflight control cells. They were subsequently used for seeding all of the experimental STL-A muscle cell cartridges. L8 is a nontumorigenic myogenic cell line originally isolated by D. Yaffe in 1969 by the selective serial passage of myoblasts isolated from primary rat skeletal muscle cell cultures prepared from newborn noninbred Wistar rats. Carcinogens were not used to establish the L8 cell line.

L8 space flown (L8SF) myoblasts. These are the stock L8 myoblasts which were inoculated and cultured in the cartridges of the STL-A flight module on the Space Shuttle mission STS-45 from 22 March–02 April 1992 (9 d in space with a total of 11 d in the STL-A). Following landing, the flight module was retrieved from the Space Shuttle and the cells were immediately removed from the cartridges with a dilute solution of trypsin-EDTA.

L8 ground control (L8GC) myoblasts. These are the stock L8 myoblasts which were inoculated and cultured at 1*g* in duplicate STL-A cartridges 3 weeks after retrieval of the STS-45 flight module. The duplicate STL-A was maintained in exact accordance to the STS-45 flight data with respect to culture duration and temperature profile. Following 11 d at 1*g*, the cells were removed from the cartridges with a dilute solution of trypsin-EDTA.

Experimental Design

L8AT cells were initially propagated in standard tissue culture flasks and fed the standard L8 growth media (see above). At 48 hr prior to flight hardware assembly, the experimental L8

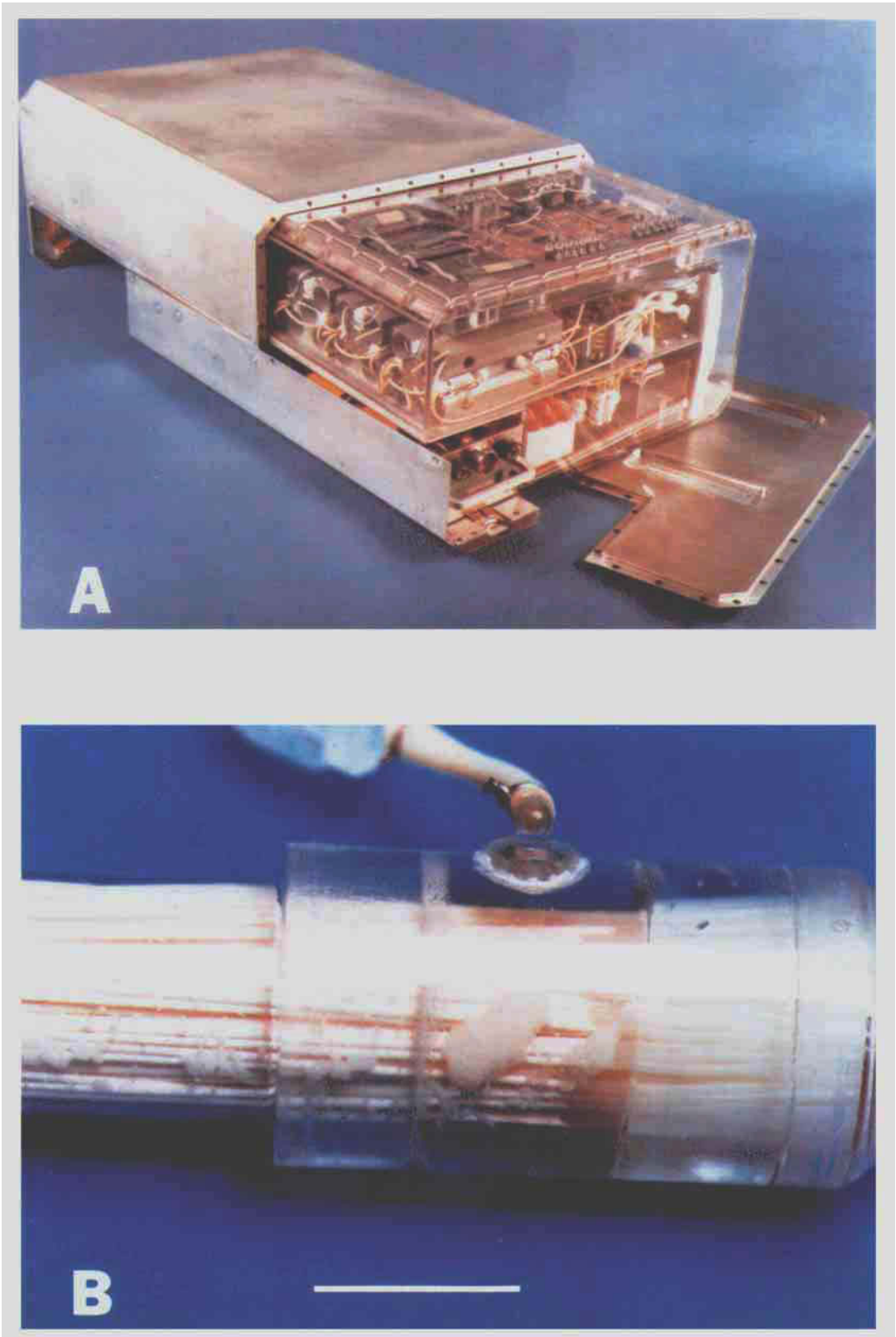

Fig. 1. **A:** External view of the Space Tissue Loss Flight Module "A" (STL-A) which flew on the Space Shuttle mission STS-45. The dimensions of the module are 48.3 cm in length, 43.2 cm in width, and 22.9 cm in height (approximate volume of 0.05 m^3). An exact duplicate system was set up and maintained on the ground as a 1*g* control matching the in-flight conditions. **B:** STL-A cell cartridge with L8SF cells growing on collagen-coated microcarrier beads (approximately 14 d of growth in space). Bar = 15 mm.

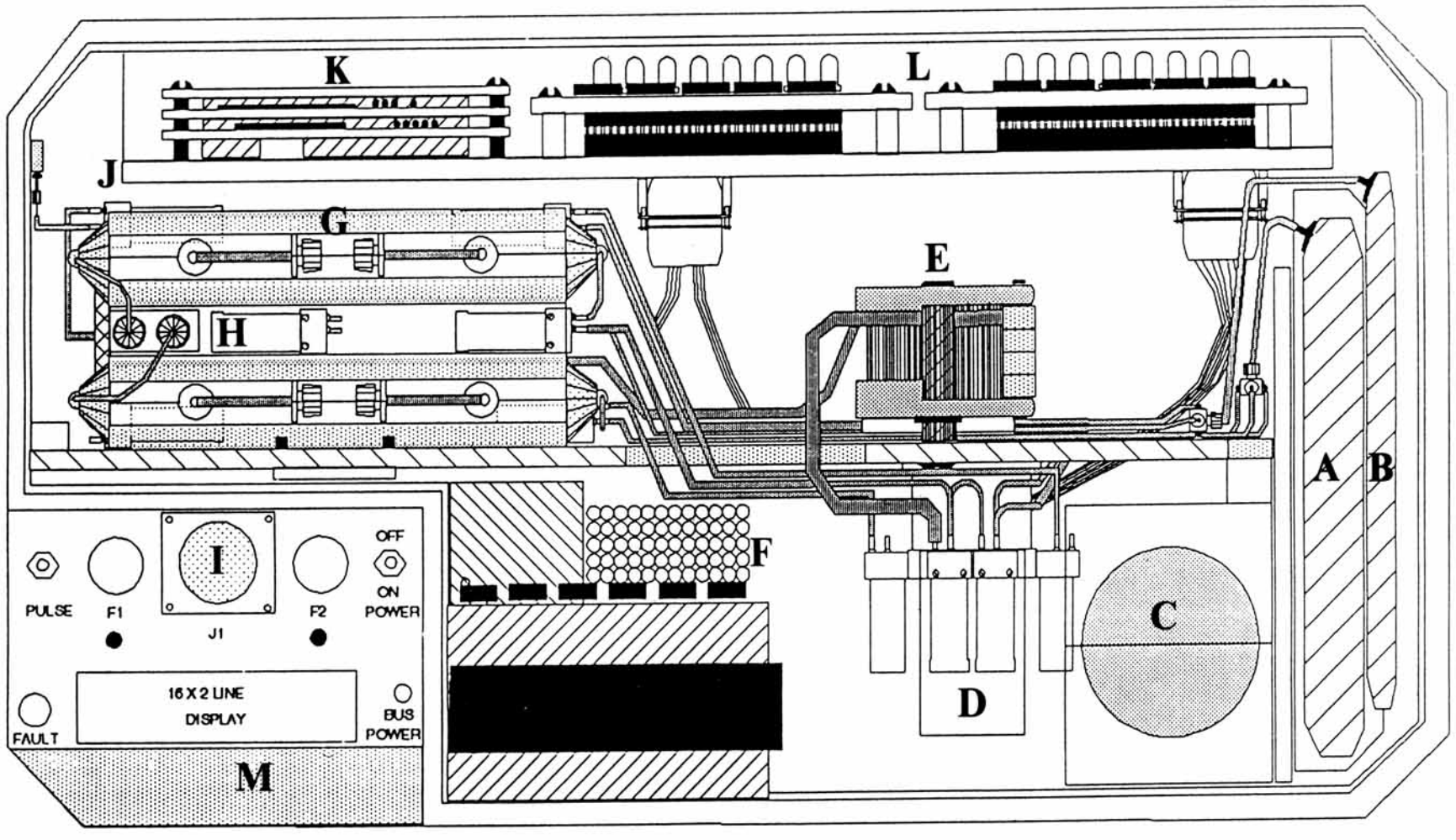

Fig. 2. Labeled internal drawing of the STL-A module which flew on the Space Shuttle mission STS-45. **A,** medium bag; **B,** sump bag; **C,** gas canister; **D,** solenoid driver valves; **E,** pump; **F,** fraction collector; **G,** rail with two visible cartridges; **H,** oxygenator; **I,** power; **J,** computer motherboard; **K,** data logger; **L,** drive boards; **M,** air vent.

myoblasts were inoculated into each cartridge at concentrations which would not permit fusion during the flight (approximately 6.7×10^6 cells/cartridge). The cartridges were maintained for 24 hr on the Cellco CELLMAX System (Cellco Inc., Germantown, MD) to permit the cells to attach to the nonporous collagen-coated microcarriers (Solo Hill) and to allow for detection of leaks and contamination. The cartridges were loaded into the actual flight rails at L −22 hr. At L −20 hr, the rails were installed into the flight hardware, handed over to NASA technicians, loaded into the mid-deck locker, attached to onboard power, and monitored by the astronauts.

Flight Log of STS-45

The STS-45 mission of Shuttle Atlantis (ATLAS-1) occurred March 24–April 2, 1992. Launch was from Launch Pad 39A with an orbit of 184 statute miles apogee and 57° inclination to the Earth's equator. This trajectory took the experiment through the south Atlantic anomaly, an area in the magnetosphere which allows significant cosmic radiation to enter the lower atmosphere. Weather delays added one extra day to the experiment duration. The flight covered more than 4.5 million miles over 180 earth orbits and landing was at the Kennedy Space Center, FL. The STS-45 unit contained three experimental groupings: NASA-sponsored experiments from University of Louisville included HL-60 and P388 immune cell lines; NASA Ames Research Center primary bone cells; and Walter Reed Army Institute of Research/Armed Forces Institute of Pathology L8 rat myoblasts.

Following landing, the flight hardware was returned to the investigator within 3 hr for processing. At no time was the system without power and gassing. Thermal setpoints were maintained until unit disassembly following landing. Three weeks after recovery of the flight data records, L8AT cells were cultured at 1*g* in an identically configured and maintained unit. These "postflight" ground control (L8GC) cells were processed and analyzed in a manner identical to the spaceflown L8 cells.

Cell Doubling and Cell Density Measurements

The CellTiter™ Non-radioactivity Cell Proliferation Assay Kit (Promega) was used in all cell proliferation experiments involving control and spaceflown cells. Standard curves were generated by assaying a series of known numbers of cells (twofold dilutions) for each cell type used. Endpoint absorbance of samples was read at 550 nm with a reference wavelength of 650 nm using an ELISA reader (Molecular Devices Thermomax Microplate Reader).

Scanning Electron Microscopy

L8 cells (L8AT, L8GC, and L8SF) growing for 14 d at 1*g* on fibers in cartridges were first gently washed in two changes of phosphate-buffered saline (PBS) (pH 7.2). The cartridges were then broken open at the ends and the fibers/cells gently removed into a 2% glutaraldehyde in PBS fixative for at least 2 hr. The fibers were then washed three times in PBS, postfixed for 30 min in 1% osmium tetroxide in PBS, washed three times (15 min each) in PBS, and dehydrated in a series of graded ethyl alcohol (50%, 70%, and 95%) followed by three changes in absolute ethanol. The samples were dried in a Tousimis Autosamdri 814 critical point drier with CO_2 and sputter coated with gold and palladium in a Emscope coating unit E 5000. Examination was accomplished in a Hitachi S-570 Scanning Electron Microscope operating at 15 kV.

Soft Agar Assay for Neoplastic Transformation

For each cell line to be assayed, wells of a six-well plate were filled with 5 ml of complete agar medium and allowed to gel. Agar medium consisted of an equal volume of either complete L8 2× medium (2× DMEM, 20% heat-inactivated horse serum, 2% chick embryo extract) or L8 2× fusion medium (2× DMEM, 2% heat-inactivated horse serum) mixed with an equal volume of a 2× agar solution (1.275% Difco Bacto Agar). A duplicate bottle of either complete agar medium or fusion agar medium was then readied for mixing just prior to the actual plating of the cells and kept at 45°C until used. Control L8 and L8SF cells were harvested into a single-cell suspension, diluted to 200 cells/ml in 5 ml of complete agar medium (final concentration of 0.34% agar), and overlaid into quadruplicate wells of the six-well plate at approximately 1000 cells/well. Visible colonies were counted and sized at 14 and 20 d of incubation to determine cloning efficiency. Statistical analysis was done using one-way Analysis of Variance. Differences were considered significant if $P < 0.05$.

RESULTS

Spaceflown L8 Myoblast Characteristics

Cell growth during flight. Temperature data from STS-45 indicated that all L8 myoblast cell cartridges were maintained continuously at 37 ± 1°C during flight. Each cartridge was fed 60 ml of media over the course of the flight with the feeding regimen consisting of equal feedings on a 6 hr cycle. All cartridges were found to be free of visible microbial and fungal contamination before and after flight. Visible plaques of cell growth were revealed following flight in the L8 cartridges (Fig. 1B). The aggregates were remarkable in that the cells grew upward in hillocks instead of the expected normal monolayer configuration. Following recovery, three of the four L8 cartridges from Rail 1 were trypsinized, placed into cryopreserving solution, and frozen as were all four L8 cartridges from Rail 2. The remaining cartridge's L8 cells were trypsinized, transferred to a standard tissue culture flask, and maintained in continuous culture. Examination of cell yields at the end of flight disclosed no visibly fused cells in any of the cultures.

Cell growth after flight. Continuously cultured L8 cells (L8AT, L8GC, and L8SF) at 1*g* on earth divided at roughly the same rate: 16.6–18.7 hr/doubling (Table I). All cells also exhibited indistinguishable morphology at initial plating densities (Fig. 3A,E,I). While the density at confluency of the spaceflown (L8SF) cells is approximately equal to preflight (L8AT) and ground control (L8GC) cells (Table I), the overall growth pattern of the L8SF cells was noticeably different: L8AT (Fig. 3B) and L8GC (Fig. 3F) seemed to form "swirl-like patterns" at confluency while L8SF (Fig. 3J) was more cobblestone-like in appearance. Cultures were continued for 32 d without passage to promote fusion. At first, no exogenous hormonal or chemical stimulation was supplied. Within 16 d, the L8AT (Fig. 3C,D) and L8GC (Fig. 2G,H) cells had achieved extensive fusion into myotubes (~75–90% of all cells in observed cultures), while no fusion into myotubes was seen in the L8SF cultures (Fig. 3K,L). However, L8SF cells did cease to proliferate upon attaining confluence in a manner similar to that of the L8AT and L8GC cells. When transferred to fusion media plus 0.3 μM insulin (Sigma), L8SF still failed to fuse while all the L8AT and L8GC cells fused as expected (data not shown). Cryopreserved L8SF specimens were recultured (see Methods) to determine if the phenomenon was observed in the other cartridges which had been subjected to slightly different media and thermal environments. In every case a stable loss of fusion competence was noted in the spaceflown L8 cells. Preliminary karyotyping of the spaceflown L8 cells indicates no obvious change in chromosome composition (data not shown).

TABLE I. Comparison of L8AT, L8GC, and L8SF Growth Characteristics*

Cell line	Approx. doubling time (hours)	Cessation at confluency	Fusion	Cell density at confluency (cells/mm²)
L8AT	17.0	Yes	Yes	395
L8GC	16.6	Yes	Yes	393
L8SF	18.7	Yes	No	439

*Averages from three experiments of quadruplicate wells.

The L8AT, L8GC, and L8SF cells were also inoculated into two cartridges each containing fibronectin-coated polypropylene fibers and maintained for 14 d in L8 growth media at 1*g* in the Cellco CELLMAX System. Scanning Electron Microscopic (SEM) examination (Fig. 4) of these cultures revealed that all cells were able to attach to and proliferate on the fibronectin-coated fibers. The L8AT (Fig. 4A,B,C) and L8GC (Fig. 4D,E,F) growth and fusion patterns resemble the normal pattern seen with these cells growing in plastic tissue culture flasks. However, the L8SF (Fig. 4G–L) growth pattern was strikingly different in that they exhibited an atypical morphology and began to pile up on top of each other.

Soft agar assay. We have also begun to investigate the possibility that spaceflight has changed the "normal" pattern of myoblast proliferation/differentiation into a phenotype exhibiting transformed-like characteristics. L8AT, L8GC, and L8SF cells were cultured for 20 d in both complete L8 growth media/soft agar and L8 fusion media/soft agar (Fig. 5). Table II presents data confirming that there was no difference in either total relative colony numbers or size distribution when these cells were grown in complete L8 growth medium (10% horse serum, 1% chick embryo extract). In contrast, when the cells were cultured in L8 fusion media (1% horse serum), L8SF cells gave rise to substantially more and larger colonies ($P < 0.05$)

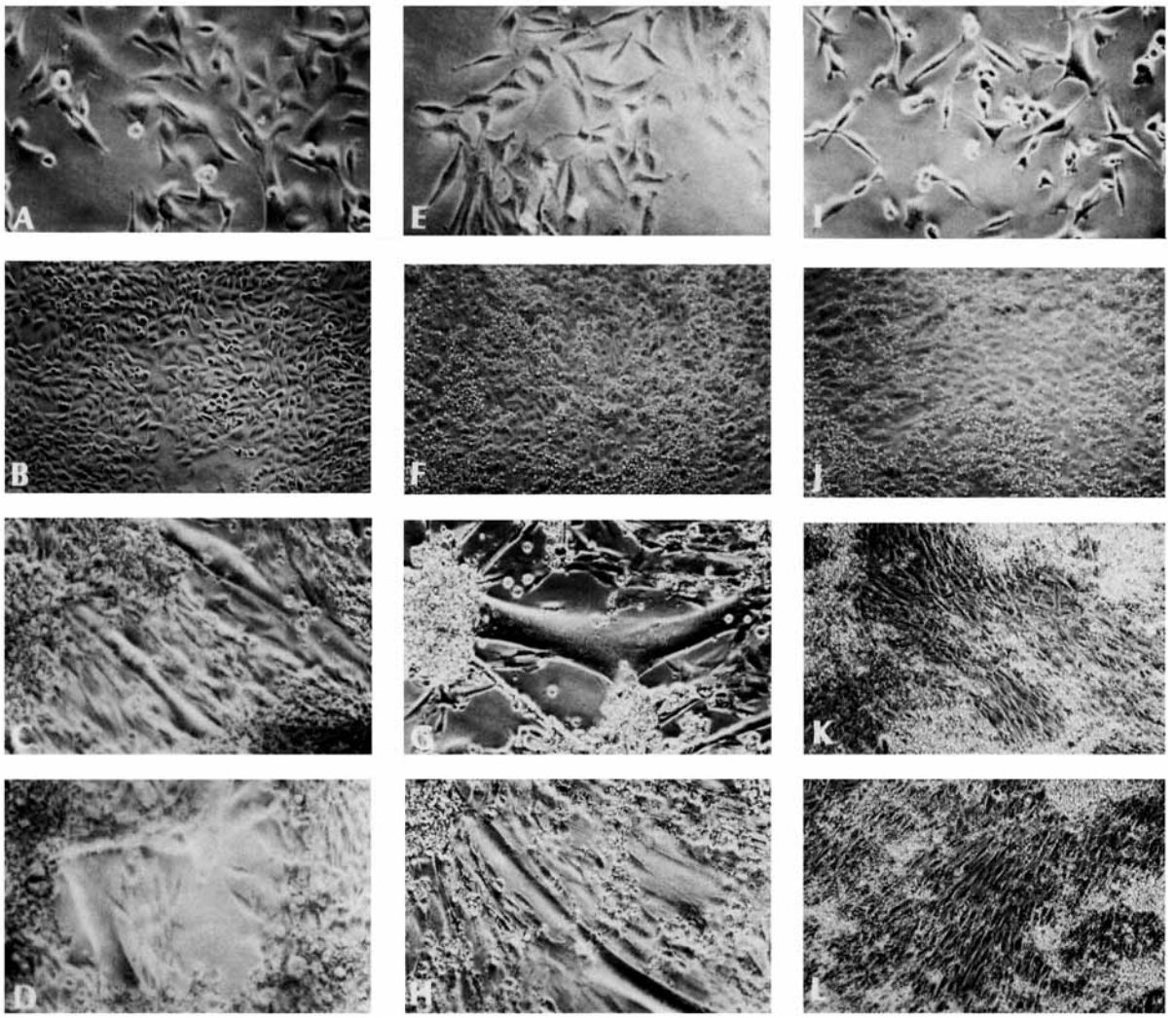

Fig. 3. L8AT, L8GC, and L8SF cell cultures. Phase contrast photographs of live L8 myoblasts (L8AT, L8GC, and L8SF) growing in plastic tissue flasks at 1*g*. Fused cells (myotubes) are those large cells which contain numerous cell nuclei. Cloning density morphology of L8AT, L8GC, and L8SF cells (**A, E, I**); confluency + 1 d of L8AT, L8GC, and L8SF cells (**B, F, J**); confluency + 10 d, myotubes of L8AT and L8GC cells (**C, D, G, H**), and nonfused L8SF cells (**K, L**). Note: A–D are L8AT cells; E–H are L8GC cells; and I–L are L8SF cells. B, F, and J are 100×, while the remaining pictures are 200×.

than did either L8AT or L8GC cells. Since the "anchorage-independent" phenotype as measured by colony-forming efficiency in soft agar is a parameter correlated with tumor formation in vivo [Shin et al., 1975] and transformation may be an alternative to normal skeletal muscle cell development [Kaufman et al., 1980], we also plan to test the tumorigenicity of all L8SF cells using the nude mouse model. Our ultimate goal is to both identify and isolate the gravity-sensing molecular target(s) using cells cultured in the STL-A.

In summary, our initial findings with L8 rat myoblasts following spaceflight in the STL-A module on the Space Shuttle mission STS-45 are: (1) discernible L8 cellular aggregates were visible to the naked eye only within spaceflown cartridges (L8SF); (2) L8SF cells continuously cultured at 1*g* on earth exhibited a plating density morphology similar to L8AT and L8GC cells when grown on tissue culture plastic; (3) all cells divide at roughly the same rate as prior to flight and all ceased proliferation upon attaining confluence; (4) the densities (cells/mm^2) of L8AT, L8GC, and L8SF cells at confluency are approximately equal, although the overall growth pattern of the L8SF cells was remarkably different from that of L8AT and L8GC cells; (5) L8SF cells exhibit a most striking and unexpected permanent phenotypic deviation in that they do

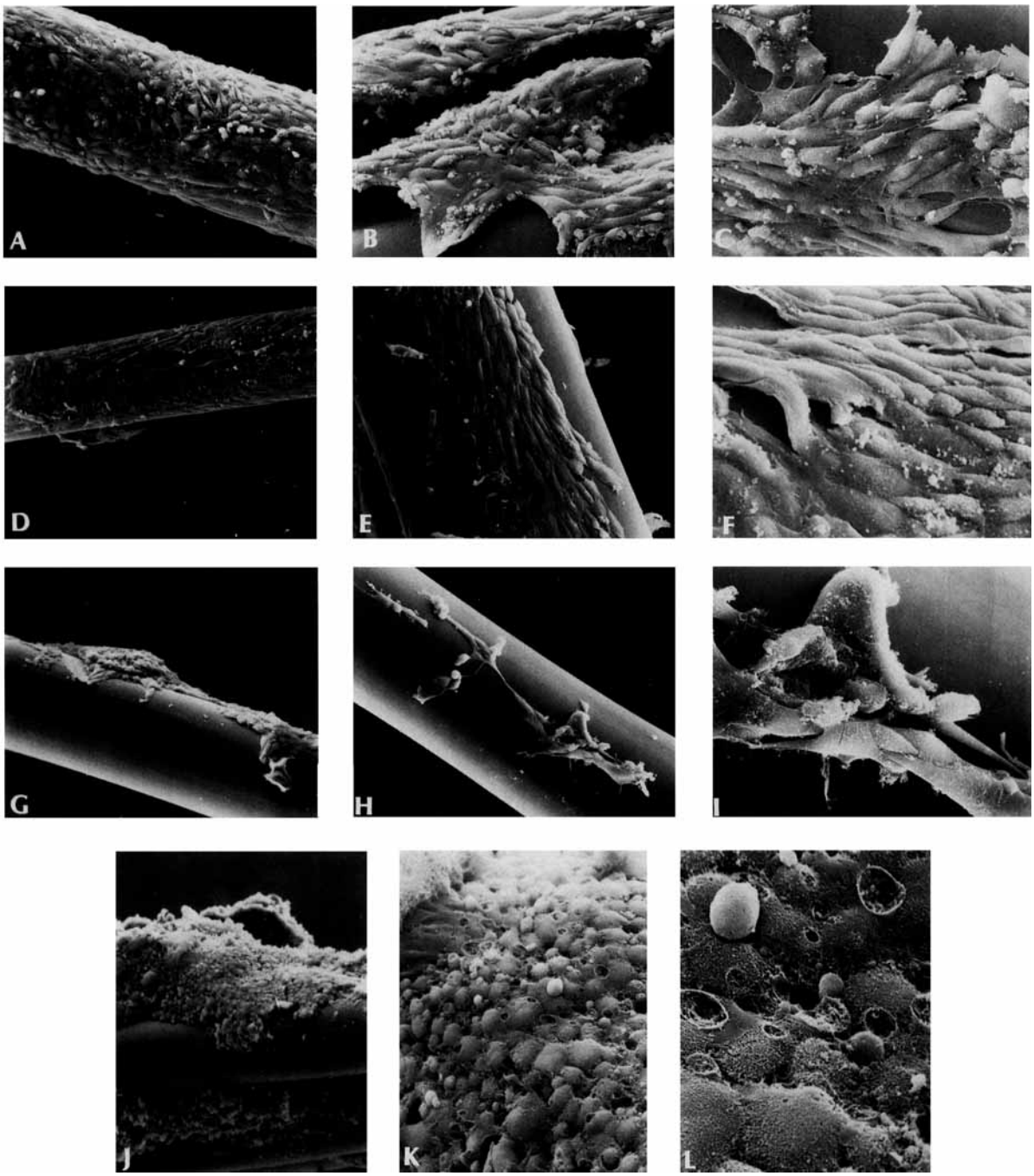

Fig. 4. Scanning electron microscopy pictures of L8 cells. Untreated L8AT, L8GC, and L8SF cells from STS-45 were cultured for 14 d on fibronectin-coated polypropylene cartridge fibers attached to the CELLMAX System, fixed, and processed for scanning electron microscopic examination. **A, B, C:** L8AT cells at/near confluency (200×, 300×, 500×, respectively); **D, E, F:** L8GC cells at/near confluency (120×, 300×, 700×, respectively); **G, H, I:** L8SF at low density (200×, 250×, 1,000×, respectively); **J, K, L:** L8SF at high density (120×, 500×, 1,500×, respectively).

not fuse when placed under in vitro fusing conditions (L8 medium with 1% horse serum), while the L8AT and L8GC cells fuse normally under these conditions; (6) fusion/differentiation media (L8 medium/1% horse serum plus 0.3 μM insulin) failed to induce fusion of the L8SF cells (data not shown); (7) SEM examination of L8 cells cultured at 1*g* revealed that only L8SF cells grew on top of each other; and (8) enhanced growth of L8SF cells in 1% horse serum/soft agar may indicate a possible shift to a transformed-like phenotype.

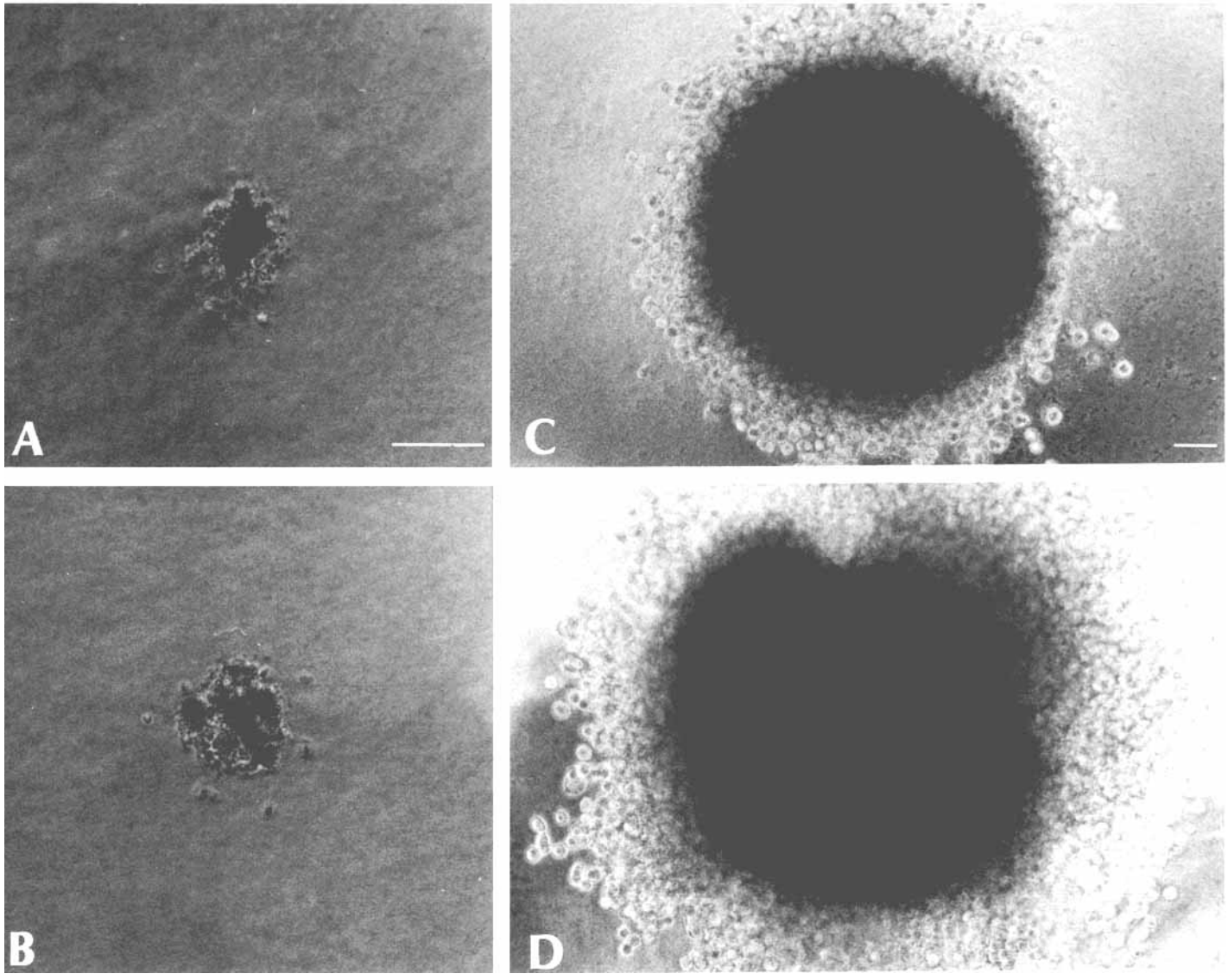

Fig. 5. Soft agar colonies of L8AT, L8GC, and L8SF cells from STS-45. **A,** L8AT cells; **B,** L8GC cells; **C, D,** L8SF cells. All cells were plated in complete L8 media with 1% horse serum/soft agar and incubated at 37°C for 20 d. Bars = 100 μm (A and B are 20×; C and D are 10×).

TABLE II. Soft Agar Neoplastic Transformation Assay

	Total visibile colonies/1,000 cells plated[a] (20 d)				
	10% Horse serum	1% Horse serum			
Cell line			S	M	L[b]
L8AT	351	22	(20	0	2)
L8GC	395	25	(21	2	2)
L8SF	327	75*	(33*	6*	26*)

[a]Averages from three experiments of duplicate wells.
[b]S = small, M = medium, L = large, where S and M could be seen only with a microscope or dissecting scope, respectively, while L could be seen with the naked eye.
*Significant difference ($P < 0.05$) when compared to either L8AT or L8GC.

DISCUSSION

The goal of our spaceflight cell culture research is to augment, at the cellular and molecular levels, the previously reported studies of the effects of spaceflight (microgravity) on human and whole animal muscular systems [for review see Grigoriev and Egorov, 1992a,b]. The cell culture system (STL-A) was specifically designed to circumvent most of the unmanageable irregularities of whole animal models: preexisting variations between and among the experimental groups, differences in food and water intake, hormonal differences and, most importantly, unmeasured stress factors. The STL-A system was designed for the continuous in-flight culturing of homogeneous populations of varying cell types while simultaneously eliminating any microgravity-generated anomalies within the microenvironment of the cell cartridges. This is critical because unintentional cell culture distress can result in dramatic and possibly artifactual changes in cellular physiology (i.e., the rapid synthesis of "stress proteins" in response to elevated temperatures, abnormally low glucose levels, and calcium or oxygen deprivation) [Morrison et al., 1992]. The STL-A module design is well suited for the culture of a diverse variety of cell types at zero gravity along with their subsequent recovery following return to the 1*g* environment. To our knowledge, this is the first investigation utilizing the STL-A to study the growth/differentiation response of muscle cells (myoblasts) following exposure to spaceflight on the Space Shuttle.

In order to evaluate the effect of spaceflight on muscle tissue, we employed rat myoblasts (L8) as our model system because many cellular and molecular characteristics of differentiating muscle can be observed in these cells during their in vitro proliferation, cessation of proliferation at confluency, and fusion into multinuclear myotubes. Following their flight on STS-45, the most striking variation displayed by the myoblast cultures within the spaceflown cartridges was their outgrowth into cellular aggregates which were visible to the naked eye. These aggregates were not visible in the control cartridges following the culturing of L8 cells in the ground-based STL-A module. While the spaceflown L8 cells (L8SF) were able to overcome their contact inhibition growth restrictions, the L8 myoblasts cultured in the ground cartridges (L8GC) formed the expected monolayer. Also, there were no fused cells recovered from either the spaceflown or ground control cartridges, thereby indicating that the cells within the cartridges were either at subconfluent densities or were not confluent long enough for fusion initiation during the culture time. A scanning EM examination of each L8 population following subculturing at 1*g* confirmed that only the L8SF cells grew on top of one another within the cartridges.

The hallmark characteristic of in vitro myoblast differentiation into skeletal muscle is the cessation of myoblast proliferation at confluency followed by the dramatic fusion of single myoblasts into nonreplicating multinucleate fibers (myotubes). This characteristic was monitored in the cells recovered from L8GC and L8SF cartridges and nonflown (L8AT) cells when they were subsequently subcultured at 1*g* in standard plastic culture dishes. While the initial proliferation profiles of the three cell lines were approximately the same, we were surprised to discover that the L8SF myoblasts exhibited a permanent phenotypic alteration whereby the spaceflown cells did not fuse and differentiate into myotubes following an extended period at confluency as did the L8GC and L8AT cells. This characteristic is also seen in many transformed muscle cell lines and in cells derived from rhabdomyosarcoma, the most common tumors of children [Tapscott et al., 1993]. These transformed clonal cell lines exhibit a variety of characteristics different from the parental cell lines [Harel et al., 1989; Kaufman and Parks, 1977; Kaufman et al., 1980; Richler and Yaffe, 1970; Yaffe and Saxel, 1977]. Many of these nonfusing variants maintain their proliferative capacity, do not form myotubes, and do not have elevated creatine kinase activity or increased myosin even though they were isolated employ-

ing a variety of protocols: serial passage of nonfusing cells present in fusing culture, agar growth clone selection, nontreated plastic dish selection, and survival of either spinner culturing or ConA treatment [Kaufman et al., 1980]. Because injection of these differentiation-defective cell lines into nude mice produced tumors [Kaufman et al., 1980], these myoblasts, which have lost the ability to control and cease proliferation, represent a transformed, potentially tumorigenic population. In addition, whereas the parental L8 cells were diploid, most of these nonfusing clones were polyploid and expressed reverse transcriptase [Kaufman et al., 1980]. However, no single in vitro characteristic was found to be a constant index of their tumorigenic capacity. Another nonfusing myoblast clone, L84 [Yaffe and Saxel, 1977], evidenced the difficulty of determining whether cells which fail to differentiate are truly noncommitted cells or whether they are just cells grown in conditions under which differentiation is not triggered [Yaffe and Saxel, 1977]. Once their fusion program is triggered (by changing to a differentiation-inducing medium containing insulin), differentiation of L84 cells seems to be qualitatively and quantitatively indistinguishable from that of the parental L8 cells. While no fusion conditions have as yet been found to "force" the differentiation of L8SF cells into myotubes, we plan to use either 5-azacytidine [Konieczny and Emerson, 1984; Taylor and Jones, 1982], which has been shown to induce the muscle phenotype, or 10^{-8}–10^{-6} M retinoic acid, which has recently been shown to induce nonfusing variants to differentiate [Garcia et al., 1993].

We assayed the growth of the L8SF cells in soft agar because colony formation in soft agar is a parameter best correlated with tumor formation in vivo [Shin et al., 1975]. Only when the cells were cultured in a soft agar with fusion media (1% heat-inactivated horse serum) were a statistically significant greater number of colonies present within the L8SF cell population as compared to L8AT and L8GC cells under identical conditions. While the entire population of L8SF cells is unable to fuse in vitro when grown on plastic tissue culture dish surfaces (we have never observed any myotubes or myofibers in the L8SF cultures), the soft agar assay indicates that there may be an L8SF cell subpopulation present which grows more vigorously in this low-serum environment. Isolation of individual subclones from the soft agar and injection of both the entire L8SF population and the various subclonal cell lines into nude mice should determine the tumorigenic potential of the respective cell populations. Our L8SF variant cells do not exhibit phenotypic properties of differentiated muscle and are distinct from the myogenic L8AT and L8GC cells by several criteria often associated with transformed myoblast growth in vitro.

It has been proposed that influencing the level of expression of genes might be a common way for gravity to exert its effect on cell proliferation and differentiation [de Groot et al., 1991a]. A family of muscle-specific regulatory genes that can both inhibit cell proliferation and initiate the complete muscle differentiation program has been discovered. They provide a mechanistic understanding of the events that underlie the establishment of the skeletal muscle phenotype [for reviews see Funk et al., 1991; Li and Olson, 1992; Olson, 1990; Tapscott and Weintraub, 1991; Weintraub et al., 1991]. These proteins, which include MyoD, myogenin, myf-5, and myf-6 (MRF4), share homologies with each other and belong to a superfamily of *Myc*-related proteins. Interestingly, myoblast cell lines that fail to fuse either do not express myogenin [Mueller and Wold, 1989] or may be deficient in a factor required for MyoD activity [Tapscott et al., 1993]. A growing body of evidence also suggests that many proto-oncogenes and possibly anti-oncogenes [p53 and retinoblastoma (Rb)] are involved in normal cell proliferation, development, and differentiation. Activated oncogenes are also known to disrupt the coordinated gene expression associated with differentiation of muscle cell types. Due to their lack of cell fusion and normal myoblast differentiation, the L8SF myoblasts offer a unique system for studying spaceflight-induced changes at the molecular level, especially during the transition from proliferating mononuclear myoblast cells to multinucleated fibers. Recent data with normal differentiating and neoplastic differentiation-deficient muscle cells showed that transformation results in the switching off of muscle-specific genes, thereby causing significant quantitative and qualitative shifts in the corresponding mRNAs [Hillion et al., 1884]. The L8SF cells cease proliferation soon after reaching confluency, which may indicate that while cessation is a necessary step for in vitro myogenesis, it may not be the "trigger" step to fusion. Genes necessary for proliferation are most likely turned off just prior to the L8SF cells reaching confluency. Because

the L8SF variant cells appear to be a permanent phenotype, an analysis of the proliferation and myogenic-related gene expression of the L8SF myoblasts should prove to be very helpful in identifying those genes specifically affected by spaceflight and possibly microgravity.

Although the mechanisms involved in spaceflight effects on cells are still unknown and a gravity sensor yet to be identified, results to date strongly suggest that cells can and do respond to microgravity [Cogoli et al., 1984]. Both c-*fos* [de Groot et al., 1991a] and c-*jun* [de Groot et al., 1991b] gene expression is altered by clinostat artificial microgravity. The message emerging appears to be that spaceflight (microgravity) affects the biological functioning of single cells, both prokaryotic and eukaryotic. The L8SF clone hails the beginning of a new direction for research into the effects of spaceflight (microgravity) on muscle cells and other cell types in general. The exact mechanism(s) by which spaceflight has altered the normal differentiation program of L8 myoblasts is as yet unknown. Whether the L8SF cells are actually a stably transformed cell line has yet to be definitively determined, although the initial growth characteristics are a clear indication that the postflight phenotype may indeed be an authentic variant phenotype. Although we are keenly aware that the isolation of other L8SF cell lines is critical, we feel that the results reported here are significant and raise several pertinent questions as to the effects of spaceflight on muscle cells.

It is known that cells respond to a sudden change in environmental conditions through the induction of a common molecular mechanism: an elevated synthesis of a family of heat shock proteins [Morimoto et al., 1990]. The abnormal expression of these stress proteins has been implicated in oxidant injury, tissue trauma, and cancer, as well as in experimental models for aging [Morimoto et al., 1990]. Therefore, it is possible that spaceflight stress (microgravity) may induce the aberrant synthesis of these proteins resulting in the L8SF phenotype. The possibility also remains that the cell culture population may have been altered by cosmic radiation. However, we feel this is unlikely because random cosmic radiation would induce foci of indiscriminate damage in cells, thereby resulting in a heterogeneous population of variant L8SF cells when cultured at 1*g*. Other unanswered questions include determining which molecular events necessary for myoblast differentiation are affected by spaceflight, at what time point pre- or postexposure to microgravity (minutes, hours, or days) these changes take place, and, most importantly, can the observed myoblast response to spaceflight be correlated with the functions of satellite cells in vivo. Satellite cells are myogenic cells, assigned the role of postnatal skeletal muscle formation and regeneration in adult muscle following injury due to exercise, stress, or injury [Allen and Rankin, 1990]. Specifically, is spaceflight and the subsequent exposure to microgravity teratogenic and capable of altering the eukaryotic differentiation/developmental programs at the cellular level, as suggested by Duke et al. [1993, in press]?

While we realize that these are initial results, and that detailed analyses in other areas need to be done, we also realize the need to repeat these experiments on similarly spaceflown L8 cells. Further biological experiments in space are needed to investigate the changes in the morphology, proliferation, and differentiation and gene expression of both whole organisms and single cells under microgravity environments. Several small scale experiments have previously been carried out utilizing spaceflown prokaryotes and single-cell eukaryotes [for a review see Cogoli and Gmünder, 1991]. Even though conclusive studies on the effect of microgravity on whole animal development must involve breeding in and prolonged exposures to the space environment (i.e., Space Station) [Duke et al., in press], experiments using the STL-A module can begin to assess the effect of microgravity on large numbers of proliferating and differentiating cell populations. With this initial data, we hope to both isolate and identify the gravity-sensing molecular target(s) utilizing spaceflown cells as cultured in the STL-A. In the near future it will be of great importance to: (1) refly L8 cells in the STL-A under similar circumstances in order to confirm the cellular data from STS-45 (the STL-A is scheduled to fly twice in 1994, at least once in 1995); (2) detail the phenotypic/cellular characteristics responsible for the L8SF deficiency of the differentiation/fusion attributes which are discrete from L8AT and L8GC cells; (3) delineate the particular molecular mechanisms of the L8SF differentiation process which may be altered by flying L8 myoblasts at zero gravity; and (4) determine the tumorigenicity of the spaceflown cells by using nude mice. Once the specific spaceflight-

regulated mechanisms are determined, the exact time sequence of effects can be established. Are certain expressed genes more susceptible to spaceflight while other sequences simultaneously remain refractory to zero gravity regulation? Are certain types of cells more susceptible to spaceflight (i.e., pluripotent vs differentiated) and is the stage of cell cycle/fetal development important (on STS-29 all 16 2-d-old chick embryos died, having stopped development during the flight) [Hullinger, 1993]? Results may have important implications for further spaceflight with respect to muscle injury, exercise, wound healing, and, most importantly, fetal development in outer space. Significant scientific knowledge will also be gained if similarities to microgravity-induced changes in space-cultured cells could be implicated in such diseases as muscular dystrophy, rhabdomyosarcoma, and cancer.

ACKNOWLEDGMENTS

This research was supported by Army Space Technology Research Office (ASTRO), United States Army Medical Research and Development Command. We express special thanks to Dr. Thora Halstead of NASA and to Col. David S. Jackson and Alice Cardamone of ASTRO for support and encouragement. D.A.K. thanks Dr. Harry Carter for his inspiring discussions about space research and TSgt Rodney Miller for his tireless paperwork management.

The opinions or assertions contained herein reflect the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army, the Department of the Air Force, the Department of the Navy, or the Department of Defense.

REFERENCES

Allaerts W (1991): On the role of gravity and positional information in embryological axis formation and tissue compartmentalization. Acta Biotheoretica 39:47–62.

Allen RE, Rankin LL (1990): Regulation of satellite cells during skeletal muscle growth and development. Proc Soc Exp Biol Med 194:81–86.

Benoff S, Nadal-Ginard B (1980): Transient induction of poly(A)-short myosin heavy chain messenger RNA during terminal differentiation of L_6E_9. J Mol Biol 140:283–298.

Caravatti M, Minty A, Robert B, Montarras D, Weydert A, Cohn A, Daubas P, Buckingham M (1982): Regulation of muscle gene expression. The accumulation of messenger RNAs coding for muscle-specific proteins during myogenesis in a mouse cell line. J Mol Biol 160:59–76.

Cogoli A, Gmünder FK (1991): Gravity effects on single cells: Techniques, findings, and theory. In Bonting SL (ed): "Advances in Space Biology and Medicine." Greenwich, CT: JAI Press, Inc., pp 183–248.

Cogoli A, Tschopp A, Fuchs-Bislin P (1984): Cell sensitivity to gravity. Science 225:228–230.

D'Amelio F, Daunton NG (1992): Effects of spaceflight in the adductor longus muscle of rats flown in the Soviet biosatellite COSMOS 2044. A study employing neural cell adhesion molecule (N-CAM) immunocytochemistry and conventional morphological techniques (light and electron microscopy). J Neuropathol Exp Neurol 51:415–431.

de Groot RP, Rijken PJ, Boonstra J, Verkleij AJ, de Laat SW, Kruijer W (1991a): Epidermal growth factor–induced expression of C-Fos is influenced by altered gravity conditions. Aviat Space Environ Med 62:37–40.

de Groot RP, Rijken PJ, Hertog JD, Boonstra J, Verkleij AJ, de Laat SW, Kruijer W (1991b): Nuclear responses to protein kinase C signal transduction are sensitive to gravity changes. Exp Cell Res 197:87–90.

Duke PJ, Montufar-Solis D, Daane B (in press): Teratogenic effects of gravitational changes. Adv Space Res.

Duke PJ, Montufar-Solis D, Daane E (1993): Teratogenic effects of delta-G: The concept of allometric growth. The Physiologist 36:S34–S37.

Emerson CP (1990): Myogenesis and developmental control genes. Curr Opin Cell Biol 2:1065–1075.

Florini JR, Ewton DZ, Ferros E, Nadal-Ginard B (1989): Muscle cell cultures. In Baserga R (ed): "Cell Growth and Division. A Practical Approach." New York: IRL Press at Oxford University Press, pp 105–120.

Funk WD, Ouellette M, Wright WE (1991): Molecular biology of myogenic regulatory factors. Mol Biol Med 8:185–195.

Garcia J, Zuniga CA, Haye KR (1993): Characterization of developmentally defective mutants of rat myogenic L8 cell line. Mol Biol Cell 4:45a.

Graeber GM, Kearney GP (1987): Space tissue loss model project ASPWS-701. Army Space Office Form.

Grigoriev AI, Egorov AD (1992a): General mechanisms of the effect of weightlessness on the human body. In Bonting SL (ed): "Advances in Space Biology and Medicine." Greenwich, CT: JAI Press, Inc., pp 1–42.

Grigoriev AI, Egorov AD (1992b): Physiological aspects of adaption of main human body systems during and after spaceflights. In Bonting SL (ed): "Advances in Space Biology and Medicine." Greenwich, CT: JAI Press, Inc., pp 43–82.

Harel J, Leibovitch MP, Guillier M, Borycki AG, Leibovitch SA (1989): Proto-oncogenes and differentiation versus transformation of striated muscle cells. Ann NY Acad Sci 567:187–207.

Hillion J, Leibovitch SA, Leibovitch MP, Raymondjean M, Kruh J, Harel J (1884): Changes in gene expression following neoplastic transformation of rat myogenic cells. Cancer Res 44:2959–2965.

Hullinger RL (1993): The avian embryo responding to microgravity of space flight. The Physiologist 36:S42–S45.

Ilyina-Kakueva EI, Portugalov VV, Kirkenkova NP (1976a): Spaceflight effects on the skeletal muscles of rat. Aviat Space Environ Med 47:700–703.

Ilyina-Kakueva EI, Portugalov VV, Krikenkova NP (1976b): Spaceflight effects on the skeletal muscles of rat. Aviat Space Environ Med 47:700–703.

Kaufman SJ, Parks CM (1977): Loss of growth control and differentiation in the fu-1 variant of the L_8 line of rat myoblasts. Proc Natl Acad Sci USA 74:3888–3892.

Kaufman SJ, Parks CM, Bohn J, Faiman LE (1980): Transformation is an alternative to normal skeletal muscle development. Exp Cell Res 125:333–349.

Kearney GP, Arnold M (in preparation): A continuous-flow perfusion culture chamber for growth of cells in orbit. In preparation.

Konieczny SF, Emerson CP Jr (1984): 5-azacytidine induction of stable mesodermal stem cell lineages from 10T1/2 cells: Evidence for regulatory genes controlling determination. Cell 38:791–800.

Li L, Olson EN (1992): Regulation of muscle cell growth and differentiation by the MyoD family of helix-loop-helix proteins. Adv Cancer Res 58:95–119.

Loughna PT, Goldspink DF, Goldspink G (1987): Effects of hypokinesia and hypodynsmia upon protein turnover in hindlimb muscles of the rat. Aviat Space Environ Med 58:A133–A138.

Mesland DAM (1992): Mechanisms of gravity effect on cells: Are there gravity-sensitive windows? In Bonting SL (ed): "Advances in Space Biology and Medicine." Greenwich, CT: JAI Press, Inc., pp 211–228.

Morimoto RI, Tissieres A, Georgopoulos C (1990): "Stress Proteins in Biology and Medicine." Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Morrison DR, Chapes SK, Guikema JA, Spooner BS, Lewis ML (1992): Experiments with suspended cells on the space shuttle. The Physiologist 35:S31–S34.

Mueller P, Wold D (1989): In vivo footprinting of a muscle specific enhancer by ligation mediated PCR. Science 246: 780–786.

Nadal-Ginard B (1990): Muscle cell differentiation and alternative splicing. Curr Opin Cell Biol 2:1058–1064.

Nicogossian AI, Parker JF Jr (1982): "Space Physiology and Medicine: NASA SP-447." Washington, DC: Government Printing Office.

Olson EN (1990): MyoD family: A paradigm for development? Gene 4:1454–1461.

Richler C, Yaffe D (1970): The in vitro cultivation and differentiation capacities of myogenic cell lines. Dev Biol 23:1–22.

Riley DA, Ellis S, Slocum GR, Satyanarayana T, Bain JLW, Sedlak FR (1985): Morphological and biochemical changes in soleus and extensor digitorum longus muscles of rats orbited in Spacelab 3. The Physiologist 28:377.

Riley DA, Ellis S, Slocum GR, Satyanarayana T, Bain JLW, Sedlak FR (1987): Hypogravity-induced atrophy of rat soleus and extensor digitorum longus muscles. Muscle & Nerve 10:560–568.

Shin S, Freedman VH, Risser R, Pollack R (1975): Tumorgenicity of virus-transformed cells in nude mice is correlated specifically with anchorage independent growth in vitro. Proc Natl Acad Sci USA 72:4435–4439.

Spooner BS (1992): Gravitational studies in cellular and development biology. Trans Kans Acad Sci 95:4–10.

Steffen JM, Musacchia XJ (1987): Disuse atrophy, plasma corticosterone, and muscle glucocorticoid receptor levels. Aviat Space Environ Med 58:996–1000.

Tapscott SJ, Weintraub H (1991): MyoD and the regulation of myogenesis by helix-loop-helix proteins. J Clin Invest 87:1133–1138.

Tapscott SJ, Thayer MJ, Weintraub H (1993): Deficiency in Rhabdomyosarcoma of a factor required for MyoD activity and myogenesis. Science 259:1450–1453.

Taylor SM, Jones PA (1982): Changes in phenotypic expression in embryonic and adult cells treated with 5-azacytidine. J Cell Physiol 111:187–194.

Weintraub H, Davis R, Tapscott S, Thayer M, Krause M, Benezra R, Blackwell TK, Turner D, Rupp R, Hollenberg S, Zhuang Y, Lassar A (1991): The *myoD* gene family: Nodal point during specification of the muscle cell lineage. Science 251:761–766.

Whalen R (1993): Musculoskeletal adaptation to mechanical forces on earth and in space. The Physiologist 36:S127–S130.

Yaffe D (1968): Retention of differentiation potentialities during prolonged cultivation of myogenic cells. Proc Natl Acad Sci USA 61:477–483.

Yaffe D, Saxel O (1977): A myogenic cell line with altered serum requirements for differentiation. Differentiation 7:159–166.