Serum deprivation induces a unique hypercontractile phenotype of cultured smooth muscle cells

Ma, Xuefei, Ying Wang, and Newman L. Stephens. Serum deprivation induces a unique hypercontractile phenotype of cultured smooth muscle cells. Am. J. Physiol. 274 (Cell Physiol. 43):C1206–C1214, 1998.—Chronic asthma is characterized by hypertrophy and hyperplasia of airway smooth muscle cells (SMC) that limit airflow by a geometric effect. Whether contractility of airway SMC is altered is not clear. Cultured cells were used as a model of hyperplasia. Phenotypic changes seen indicated conversion to a synthetic, weakly contractile type. At confluence, although limited reversal of protein changes was seen, no restoration in contractility occurred. Phenotypic modulation of postconfluent cultured airway SMC under prolonged serum deprivation (arrested cells) is reported here. Two phenotypically distinct groups of cells were identified in primary airway SMC cultures: 1) elongated spindle-shaped cells, which expressed large amounts of smooth muscle contractile and regulatory proteins, and 2) flat and stellate cells, which expressed very little. The first group showed a surprising shortening capacity and a velocity that was even greater than that of the freshly isolated cells, whereas the second group became spherical and noncontractile. Even more surprising was that the myosin heavy chain (MHC) isoform (SM-B) generally said to be associated with the higher shortening velocity disappeared from the cell, while the content of the key rate-limiting regulating enzyme, myosin light chain kinase (MLCK), increased 30-fold. We conclude that a functional, contractile phenotype of airway SMC can be obtained by prolonged serum deprivation. We speculate that the increased contractility could be the result of increased phosphorylation of the 20-kDa myosin light chain resulting from increased content of smooth muscle MLCK rather than any increase in endogenous MHC ATPase activity. This model may be useful for study of SMC differentiation and contraction.

smooth muscle myosin light chain kinase; smooth muscle myosin heavy chain isoform; airway; asthma

SMOOTH MUSCLE CELLS (SMC) exhibit a high degree of plasticity and do not undergo terminal differentiation (15, 24). The principal function of mature SMC is contraction, which depends on expression of a large number of different contractile and regulatory proteins present in precisely controlled concentrations (8). These cells undergo rapid phenotypic modulation in primary culture, manifested by a marked decrease in content of smooth muscle-specific contractile and regulatory proteins and by an increase of nonmuscle proteins (6, 20). Retention of differentiated contractile and regulatory proteins and functional surface receptors were reported in cultured SMC (18), and reexpression of smooth muscle marker proteins was identified in postconfluent cultures (6), but redevelopment of contractility of proliferative SMC has not been shown. Studies using magnetic twisting cytometry and atomic force microscopy suggested that cultured SMC retain the ability to stiffen in response to contractile agonists (9, 10). Ca\textsuperscript{2+} transients were also reported in cultured SMC in response to a variety of contractile agonists (13, 17). However, this indirect evidence does not prove the existence of contraction in cultured SMC. Increased stiffness of cultured cells as revealed by magnetic twisting cytometry and atomic force microscopy may well be the result of alteration of non-contractile-related cytoskeletal structures. Dissociation of the Ca\textsuperscript{2+} signal from contraction may occur in cultured SMC. Therefore, these reported properties of cultured SMC cannot be cited as clear evidence for the existence of contraction. Presently it is believed that cells in culture undergo dedifferentiation that only partially reverses at confluence. Functionally, they remain very poorly contractile. Bowers and Dahm (3) reported that the contractile phenotype of freshly isolated SMC could be maintained in a defined medium in which the proliferation of cells was minimal, but once the cells were cultured in proliferative media their contractility was lost rapidly. Gunther et al. (5) and Birukov et al. (2) reported a transient maintenance of smooth muscle contractility in proliferative cultures, but again contractility was lost with increased duration of cell growth. In contrast, with striated muscle cells, relatively little is currently known about molecular mechanisms that control smooth muscle differentiation, due in part to the extreme plasticity of this type of cell and to limitations with respect to the inducibility and/or retention of the differentiated phenotype in cultures.

Therefore, establishment of a contractile SMC culture would facilitate studies of smooth muscle differentiation and control of SMC contraction. This was the major objective of our studies. Phenotypic modulation of primary tracheal SMC culture was examined during prolonged serum deprivation. Contractility of cultured tracheal SMC was estimated by direct measurement of zero-load shortening in lifted cells in response to contractile agonists and electrical stimulation. Contractility of smooth muscle is regulated by a variety of contractile and regulatory proteins (8). Smooth muscle myosin light chain kinase (MLCK) is believed to be the major protein that regulates smooth muscle contraction. Recently, a new smooth muscle myosin heavy chain (MHC) isoform (SM-B) that possesses an additional insert of seven amino acids in the NH\textsubscript{2} terminus was identified and reported to be important in determining smooth muscle mechanical properties (7, 12). Other proteins such as smooth muscle \alpha-actin, total smooth muscle MHC, tropomyosin, caldesmin, and calponin are also important in determining SMC contractility. Restoration in expression of these proteins may be a
necessary preparation for recovery of cultured SMC contractility. Therefore, temporal changes in expression of these proteins were also examined during prolonged serum deprivation in airway SMC cultures. Our results demonstrated that prolonged serum deprivation resulted in emergence of spindle-shaped phenotype in cultured tracheal SMC. These cells expressed considerably increased amounts of smooth muscle contractile and regulatory proteins. They remained elongated after being lifted from the plate and demonstrated very similar morphology to that of freshly isolated contractile cells; they shortened in response to contractile agonists and electrical stimulation. Surprisingly, these cells showed a contractility that was even greater than that of freshly isolated cells. These data indicate that a functional contractile phenotype can be induced in tracheal SMC cultures through prolonged serum deprivation.

METHODS AND MATERIALS

SMC Culture

Tracheae were excised from anesthetized, 6- to 12-mo-old mongrel dogs and placed into ice-cold Ca²⁺-free Krebs solution. Trachealis muscle was dissected, cleaned of serosa, vasculature, and epithelia at room temperature, and washed four times in Hanks’ balanced salt solution (HBSS) containing 100 mg/ml streptomycin and 100 U/ml penicillin under aseptic conditions. The muscle was then minced thoroughly with fine scissors and resuspended in digestion buffer (HBSS containing 600 U/ml collagenase ( Gibco BRL), 8 U/ml type IV elastase (Sigma), and 1 U/ml type XXVII Nagarse protease (Sigma)). Cells were isolated by serial digestion (3 stages, 45 min each) with vigorous shaking at 37°C. The fractions were pooled, filtered through 70-µm nylon mesh, and then washed twice by resuspension in ice-cold PBS following centrifugation. Crude protein homogenates were prepared in washed twice by resuspension in ice-cold PBS following centrifugation. Crude protein homogenates were prepared in

Preparation of SMC Protein Homogenates

Cultured SMC were collected by trypsinization beginning from the day of confluence and every three days during serum deprivation thereafter up to day 15. The collected cells were washed twice by resuspension in ice-cold PBS following centrifugation. Crude protein homogenates were prepared in Tris lysis buffer containing 1.5% Nonidet P-40 (NP-40; pH 7.6) to which protease inhibitors (leupeptin, phenylmethylsulfonyl fluoride, and soybean trypsin inhibitor) were added freshly. Cells were disrupted by pipetting several times. Samples were finally stored at −20°C until used for electrophoretic analysis. The storage period did not exceed 1 mo. The protein content of all samples was measured using the Bio-Rad protein assay kit with a Milton Roy Spectronic 1001 Plus spectrophotometer. BSA (fraction V, Sigma Chemical, St. Louis, MO) was used as a relative protein standard for all assays.

Western Blot Analysis

Proteins in cell homogenates were fractionated by SDS-PAGE on 8 × 10-cm minigels and then transferred to nitrocellulose, as described by Stephens et al. (22). Blots were blocked overnight at 4°C in 0.1% Tween 20–10 mM Tris-buffered saline (TTBS) containing 3% nonfat dried milk powder. Blots were then incubated in primary antibody [1:1,000 monoclonal anti-smooth muscle MLCK (Sigma); 1:10,000 monoclonal anti-smooth muscle α-actin (Boehringer); 1:10,000 polyclonal rabbit anti-smooth muscle MHC (both SM-1 and SM-2; Groeschel-Stewart); 1:1,000 polyclonal rabbit anti-SM-B, a gift from Dr. R. Low (University of Vermont, Burlington, VT); 1:7,500 rabbit anti-nonmuscle MLCK polyclonal antibody, a gift from Dr. T. Daigle (Indiana University, Bloomington, IN); and 1:10,000 rabbit anti-nonmuscle myosin heavy chains (Sigma)]. Cells were then washed with TTBS containing 1% milk three times and incubated for 40 min at room temperature with biotinylated secondary antibodies (Amersham Life Science, Oakville, ON) diluted 1:1,000 in TTBS containing 1% milk powder. Blots were rinsed again with TTBS and finally incubated for 40 min at room temperature with streptavidin-horseradish peroxidase conjugate (Amersham) diluted 1:5,000 in TTBS. Semi-quantitative staining of specific proteins was achieved using chemiluminescence detection; blots were washed in TTBS and subsequently dipped for 1 min into luminol substrate solution (Amersham). Chemilumigrams were developed on Hyperfilm ECL (Amersham); the normal exposure times ranged from 30 s to 5 min. An LKB Ultroscan XL laser densitometer was employed to scan the developed films for estimation of protein content. No detectable cross-reactivity of the muscle and nonmuscle protein isoforms was found using antibodies for nonmuscle MHC, nonmuscle MLCK, smooth muscle MHC, and smooth muscle MLCK. All values were normalized to total protein loaded onto the gels to allow for comparison between samples.

Fluorescent Immunocytochemistry

Freshly isolated cells were plated in six-well dishes containing 22 × 22-mm rat tail collagen, and collagen type I-coated glass coverslips (Becton Dickinson). When the cells attained confluence, they were arrested for 10 days by withdrawing serum and the coverslips and attached cells were rinsed with PBS and fixed in 1% paraformaldehyde-PBS (pH 7.6) for 15 min at 4°C. They were subsequently permeabilized using 0.1% Triton X-100 in PBS for 15 min at 4°C and then rinsed with PBS and used immediately for immunostaining or stored in PBS containing 0.05% sodium azide for a maximum of 10–14 days. For immunostaining, the coverslips were incubated in blocking solution (PBS containing 5% normal goat serum and 0.1% Tween 20) for 2–4 h at 4°C in a humidified chamber. After rinsing with PBS containing 1% BSA and 0.1% Tween 20, the coverslips were incubated with primary antibodies diluted in PBS containing 1% BSA and
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Measurement of Single Cell Shortening

Measurement of single cell shortening was conducted at room temperature. A drop of cell preparation was transferred to a custom-designed chamber containing 1 ml aerated Krebs-Henseleit solution and allow to settle for 5 min, after which the chamber was slowly perfused with fresh Krebs-Henseleit solution. Cell length was measured with an inverted microscope. Maximum shortening of cells was elicited by applying bipolar electric pulse stimulation (10 Hz, 40 V, 1 ms width), with one spot electrode placed ~10 μm away from the cell and the other at a random position in the chamber. Throughout the experiment, images of the cells were monitored and recorded via a charge-coupled device video camera mounted on the microscope. Cell shortening was then analyzed with a computer program (peak5) for maximal shortening capacity (∆Lmax) and maximal velocity of shortening (V0). The shortening velocity and capacity in response to electrical field stimulation “hypercontractility.”

Data Analysis

Data are expressed as means ± SE. V0 and ∆Lmax were normalized with respect to initial cell length. ANOVA was employed to analyze the differences of mean values at different time points. Duncan’s new multiple-range test was used as a complementary analysis to the ANOVA so as to determine which points were responsible for differences indicated by ANOVA, with P < 0.05 considered significant. The unpaired two-tailed Student’s t-test was used to compare mean values between two groups as needed.

RESULTS

Morphologically, two distinct groups of cells appeared in postconfluence primary SMC culture during long-term serum deprivation (Fig. 1): one group of cells appeared flat and bright under the inverted microscope; these comprised almost all the cells that were present before serum deprivation. The second group demonstrated the normal elongated spindle shape and were aligned side by side in bundles in most cases; they were dark but possessed a shining sarcolemma. This group began to appear after 2 days of serum deprivation and increased in number as deprivation was prolonged. After 15 days of deprivation, they comprised 28.5 ± 4.6 (SE) of all cells present but occupied almost 40% of total area of the culture dish. When cells were lifted using trypsin-EDTA, those in the second group retracted but remained elongated. Their average length was similar to that of the freshly isolated tracheal SMC (110 ± 8 μm).

These elongated SMC showed normal reversible contraction on stimulation with optimal doses of ACh (10⁻⁵ M), histamine (10⁻⁵ M), KCl (10 mM), and...
low-level single-pulse electrical stimulation (10 V; Fig. 2). Maximal contraction could be induced with repeated pulse electrical stimulation. Figure 3 shows typical curves of unloaded shortening of freshly isolated and the newly induced contractile single tracheal SMC under repeated pulse electrical stimulation. Surprisingly these newly induced contractile cells evinced hypercontractility to electrical stimulation (Fig. 4). Thus they shortened maximally by 50% of their original length at room temperature on repeated electrical stimulation, whereas freshly isolated cells only shortened by 27%. These cells shortened faster, with the $V_0$ at 8% of cell length/s, almost double the value for freshly isolated cells. In addition, arrested cells responded to electrical stimulation at lower current intensity than freshly isolated cells, indicating a higher sensitivity to electrical stimulation. The voltages inducing half-maximal contraction for freshly isolated and arrested contractile cells were $18 \pm 3$ and $11 \pm 2$ V, respectively. These were significantly different ($P < 0.05$).

In correspondence with the time-dependent increase in number of elongated spindle-shaped cells, expres-

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**Fig. 1.** Induction of 2 distinct phenotypes of cells in primary airway smooth muscle cell (SMC) culture under prolonged serum deprivation. A: phase-contrast microscopy of confluent cultured cells, showing a relatively homogeneous population and hill-and-valley pattern. B: microscopy of 10-day arrested cultured cells, showing 2 distinct groups of cells: flat circular cells, which appear bright, and spindle-shaped elongated cells with phase-lucent sarcolemma, which appear dark and are aligned side by side in most cases.

**Fig. 2.** Typical images showing contraction and relaxation of a single airway SMC. A, B, and C: images of a freshly isolated cell fully relaxed, partially contracted, and 10 min after withdrawal of stimulation, respectively. D, E, and F: images of an arrested contractile cell fully relaxed, partially contracted, and 10 min after withdrawal of stimulation, respectively. Cells were stimulated electrically. Dark spot near cell is one of the electrodes; the other was located at other side of cell.
sion of contractile and regulatory proteins increased, as revealed by Western blot analysis (Fig. 5). After 15 days of arrest, total smooth muscle MHC content increased 10.8 ± 1.1-fold, smooth muscle α-actin content increased 5.9 ± 1.0-fold, and smooth muscle MLCK increased by 62.9 ± 13.5-fold compared with contents of cells in nonarrested confluent cultures. The content of smooth muscle type MHC reached the same level as in freshly isolated cells. Surprisingly, the contents of smooth muscle MLCK and α-actin were significantly (30 and 2 times, respectively) higher than those of Fig. 3. Typical curves for unloaded shortening of single tracheal SMC. Arrest, arrested contractile cells; Fresh, freshly isolated contractile cells. An increased contractility was found in arrested cells. Single cells were stimulated maximally with repeated pulse electrical stimulation at room temperature. L_cell, cell length.

Fig. 4. Comparison of mechanical properties of arrest-induced contractile cells in tracheal SMC culture with those of freshly isolated cells. A: maximal velocity of shortening (V_max). B: maximal shortening capacity (ΔL_max). **Significantly increased contractility (V_max and ΔL_max, P < 0.001) was identified in arrest-induced contractile cells compared with freshly isolated cells.

Fig. 5. Temporal changes in contents of smooth muscle contractile and regulatory proteins in cultured tracheal SMC during prolonged serum deprivation. A: smooth muscle α-actin (sm-α-actin) and smooth muscle total myosin heavy chain (sm-MHC). B: smooth muscle myosin light chain kinase (sm-MLCK). Progressive increases in expression of these proteins in cultured SMC were identified under prolonged serum deprivation.

Fig. 6. Comparison of contents of smooth muscle contractile and regulatory proteins among freshly isolated (F), cultured confluent (C), and cultured 15-day arrested (A) tracheal SMC. Significant decrease of contractile protein content in confluent cells and restoration in arrested cells were found. Surprisingly, contents of sm-α-actin and sm-MLCK in arrested cells were even significantly higher than those in freshly isolated cells. *P < 0.05, **P < 0.01, and ***P < 0.001, compared with freshly isolated cells.
Days of Serum Deprivation

Fig. 7. Messenger expression of smooth muscle myosin head isoform (SM-B) in tracheal SMC culture during prolonged serum deprivation. No SM-B mRNA were detected in cultured cells from confluence to 15 days of serum deprivation using RT-PCR. F, freshly isolated cells; Mr, molecular marker.

freshly isolated cells (Fig. 6). The expression of SM-B (the 7-amino acid “inserted” isoform of smooth muscle MHC) was not detected at all tested time points, both at protein and message level (Fig. 7).

To further confirm that the newly emerging spindle-shaped cells express more smooth muscle contractile and regulatory proteins, an immunocytochemical study was employed. Results of experiments employing specific antibodies showed that spindle-shaped cells stained intensively for smooth muscle type MHC, \( \alpha \)-actin, and smooth muscle MLCK, whereas the flat cells stained quite faintly with smooth muscle MLCK and were almost negative to smooth muscle MHC and \( \alpha \)-actin staining (Fig. 8). In addition, these cells also stained strongly for nonmuscle MLCK and \( \beta \)-tubulin (Fig. 9). Centrally located cigar-shaped nuclei were identified in spindle-shaped cells, but round nuclei were seen in flat ones (Fig. 8).

The above evidence demonstrated that serum deprivation resulted in phenotypic modulation of cultured airway SMC from synthetic to contractile, in spite of the fact that SM-B, the isoform reported to be responsible for conferring \( V_{o_r} \), was considerably downregulated.

DISCUSSION

The concept of plasticity of SMC is widely accepted and considered to be a necessary part of the SMC differentiation program that evolved because it conferred a survival advantage to the organism (19). It is well established that the SMC can change its phenotype from contractile to synthetic, and vice versa, in response to environmental influences (15). The mature contractile SMC was reported to undergo a rapid modulation of its phenotype to the immature synthetic type during culture in the presence of serum (6, 20). This was demonstrated by a considerable decrease in smooth muscle-specific contractile and regulatory proteins and an increase in nonmuscle type proteins. Direct evidence for reversal of modulation of cultured SMC, i.e., redevelopment of contractile properties, has never been demonstrated, although reaccumulation of smooth muscle MHC was found in postconfluent cultures (this is the biochemical concomitant of contractile...
phenotype) (6). Recovery of contractile responsiveness was reported in cultured SMC (13, 17). However, increased intracellular Ca$^{2+}$ transient and/or myosin light chain (MLC) phosphorylation in response to contractile agonists were used as indexes of contractility. These are indirect methods for inferring contractility and have limited usefulness. Our results demonstrate that mature contractile SMC can be induced in postconfluent primary cultures under long-term serum deprivation. After "lifting," these cells retained their normal spindle shape and shortened isotonically in response to contractile agonists such as ACh, histamine, KCl, and electrical stimulation, which also indicates that appropriate receptors were present. The data demonstrate that cultured airway SMC (synthetic phenotype) retain the capability to reverse their phenotype and undergo differentiation to contractile phenotype as a result of serum deprivation. Our studies provide novel direct evidence that under appropriate conditions cultured noncontractile SMC developed into a contractile phenotype.

Molecular mechanisms that control the differentiation program of SMC have not yet been identified. The SMC is believed to be remarkably plastic in that it can undergo rapid and reversible changes of its phenotype in response to a variety of different stimuli. Consistent with this property, differentiation of SMC appears to be highly dependent on environmental influences (16). Our results demonstrated that the differentiated phenotype of cultured airway SMC could be induced and maintained as the result of serum deprivation. This implicates the importance of growth arrest in determining SMC differentiation and may provide a model to study differentiation of SMC. The redevelopment of normal contractility of cultured SMC was accompanied by reappearance of smooth muscle contractile, structural, and regulatory proteins, such as smooth muscle MHC, $\alpha$-actin, and smooth muscle MLCK, and morphological reversion to normal spindle shape. Our data also show that the contents of nonmuscle MLCK and $\beta$-tubulin remain elevated in these contractile cells. This provides an additional difference between arrested contractile and freshly isolated cells. This suggests that downregulation of nonmuscle type proteins may not be required in induction of contractile type of cells from cultured noncontractile SMC under serum deprivation. Communication among cells may also be important in differentiation under serum deprivation, because induced contractile cells were found in most cases formed into bundlelike clusters aligned in parallel. Instead of invoking communication between cells, the grouping of the contractile cells in bundles can also be attributed to their forming a colony that originated from a common precursor. We currently cannot distinguish these two possibilities.

Induction of contractile phenotype in cultured tracheal SMC was found to be accompanied by considerable increased expression of smooth muscle $\alpha$-actin, smooth muscle MHC, and smooth muscle MLCK during prolonged serum deprivation. Surprisingly, we did not find corresponding changes in expression of the smooth muscle MHC isoform (SM-B) that possesses a seven-amino acid insert in its NH$_2$ terminus. Actually, we did not detect any expression at all of SM-B at either protein or message levels in cultured tracheal SMC before or during serum deprivation, but it was prominently detected in freshly isolated tracheal SMC. Expression of SM-B has been reported to be important in determining smooth muscle mechanical properties (25). It confers a cycling velocity on the muscle that is three times faster than those of the other isoforms (12). Our finding seems contradictory to this notion. The newly induced spindle-shaped cultured tracheal SMC showed an elevated contractility as a result of arrest but did not express SM-B, whereas freshly isolated cells did express SM-B isoform but showed lower contractility, indicating that proteins other than SM-B are responsible for the supercontractility of newly induced contractile cells. Dissociation of the content of SM-B expression from contractile properties of smooth muscle was also found by other investigators. Haase and Morano (7) reported a decrease of SM-B expression while the $V_o$ of smooth muscle increased in pregnant rat myometrium fibers. Siegman et al. (21) recently found that no...
correlation between the amount of SM-B and shortening velocity existed in mouse megacon. The latter pointed out that to compare the velocities regulated by the different MHC isoforms full phosphorylation of the 20-kDa MLC (MLC_{20}) must be ensured. When this was carried out using adenosine 5′-O-(3-thiotriphosphate), no difference in velocity due to the different isoforms was seen. The correlation reported by others between V₀ and MHC isozyme is therefore not the effect of the isozyme itself.

Smooth muscle MLCK is an important candidate that could be responsible for the correlation between V₀ and MHC isozyme activity. Considerable increase in smooth muscle MLCK content was found in newly induced contractile cells. After 15 days of serum deprivation, smooth muscle MLCK content in cultured cells increased 62.9-fold compared with cells in nonarrested confluent culture. Our previous studies showed that the content of smooth muscle MLCK in confluent tracheal SMC culture decreased by 50% compared with that of freshly isolated cells (6). Arrested cells expressed smooth muscle MLCK at a content of 30 times that of freshly isolated cells; this may contribute to the increased contractility of arrested cells. Smooth muscle MLCK is known to be a primary regulator of smooth muscle contraction through Ca^{2+}/calmodulin-dependent phosphorylation of regulatory MLC (MLC_{20}). Increased smooth muscle MLCK content and activity would lead to increased activity of MLC_{20} phosphorylation with concomitant increase in velocity during smooth muscle activation. The importance of smooth muscle MLCK in regulating smooth muscle contractility was supported by our previous studies on ragweed pollen-sensitized canine airway smooth muscle, in which an increased smooth muscle contractility was found to be closely correlated with the increased content and activity of smooth muscle MLCK (11). Stephens and J. Jiang (23) recently reported on the basis of a motility assay that in vitro motility of the smooth muscle myosin head increased with increase of smooth muscle MLCK concentration, which further demonstrated the significance of smooth muscle MLCK in regulating smooth muscle contractility. Increased expression of smooth muscle α-actin may also contribute to the increased contractility, by providing more attachment sites for myosin heads.

In conclusion, our data demonstrate that a functional, fully contractile phenotype is induced in cultured tracheal SMC as a result of prolonged serum deprivation. These newly induced contractile SMC possess even greater contractility than freshly isolated tracheal SMC. This model could provide a tool for studies to determine the relationship between smooth muscle differentiation and contraction. The development of hyperreactivity in cultured SMC, stemming from growth arrest as a result of serum deprivation, represents a novel finding. The responsible mechanisms need to be investigated. The existence of cellular heterogeneity also requires that these mechanisms be delineated for the different subpopulations of cells.

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