Cell culture retains contractile phenotype but epigenetically modulates cell-signaling proteins of excitation-contraction coupling in colon smooth muscle cells

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Shi XZ, Sarna SK. Cell culture retains contractile phenotype but epigenetically modulates cell-signaling proteins of excitation-contraction coupling in colon smooth muscle cells. Am J Physiol Gastrointest Liver Physiol 304: G337–G345, 2013. First published December 13, 2012; doi:10.1152/ajpgi.00369.2012.—Smooth muscle cell cultures are used frequently to investigate the cellular mechanisms of contraction. We tested the hypothesis that cell culture alters the expression of select cell-signaling proteins of excitation-contraction coupling in colon smooth muscle cells without altering the contractile phenotype. We used muscularis externa (ME) tissues, freshly dispersed cells (FC), primary cell cultures (PC), and resuspensions of cell cultures (RC). Colon smooth muscle cells retained their phenotype in all states. We investigated expression of 10 cell-signaling proteins of excitation-contraction coupling in all four types of tissue. Expression of all these proteins did not differ between ME and FC (P > 0.05). However, expression of the α,c-subunit of Ca,1,2b, myosin light chain kinase, myosin phosphatase target subunit 1, and 17-kDa C kinase-potentiated protein phosphatase-1 inhibitor (CPI-17) decreased in PC and RC vs. ME and FC (all P < 0.05). Expression of Goi3, serine/threonine protein phosphatase-1 β-catalytic subunit, and Rho kinase 1 increased in PC and RC vs. ME and FC (all P < 0.05). Cell culture and resuspension downregulated expression of α-actin and calponin, but not myosin heavy chain. The net effect of these molecular changes was suppression of cell reactivity to ACh in RC vs. FC. Overexpression of CPI-17 in PC partially reversed the suppression of contractility in resuspended cells. Methylation-specific PCR showed increased methylation of the CPI-17 gene promoter in PC vs. ME (P < 0.05). We concluded that smooth muscle cells retain their contractile phenotype in culture. However, reactivity to ACh declines because of altered expression of specific cell-signaling proteins involved in excitation-contraction coupling. DNA methylation of the CPI-17 promoter may contribute to its gene suppression.

organism develop from a single zygote by successive meiotic and mitotic cell divisions. During each cell division, the genomic and epigenetic codes are transferred from the parent to the daughter cells by complex and incompletely understood mechanisms (4, 13, 20). The error rates of DNA replication are usually very low because of the proofreading abilities of the DNA polymerases (5). However, any unchecked errors have the potential to change the phenotype of daughter cells. By contrast, transfer of epigenetic codes to daughter cells is highly plastic, and the inherited codes in daughter cells are subject to changes in the cellular microenvironment.

The investigation of cellular mechanisms often requires cultured cells. However, the environment in which the terminally differentiated cells proliferate in culture markedly differs from that in situ. Therefore, the epigenetic mechanisms that take cues from the cellular microenvironment to establish the transcription rates of the active genes may set the transcription rates of some genes differently from those in the intact organism. The goal of this study was to investigate whether colon smooth muscle proliferation in the unnatural environment of culture medium alters the smooth muscle contractile phenotype by epigenetic programming. We found that smooth muscle cells retained their phenotype when they stopped proliferating in confluent cultures. Despite the differences between the microenvironments of cell cultures and intact organisms, the epigenetic mechanisms retained the active status of all genes that encode the proteins of excitation-contraction coupling and the smooth muscle-specific contractile proteins. However, expression levels of some of these proteins decreased, while those of others increased or showed no change. Overall, these changes suppressed the contractile function of resuspended cultured smooth muscle cells. Colon smooth muscle cells proliferate in situ during inflammation induced by trinitrobenzene sulfonic acid (TNBS). We found that, after the inflammation had subsided, none of the genes showed decreased expression and some showed increased expression, while most were unaffected.

EXPERIMENTAL METHODS

Rat colon circular smooth muscle dispersion. The Institutional Animal Care and Use Committee of the University of Texas Medical Branch at Galveston, Texas, approved the procedures used in this study on Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN). Distal colons were obtained from 3- to 4-wk-old male Sprague-Dawley rats. The longitudinal muscle-myenteric plexus layer and the mucosal-submucosal layers were removed. The circular muscle layer was collected in HEPES buffer (in mmol/l: 120 NaCl, 2.6 KH₂SO₄, 4 KCl, 2 CaCl₂, 0.6 MgCl₂, 25 HEPES, 14 glucose, and 2.1% essential amino acid mixture, pH 7.4). Two successive digestions with papain and collagenase dispersed smooth muscle cells, as described previously (23, 25). After digestion, freshly dispersed colon circular smooth muscle cells from each rat were divided into three portions for 1) protein extraction, 2) cell contractility in response to ACh, and 3) cell culture.

Primary culture of rat colon circular smooth muscle cells. The dispersed cells were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS in the presence of 100 U/ml of penicillin
G, 100 µg/ml of streptomycin sulfate, and 0.25 µg/ml of amphotericin B. The cells from each rat were cultured in two of the six-well plates for 8 or 9 days until they were ~90% confluent. The cells were then cultured in 1% FBS for another 24 h. The cultured cells in one plate were harvested in lysis buffer for protein extraction; those in the other plate were gently scraped with a rubber policeman and incubated in serum-free DMEM with occasional shaking. After incubation for 24 h, the cells were dispersed and their contractile response to ACh was monitored.

Cell contractility assay. Freshly dispersed cells or the cultured cells resuspended in serum-free medium for 24 h were relaxed at rest; they responded with shortening in the presence of ACh (10⁻⁹–10⁻⁵ M). To quantitate muscle contraction, an aliquot (0.45 ml) of cells at ~10⁴ cells/ml was exposed to 50 µl of ACh or vehicle control for 40 s at 31°C and fixed with 1% acrolein. The lengths of 30 consecutive intact healthy cells were measured by scanning micrometry, as described previously (24). NIH Image 1.61 was used to measure cell length.

Overexpression of CPI-17 in cultured colon smooth muscle cells. Rat Cpi-17 cDNA (NM_053890) (38) was cloned into pCMV6 vector (Oregene, Rockville, MD) to test the effect of its overexpression in cultured colon smooth muscle cells. Cpi-17 cDNA was transfected into the primary culture of rat colon circular smooth muscle cells on day 8 with the transfection reagent FuGENE 6 (Roche, Mannheim, Germany). After 24 or 48 h, cells were harvested for biochemical and contractility studies.

Western blotting. Protein extracts were processed as described previously (23, 25). The proteins in the samples were resolved by standard immunoblotting using equal loading (10 or 20 µg) in each lane; β-actin was used as an internal control. The antibody (1:200–1:400 dilution) for the α₁C-subunit of Ca₁,1.2b channels (catalog no. ACC-003) was purchased from Alomone Laboratories (Jerusalem, Israel), and the antibody against Goαq was obtained from Calbiochem (Billerica, MA); other antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Methylation-specific PCR. One microgram of extracted DNA was subjected to sodium bisulfite modification using the MethylDetector kit (Active Motif, Carlsbad, CA) following the manufacturer’s protocol. The modified DNA was PCR-amplified with two primer sets designed for the sodium bisulfite-treated modified DNA was PCR-amplified with two primer sets designed for the unmethylated Cpi-17 promoter sequence [5′-CGAT-TATTTTTTATAGAGAAAGATAC-3′ (forward) and 5′-GCC-GAAACTTACACTACAAAACGA-3′ (reverse)]. The primers for amplification of the unmethylated Cpi-17 promoter were 5′-TTGATTATTTTTATAGAGAAAGATAC-3′ (forward) and 5′-ACCAAAACACTTACTACAAAACAAA-3′ (reverse).

Statistics and data analysis. Values are means ± SE. Statistical analysis was performed by analysis of variance with nonrepeated measures. Multiple comparisons were made with the Student-Newman-Keuls test. The difference between two means was tested by t-test. P < 0.05 was considered statistically significant. All analyses were conducted using SPSS version 12.0 (SPSS, Chicago, IL).

RESULTS

Morphology, phenotype, and reactivity of smooth muscle cells to ACh. The freshly dissociated smooth muscle cells, cells in culture, and resuspended cultured cells were immunoreactive to smooth muscle-specific α-actin (Fig. 1). The freshly dissociated smooth muscle cells had an elongated spindle-like shape (Fig. 1A), the cells in culture had an elongated irregular shape (Fig. 1B), and the resuspended cultured cells were cylindrical (Fig. 1C). ACh concentration dependently contracted the freshly dispersed and resuspended cultured cells along their long axis (Fig. 2A); shortening of the resuspended cells was significantly less than that of the freshly dissociated cells (Fig. 2A).

Phosphorylation of the 20-kDa regulatory light chain. The cell signaling pathways activated by binding of ACh to muscarinic M3 receptors on colon smooth muscle cells converge on myosin light chain kinase (MLCK), the activation of which phosphorylates the 20-kDa regulatory light chain (RLC20), and on myosin light chain phosphatase (MLCP), the activation of which dephosphorylates RLC20 (15, 21, 31). The net phosphorylation of RLC20 relates to the strength of smooth muscle contraction. We investigated whether the intensity of RLC20 phosphorylation in response to 10⁻⁵ M ACh in smooth muscle cells changed in transition from muscle strips to freshly dissociated cells and to resuspension of cultured cells. Western blotting with anti-phosphorylated RLC20 (pRLC20) antibody showed that the basal level of pRLC20 decreased progressively from muscle strips to freshly dissociated cells to resuspended cultured cells (Fig. 2B). However, the time of increase in pRLC20 normalized to the respective basal level following exposure to 10⁻⁵ M ACh was similar in all three states of smooth muscle cells (Fig. 2C).

Fig. 1. Immunofluorescence staining of smooth muscle-specific α-actin in freshly isolated (A), cultured (B), and resuspended cultured (C) cells. Images are representative of 3 or 4 independent experiments.
Alterations in expression of cell-signaling proteins important in excitation-contraction coupling in different states of smooth muscle cells. We investigated whether cell culture altered expression of 10 key cell-signaling proteins important in excitation-contraction coupling and three smooth muscle-specific contractile proteins in gut smooth muscle cells (15, 21, 22). Four of the cell-signaling proteins, M₁ muscarinic receptor, the pore-forming \( \alpha_{1C3} \)-subunit of \( Ca_{2+} \) (L-type) \( Ca_{2+} \) channels, \( G_{\alpha_q} \), and \( G_{\alpha_\beta} \), are located at the beginning of cell-signaling cascades (group A proteins). Another four proteins, RLC₂₀, MLCK, myosin phosphatase target subunit 1 (MYPT₁), and serine/threonine protein phosphatase (PP)-1 β-catalytic subunit (PP1c), are located toward the end of the signaling cascades (group B proteins). The last two proteins, 17-kDa C kinase-potentiated protein phosphatase-1 inhibitor (CPI-17) and Rho kinase 1 (ROK1), are located between groups A and B (group C). We also investigated the effects of cell culture on expression of the smooth muscle-specific contractile proteins smooth muscle \( \alpha \)-actin, smooth muscle myosin heavy chain (MHC), and calponin. Expression of all these proteins in muscularis externa (ME) tissue, primary cell culture, and resuspensions of cell culture was compared with their expression in fresh cells.

Western blotting with respective antibodies showed that cell dissociation had no significant effect on expression of any of the cell-signaling proteins in groups A, B, and C (Fig. 3). By contrast, cell culture and resuspension of cultured cells altered expression of select proteins in each group. In group A, cell culture or resuspension of cultured cells did not affect expression of M₃ muscarinic receptor and \( G_{\alpha_q} \) protein (\( P > 0.05 \)); however, both processes suppressed expression of the \( \alpha_{1C3} \)-subunit (\( P < 0.05 \)) but enhanced expression of \( G_{\alpha_\beta} \) (\( P < 0.05 \)). In group B, cell culture or resuspension of cultured cells had no effect on expression of RLC₂₀ (\( P > 0.05 \)); however, both processes suppressed expression of MLCK and MYPT₁ (\( P < 0.05 \)) and enhanced expression of PP1c (\( P < 0.05 \)). Finally, in group C, cell culture or resuspension of cultured cells suppressed expression of CPI-17 (\( P < 0.05 \)) and enhanced expression of ROK1 (\( P < 0.05 \)). Expression of these proteins did not differ between primary cultures and resuspension of cells (Fig. 3).

Cell culture or resuspension of cultured cells also suppressed expression of smooth muscle-specific \( \alpha \)-actin and calponin (\( P < 0.05 \)) but had no effect on expression of MHC (Fig. 3). Cell dispersion had no effect on expression of any contractile protein (Fig. 3).

Effects of enteric neurotransmitters on expression of cell-signaling and contractile proteins. Recent studies show that spontaneous release of enteric neurotransmitters such as VIP maintains transcription of specific genes encoding the cell-signaling proteins of excitation-contraction coupling in colon smooth muscle cells (23, 26). We investigated whether the prolonged absence of neurons and their neurotransmitters in the culture medium accounts for changes in expression of select cell-signaling proteins important in excitation-contraction coupling. Generally, investigators reduce the serum or buffer prior to using them for cell-shortening measurements or Western blotting. We investigated whether the absence of neurotransmitters or serum altered expression of cell-signaling proteins in primary cell cultures.

We incubated the primary cultures with \( 10^{-5} \) M methacholine (MCh), \( 10^{-5} \) M VIP, 25 \( \mu \)M \( S \)-nitrosothiol (GSNO), or 10% bovine serum for 24 h prior to Western blotting. In addition, we incubated cell suspensions with all three neurotransmitters or their donor (MCh, VIP, and GSNO) together. Expression of each protein in freshly dissociated cells served as control. Expression of select proteins changed in primary cultures, as noted in Fig. 3. Incubation of cell cultures with MCH or GSNO had no further effect on expression of any of the cell-signaling proteins compared with that in freshly dispersed cells (Fig. 4). Incubation with VIP or VIP + MCh + GSNO enhanced expression of the \( \alpha_{1C3} \)-subunit, without affecting expression of other proteins (Fig. 4). Incubation of cell cultures with 10% serum did not affect expression of any cell-signaling protein. By contrast, incubation of primary cultures with MCh, VIP, GSNO, or MCh + VIP + GSNO
significantly enhanced expression of α-actin (Fig. 5); incubation with 10% serum had no effect. These incubations had no effect on expression of MHC or calponin (Fig. 5).

Effect of CPI-17 overexpression on contractility of resuspended smooth muscle cells in primary cultures. Among all proteins that showed a decrease in expression in cell culture and resuspended cells, expression of CPI-17 decreased the most (Fig. 3). We investigated whether suppression of CPI-17 contributed to the deficit in reactivity of the smooth muscle cells to ACh in suspension. Transfection of Cpi-17 cDNA in primary cultures increased CPI-17 expression (Fig. 6), which significantly reversed suppression of the contractile response to ACh in resuspended smooth muscle cells (Fig. 6B).

Epigenetic dysregulation in primary cultures. DNA methylation of promoters, particularly CpG islands in the promoter, has the potential to suppress gene transcription. We investigated whether DNA methylation of the Cpi-17 promoter increased during cell division in cell culture. MethPrimer analysis identified two CpG islands on the Cpi-17 promoter at −371 to −220 and −164 to −55. Methylation-specific PCR showed a significant increase in methylation of the Cpi-17 promoter during cell dispersion (157 ± 11% vs. ME tissue, P < 0.05) and a further significant increase during culture (203 ± 16% vs. ME tissue, P < 0.05 vs. ME tissue and fresh cells; Fig. 7).

Effect of in situ cell proliferation on gene expression. TNBS inflammation induces cell hyperplasia in colon smooth muscle cells, resulting in their thickening (16, 28). We investigated whether proliferation of fully differentiated smooth muscle cells in situ also alters expression of the cell-signaling and contractile proteins. We performed these experiments 35 days after TNBS insult, when the inflammatory response has subsided (16). We found that in situ cell proliferation enhanced expression of Gq_{11} and MYPT1 (Fig. 8). By contrast, MYPT1 was suppressed during cell proliferation in culture (Fig. 3). Similarly, cell proliferation in situ upregulated expression of α-actin (Fig. 8), while cell proliferation in vitro suppressed it (Fig. 3). In situ smooth muscle proliferation did not affect expression of other cell-signaling or contractile proteins.

DISCUSSION

Fully differentiated smooth muscle cells switch phenotype from contractile to synthetic during the proliferative stage to secrete key enzymes and promote cell division and duplication. However, before using cultures to investigate cellular mechanisms of contractility, one must first establish that the cultured cells reestablish their contractile phenotype. Most studies have confirmed the contractile phenotype of cultured gut smooth muscle cells by demonstrating that they express smooth muscle-specific contractile proteins by immunostaining (17, 19, 27). Some studies found reduced expression of several contractile proteins in colon (17) and vascular (18) smooth muscle cells but did not investigate the alterations in expression of the cell-signaling proteins involved in excitation-contraction coupling; the contractile proteins receive signals from the cascade of cell-signaling proteins activated during excitation-contraction coupling telling them to contract. These signals, initiated by binding of an agonist, such as ACh, to M3 receptors in gut smooth muscle cells, define the profile of contraction, such as amplitude and duration. A change in the contractility of

Fig. 3. Expression levels of 3 groups of cell-signaling proteins (groups A, B, and C) and contractile proteins in muscularis externa (ME) tissue, freshly dissociated cells (FC), cells in primary culture (PC), and resuspended cultured cells (RS) detected by Western blotting. Values (means ± SE) are shown as fold change vs. FC; n = 4 or 5. M3, M1 muscarinic receptor; Gq_{11}, and G3, G proteins; α_{1c}, α_{1c}-subunit; MLCK, myosin light chain kinase; MYPT1, myosin phosphatase target subunit 1; PP1c, Ser/Thr protein phosphatase 1c-subunit; MLCK, myosin light chain kinase-potentiated protein phosphatase inhibitor; ROK1, Rho kinase 1; MHC, myosin heavy chain. *P < 0.05 vs. FC.
cells in vivo signifies potential motor dysfunction (3, 23, 26). Our findings show that cell culture modifies expression of select contractile proteins, as well as expression of proteins of excitation-contraction coupling, indicating modification of the contractile phenotype and smooth muscle function.

The process of enzymatic dispersion of smooth muscle cells from muscle strips followed by cell culture and resuspension progressively suppressed the relative phosphorylation of RLC20 per unit of the housekeeping protein β-actin, which indicates a progressive decrease in force generation by smooth muscle cells (12). However, the time course of pRLC20 did not change, suggesting intact morphology of contraction. The decrease in force generation in freshly dispersed cells occurred without a relative change in expression of cell-signaling proteins active during excitation-contraction coupling or the contractile proteins we tested. We cannot rule out the effect of differences in the microenvironment, cytoskeleton, and extracellular matrix between freshly dissociated cells and intact muscle strips as an additional probable cause of the lower force generated in freshly dissociated cells from muscle strips followed by cell culture and resuspension.

ACh, acting on M3 muscarinic receptors coupled to the G proteins Gq and G13, is the physiological neurotransmitter that stimulates spontaneous colon contractions in vivo (21, 22). Activation of these G proteins stimulates at least three signaling cascades that regulate the amplitude and duration of smooth muscle contraction (15, 21, 22). The opening of Ca1.2b (L-type) Ca2+ channels induces Ca2+ influx and release of Ca2+ from the endoplasmic stores; the Ca2+-calmodulin complex activates MLCK to phosphorylate RLC20, which increases phosphorylation of RLC20, resulting in an increase in contraction amplitude without a change in intracellular Ca2+ concentration (Ca2+-independent contraction) (8, 9, 30, 33). Note that the presentation of these pathways is simplified; they may cross talk, and some additional kinases may activate/deactivate MLCK and MLCP, respectively (7, 12, 15, 29). Yet another way to alter the contractility of smooth muscle cells is to modulate expression of the proteins in the above-described signaling cascades (23, 26, 28). We found that cell culture differentially modulates expression of key signaling proteins in all three signaling cascades.

**Fig. 4.** Expression levels of 3 groups of cell-signaling proteins (groups A, B, and C) in cultured cells under different conditions: culture (C), methacholine [MCh (M), 10⁻⁷ M], VIP (V, 10⁻⁷ M), nitric oxide donor S-nitrosoglutathione [GSNO (G), 25 μM], MCh + VIP + GSNO (A), and 10% serum (S). Values (means ± SE) are shown as fold change vs. fresh cell control (F); n = 4. *P < 0.05 vs. F. #P < 0.05 vs. C.
of excitation-contraction coupling in colon smooth muscle cells to alter cell contractility. Cell culture downregulates expression of the pore-forming $\alpha_{1C}$-subunit of CaV1.2b channels, reducing Ca$^{2+}$ influx and suppressing cell contractility (10, 28). Downregulation of CPI-17 in the PKC pathway in cell cultures enhances activation of MLCP by ACh to decrease pRLC$_{20}$, thereby reducing cell contractility. By contrast, upregulation of PP1c and ROK1 reduces MLCP activation in response to ACh, thereby enhancing pRLC$_{20}$ and cell contractility. The net total effect of the opposing alterations in gene expression of the cell-signaling proteins involved in excitation-contraction coupling in colon smooth muscle cells is suppression of contractility.

The cell culture of vascular smooth muscle and tracheal cells also alters expression of cell-signaling proteins; it appears, however, that these alterations may be cell-specific. ROCK1 expression increased in colon smooth muscle cultures but decreased in vascular smooth muscular cell cultures; MYPT1 expression decreased in colon cells and increased in vascular cells (2, 6, 36); expression of other proteins, such as CPI-17, MLCK, and the $\alpha_{1C}$-subunit, decreased and expression of PP1c increased in both cell types. The vascular studies did not compare the contractility of resuspended vascular smooth muscle cells with that of freshly dispersed cells to determine whether culture suppresses or enhances agonist-induced contractility.

TNBS-induced inflammation in intact rats causes hyperplasia and hypertrophy (28). A previous study found similar changes in gene expression of the cell-signaling proteins during the 7-day period after induction of inflammation, in

Fig. 5. Expression levels of contractile proteins $\alpha$-actin, calponin, and myosin heavy chain (MHC) in cultured cells under conditions described in Fig. 4 legend. Values (means ± SE) are shown as fold change vs. F; n = 4. *P < 0.05 vs. F. #P < 0.05 vs. C.

Fig. 6. A: transfection of primary culture of rat colon circular smooth muscle cells with Cpi-17 cDNA significantly restored CPI-17 protein expression in cultured cells. B: increase in expression of CPI-17 significantly improved cell contractility. Values are means ± SE; n = 3 or 4 independent experiments. *P < 0.05 vs. fresh cells. #P < 0.05 vs. cells in culture (Ctr).
which colon smooth muscle cells proliferate; α1C-subunit, CPI-17, and Goq were downregulated, while M3 receptor protein was unaffected (28). However, downregulation of these proteins recovered fully 35 days after induction of inflammation. In fact, expression of MYPT1 and Goq, downregulated during cell proliferation, was upregulated at 35 days. Despite the persistence of these changes, the contractile response of smooth muscle cells was normal at this time (35). By contrast, expression of contractile proteins continues to deteriorate in the in vitro environment, as they are cultured repeatedly (16). These differences suggest that, within the environment of the intact colon, the epigenetic mechanisms more or less reset after inflammatory injury to ensure normal function. Taken together, these findings suggest that the differences in the environment of the intact colon and cell cultures may not affect short-term changes in expression of smooth muscle cell-signaling proteins. However, the natural environment in the intact colon ensures recovery from injury, while repetitive proliferation in cell cultures may eventually change the smooth muscle phenotype.

Our findings show that, among the major neurotransmitters of the motor neurons we tested, only VIP regulates expression of the α1C-subunit of Cav1.2b channels. Expression of the rest of the cell-signaling proteins was not affected by incubation with VIP, ACh, or the nitric oxide donor GSNO. Addition of serum to resuspended cells also did not alter expression of any cell-signaling protein.

Our findings provide proof of principle that epigenetic mechanisms triggered by the unnatural microenvironment under which cells proliferate in cultures may alter expression of select cell-signaling proteins involved in excitation-contraction coupling. DNA methylation of CpG islands on promoters suppresses gene expression (14). We found that in vitro cell proliferation methylated the CpG islands on the Cpi-17 promoter and suppressed expression of CPI-17, in turn contributing to impaired cell contractility. Overexpression of CPI-17 partially reversed this impairment. Successful passaging of cells in culture eventually transforms the smooth muscle contractile phenotype to the secretory phenotype (17). It is likely that, by 35 days after inflammatory insult, the smooth muscle cells have undergone several generations of proliferation. We found, however, that at this time the contractile phenotype remained intact, with only
minor alterations in expression of cell-signaling and contractile proteins. Note that epigenetic reprogramming in cell cultures upregulated expression of some signaling proteins; this is likely due to acetylation of lysine residues on histone proteins in the promoters of the genes encoding those proteins (11, 37). Investigation of epigenetic modulation of all cell-signaling proteins is outside the scope of this work.

We conclude that cell dispersion, primary cultures, and resuspensions of primary cultures retain the colon smooth muscle contractile phenotype. However, epigenetic dysregulation during cell proliferation in primary culture significantly down regulates expression of select cell-signaling proteins involved in excitation-contraction coupling and contractile proteins, while up regulating or not affecting others. The net effect of these modulations is to suppress smooth muscle reactivity to ACh. Cell culture and resuspension decreases expression of RLC, but the temporal profile of RLC phosphorylation by ACh does not change. Overexpression of CPI-17 in cultured cells partially restores the reactivity of resuspended smooth muscle cells to ACh. By contrast, in situ cell proliferation induced by TNBS inflammation does not produce major epigenetic modulation of cell-signaling proteins, once the short-term effects of inflammatory mediators have subsided (28). Retention of the contractile phenotype in cell cultures allows such cells to be used to investigate the cellular mechanisms of smooth muscle contraction. However, relative changes in expression of cell-signaling proteins involved in excitation-contraction coupling need to be taken into account in interpretation of the findings.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

X.-Z.S. performed the experiments; X.-Z.S. analyzed the data; X.-Z.S. prepared the figures; X.-Z.S. drafted the manuscript; S.K.S. is responsible for conception and design of the research; S.K.S. interpreted the results of the experiments; S.K.S. edited and revised the manuscript; S.K.S. approved the final version of the manuscript.

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