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Gravitational force modulates G_2/M phase exit in mechanically unloaded myoblasts

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Abbreviations: CaM, calmodulin; CaMKII, CaM-modulated kinase II; CFSE, carboxyfluorescein diacetate, succinimidyl ester; FACS, fluorescence activated cell sorting; FB,: fetal bovine serum; GM, growth media; HG, hypergravity; IGF-1, insulin-like growth factor 1; MFI, median fluorescence intensities; PBS, phosphate-buffered saline; PI, propidium iodide; RPM, random positioning machine; SM, simulated microgravity; TRPC1, transient receptor potential channel 1

Prolonged spaceflight gives rise to muscle loss and reduced strength, a condition commonly referred to as space atrophy. During exposure to microgravity, skeletal muscle myoblasts are mechanically unloaded and respond with attenuated cell proliferation, slowed cell cycle progression, and modified protein expression. To elucidate the underlying mechanisms by which muscle mass declines in response to prolonged microgravity exposure, we grew C2C12 mouse muscle cells under conditions of simulated microgravity (SM) and analyzed their proliferative capacity, cell cycle progression, and cyclin B and D expression. We demonstrated that the retarded cell growth observed in SM was correlated with an approximate 16 h delay in G_2/M phase progression, where cells accumulated specifically between the G_2 checkpoint and the onset of anaphase, concomitantly with a positive expression for cyclin B. The effect was specific for gravitational mechanical unloading as cells grown under conditions of hypergravity (HG, 4 g) for similar durations of time exhibited normal proliferation and normal cell cycle progression. Our results show that SM and HG exert phenomenological distinct responses over cell cycle progression. The deficits of SM can be restored by terrestrial gravitational force, whereas the effects of HG are indistinguishable from the 1 g control. This suggests that the mechanotransduction apparatus of cells responds differently to mechanical unloading and loading.

Introduction

Macroscopic mechanical forces are key in tissue anabolism, particularly of skeletal muscle. An often underappreciated form of mechanical input of profound developmental consequence is the unyielding force of gravity. Skeletal muscle has evolved to confer movement and stability against the constant force of gravity. This capacity of skeletal muscle is largely endowed by our antigravity (postural) muscles, such as the gluteal and abdominal muscles, but particularly the soleus, that allow us to sustain an upright position for prolonged periods while experiencing relatively little fatigue. Our antigravity muscles are functionally adapted to this task via their higher expression of slow muscle fibers, also known as oxidative or red fibers, reflecting their rich blood supply, myoglobin content, enrichment in mitochondria, and, consequently, elevated aerobic energy substrate utilization. Importantly, slow, oxidative muscles are preferentially sacrificed by the removal of gravitational force, whereas predominantly fast-twitch, glycolytic muscles such as the extensor digitorum longus and gastrocnemius, are relatively spared.[1,2] The significant loss of oxidative muscle mass that results during space travel limits the capability of humans to undertake long-term missions.[3] The muscle loss ensuing during gravitational mechanical unloading is a combined consequence of decreased protein synthesis, increased protein degradation, and reduced regenerative capacity at the level of the muscle progenitor cell pool.[1]

Succinctly, cells convert mechanical signals into biochemical responses, a process that is short-circuited in cases of systemic mechanical unloading, as arises during space travel characterized by reduced gravitational force. We previously demonstrated that simulated microgravity (SM) results in a deceleration of the cell cycle downstream of mitigated mechanically induced calcium entry.[4] In this report, we extended the analysis by examining cyclin expression in cells grown under SM as well as hypergravity (HG). The turning of the cell cycle is facilitated by calcium and is divided into 4 stages: G_1, S, G_2, and M phases. A restriction point exists within G_1 that dictates if cells continue to divide and transit into the S phase, or if they exit from the cell

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cycle and enter a quiescent state called G_0, where proliferation is restricted, as a segue to differentiation. G_1 and G_2 are gaps, serving mainly as regulatory stages into subsequent phases. During the S phase, DNA is replicated (post-G_1). In the M phase (post-G_2) cell duplication occurs, characterized by mitosis and cytokinesis. The cell cycle kinases regulate the progression through the cell cycle and are activated upon association with cyclins that are themselves regulated at the level of synthesis and degradation by cell cycle-specific ubiquitin ligases expressed at specific stages of the cell cycle.[5] The expression of certain cyclins hence serve as a manner to determine the position of cells within the cell cycle. For instance, cyclin B is synthesized at the exit of S phase and is degraded at the end of mitosis, whereas cyclin D is typically expressed during G_1.[6] It has been previously shown that the degradation of cyclin B is a requirement for the termination of cytokinesis at the end of mitosis.[7]

Calmodulin (CaM) is a fundamental calcium-activated regulatory protein governing many calcium-dependent processes, including the cell cycle at several key locations. CaM sequentially participates in the transition of cells from G_1 to S phase, from G_2 to mitosis and from anaphase to metaphase. Indeed, reduced expression of CaM results in cell cycle arrest in G_1, G_2, or metaphase, depending on when depression is evoked.[8] Although the instigating source of calcium can be varied depending on cell type and state of differentiation, calcium has been shown to induce the expression of CaM-modulated kinase II (CaMKII), which, in turn, activates cyclin B, allowing the transition from G_2 to M phase.[9] This same mitotic kinase, after a delay, activates proteasomal degradation of cyclin B, required for the metaphase/anaphase transition.[10] We have previously shown that SM reduces the expression of the mechanically regulated cation channel, transient receptor potential channel 1 (TRPC1), as well as 2 isoforms of insulin-like growth factor 1 (IGF-1), transcriptional events regulated by calcium.[4] We hypothesized that the resultant depression of calcium entry into the cells as a result of SM would alter the balance of CaMKII activation and would be reflected as a change in G_2/M phase progression downstream of altered cyclin B expression.

In this study, we used SM as a method to mechanically unload muscle cells to help understand the underlying molecular mechanisms, giving rise to muscle atrophy in response to disuse on earth or arising from long-term travel in space. To attain a more comprehensive understanding of the role that gravitational forces play on muscle development, we also grew myoblasts in the presence of HG. Surprisingly, HG exerts no effect over cell cycle regulation, unlike the noticeable accumulation of cells within the G_2/M phase of the cell cycle observed with SM. Being exquisitely mechanosensitive, skeletal muscle tissue is robustly responsive to changes in intracellular calcium concentration. The strong dependence of skeletal muscle maintenance on mechanical loading and the relevance of skeletal muscle to human well-being create a socio-economic urgency to develop therapeutic strategies to counteract muscle loss as a result of advanced aging or immobilization due to disease or injury. This process inevitably commences with the understanding of the molecular mechanisms compromised by mechanically unloading muscle cells.

Results

SM inhibits cell proliferation

We sought to investigate the underlying mechanism by which muscle mass declines in response to microgravity exposure. C2C12 mouse muscle cells were cultured under SM or at 1 g and their growth assessed by CFSE staining, where fluorescent intensity is inversely related to the ability of a cell population to expand (see "Materials and Methods", Cell proliferation). No difference was observed between our 1 g control conditions, cells maintained within the random positioning machine (RPM) environment (RPM control), or inside a standard tissue culture incubator (incubator control). Conversely, cells placed onto the RPM, and thus experiencing SM, showed an overall higher fluorescence intensity (less dilution of the dye content per cell i.e., less cell division) and, as a population, exhibited a relatively smaller shift to lower fluorescence values (leftward) with time in culture when compared with either control state, indicating attenuated expansion under conditions of gravitational mechanical unloading (**Fig. 1A**). Although a trend for slowed cell expansion was apparent as early as 6 h after initiating the experiment, the difference between conditions became more apparent with time. Examining the median fluorescence intensities (MFI) normalized to the 6 h RPM control, both controls exhibited increasing statistical significance, whereas the RPM sample did not (**Fig. 1B**), demonstrating that the cells were dividing much more slowly on the RPM. Comparing the cells cultured under SM and the time-matched controls, a significant difference was observed at 12 h, but not at 24 h, meaning that cell propagation is slowed, although not completely halted in SM. From our previous results, we know that this decrease in propagation is not due to a reduction in cell survival, but to a change in cell cycle progression.[4]

SM accumulates cells at G_2/M phase

To investigate the cause for the slowed proliferation observed in SM, we monitored the time course of cell cycle progression. Culturing cells on the RPM for 6 and 12 h resulted in their depletion from the G_0/G_1 and S phases (**Fig. 2A**), concomitant with their accumulation in the G_2/M phase. After 6 and 12 h of commencing SM culturing conditions, 36.63 ± 6.08% and 44.61 ± 2.15% of the cells remained within G_2/M, respectively (**Fig. 2B**), corresponding to a 2.62- and 2.44-fold increase over their respective control samples. After 24 h, in contrast, the cells cultivated under SM recovered and exhibited a similar cell cycle profile as cells cultivated at 1 g. This could be explained if cells in SM overcame the blockage by some other means, or by the eventual accumulation of calcium to achieve a threshold necessary for cell cycle progression; hence, progression through the cell cycle would be retarded, not prevented. No difference was observed between either 1 g controls (incubator or RPM environment) throughout the time course.

G_2/M phase exit is selectively delayed by approximately 16 h under SM

We next set out to determine the exact duration of the blockade temporarily imposed by SM. To this end, cells were pharmacologically synchronized in the G_2/M phase, after which they were placed directly onto the RPM, where they were allowed to

progress to the G_0/G_1 checkpoint. At 2, 12, 18, and 24 h of culture, cellular DNA content was analyzed by FACS to monitor cell cycle progression under 1 g and SM. Whereas the majority of the cells cultivated under SM were retained in the G_2/M phase for approximately 18 h before proceeding to the G_0/G_1 checkpoint, control cells commenced their transit to G_0/G_1 already by the first time point, 2 h (**Fig. 3A**). Indeed, the SM samples did not show an appreciable increase in the size of the G_0/G_1 peak (shaded gray) until after 18 h. Explicitly, after 2 h of release from synchronization, 59.71 ± 6.62% of the cells on the RPM remained within the G_2/M phase, whereas 46.13 ± 3.30% of control cells remained therein and yet, comparable in magnitude (49.43 ± 9.18%) to the percentage of cells cultivated in SM retained within G_2/M and released 18 h earlier (**Fig. 3B**). This data indicates that there is an approximately 16 h delay in the G_2/M phase progression when cells are grown under SM.

G_0/G_1 and S phase progressions are not altered under SM

To determine if G_0/G_1 and S phase progression are similarly affected by SM, cells were synchronized at the G_0/G_1 checkpoint and then placed on the RPM or at terrestrial gravity, and their cell cycle profile was analyzed 2, 4, and 6 h after their release from pharmacological blockade. As apparent from the representative histograms, the majority of cells in all samples traversed to the S phase after 2 h, indicating that there was no obvious alteration in G_0/G_1 progression in cells cultured in SM compared with terrestrial gravity (**Fig. 4**). In addition, all cells reached the G_2/M phase after 6 h of being released from the blockade. Based on these results, it appears that the progression through G_0/G_1 and S phases is not disturbed by SM. This observation further corroborates our earlier assertion that microgravity simulated on the RPM is not generally cytotoxic to cells and validates our model system of environmental mechanical unloading.[4]

Cyclin B, but not cyclin D, expression is augmented by SM

The expression of certain cyclins serves as a reference for determining the general location of a population of cells within the cell cycle.[6] To determine the exact sub-phase where cells grown under SM are accumulated within the G_2/M phase, we followed the expression of cyclin B. The expression of cyclin B commences to increase at the exit from S phase, reaches its peak as cells enter mitosis, and then recedes at the transition of

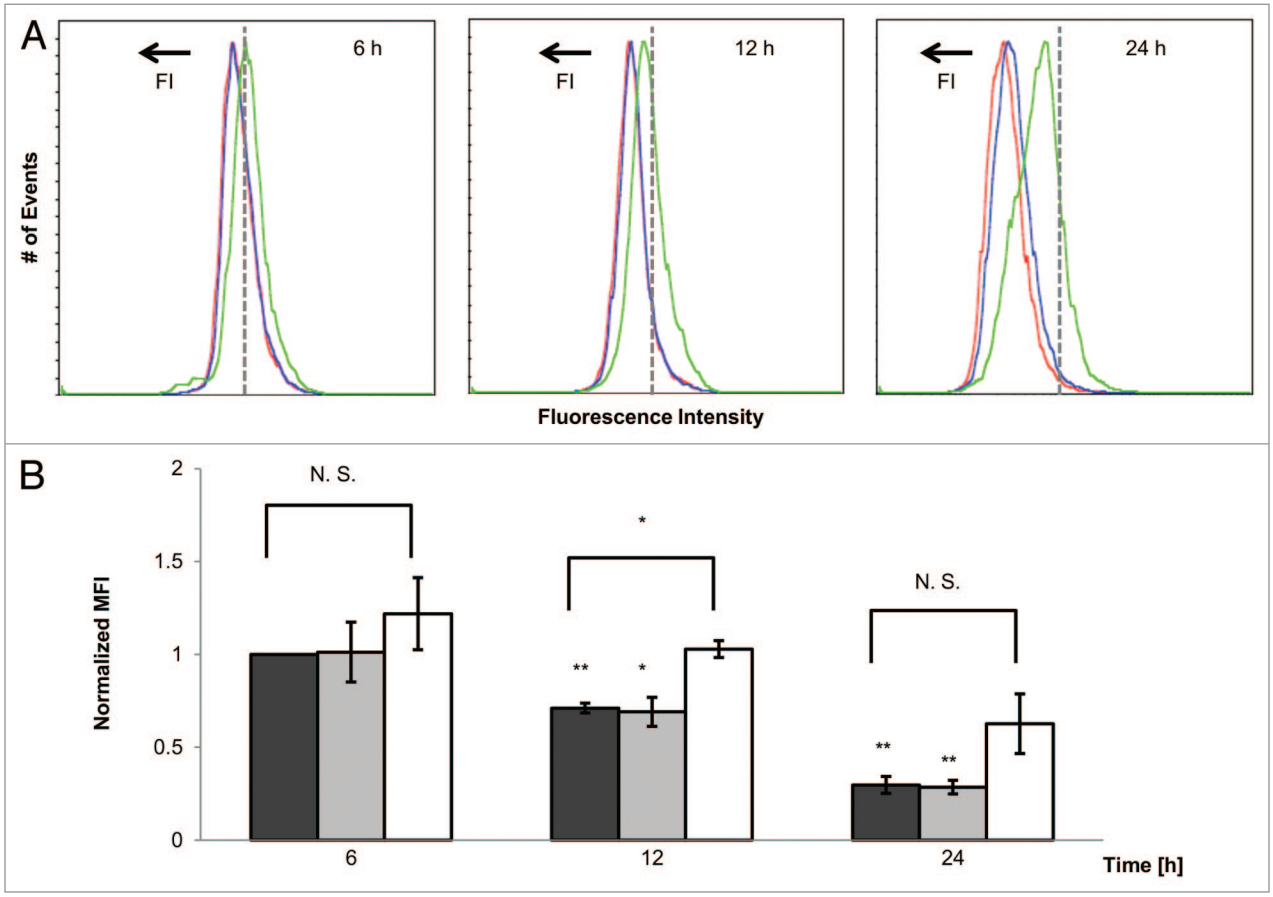

Figure 1. Simulated microgravity inhibits cell proliferation. Cell growth was assessed by collecting CFSE-stained cells 6, 12, and 24 h after culture at 1 g or simulated microgravity (SM). Representative histograms of the fluorescence intensities are shown (color online): random positioning machine (RPM) control in blue, incubator control in red and SM in green (**A**). The dashed gray line is placed on the same position on each histogram to help visualize the shifts, symbolized by an arrow pointing to decreased fluorescence intensity (FI) values. The graph with the data from all 3 experiment repetitions illustrates that as cells divide, they lose fluorescence intensity (**B**). The SM (white columns) samples were brighter than the 1 g samples: RPM control (dark gray columns) and incubator control (light gray columns). The median fluorescence intensities (MFI) were normalized to the RPM control in the first time point (6 h). Means ± standard deviations were obtained from 3 independent series of experiments. NS, not significant; *: $P < 0.05$; **: $P < 0.005$ against the 6 h RPM control unless described as against each RPM control.

metaphase to anaphase due to calcium-dependent proteolytic degradation.[11,12] Our analysis showed that after 6 and 12 h, 40.37 ± 1.01% and 42.53 ± 3.95% of the cells, respectively, were cyclin B-positive in the RPM samples (**Fig. 5A**). These values are dramatically elevated compared with control samples exhibiting 22.33 ± 2.16% and 24.17 ± 1.30% of cyclin B-positive cells after 6 and 12 h, respectively. After 24 h, the statistical significance disappeared as the percentage of positive cells expressing cyclin B decreased to 20.07 ± 3.45% in cells grown under SM, close to the value of 22.5 ± 0.79% observed in the control cells. This effect paralleled our previous cell cycle analysis, where cells accumulated within the G_2/M phase after 6 and 12 h of SM, but recovered after 24 h. More precisely therefore, this analysis indicates that it is the progression from the G_2 checkpoint to metaphase that is being specifically delayed under conditions of SM. Again, there was no difference detected between the distinct 1 g controls (incubator or RPM environment) throughout the entire time course.

As cells accumulated in the G_2/M phase of the cell cycle as a result of cultivation in SM were positive for cyclin B for up to 12 h, we tested the possibility that these same cells would have reduced expression of other cyclin classes characteristic of other phases of the cell cycle. Cyclin D is commonly expressed in early G_1, marking the commitment of cells to transit into S phase.[6] There was no significant difference, however, in the samples placed on the RPM or in 1 g controls regardless of time of inspection with reference to cyclin D (**Fig. 5B**). Indeed, nearly all the cells, irrespective of their cell cycle position, were positive for cyclin D, suggesting that cyclin D levels are less vulnerable to decrements in cytosolic calcium and more widely distributed throughout the cell cycle.[12,13]

HG does not affect cell proliferation, cell cycle progression, or cyclin B and D expression

In an attempt to gain a wider understanding of the gravity threshold sensed by cells, we grew cells under conditions of HG. We tested the possibility that HG would have the opposite effect of SM, promoting cell proliferation, and thus becoming a potential therapy for muscle atrophy. However, no significant difference in cell growth or cell cycle profile was observed after 6, 12, or 24 h of exposure to 4 g when compared with the 1 g controls (**Figs. 6 and 7A and B**). The G_0/G_1, S and G_2/M phase progressions were also analyzed under HG and proved identical results to terrestrial controls (**Fig. 8A and B**). To complete the analysis, cyclin B and D expressions were also investigated, although no effect was observed (**Fig. 9A and B**). In conclusion, based on these results, 4 g did not provoke any effect on cell growth, cell

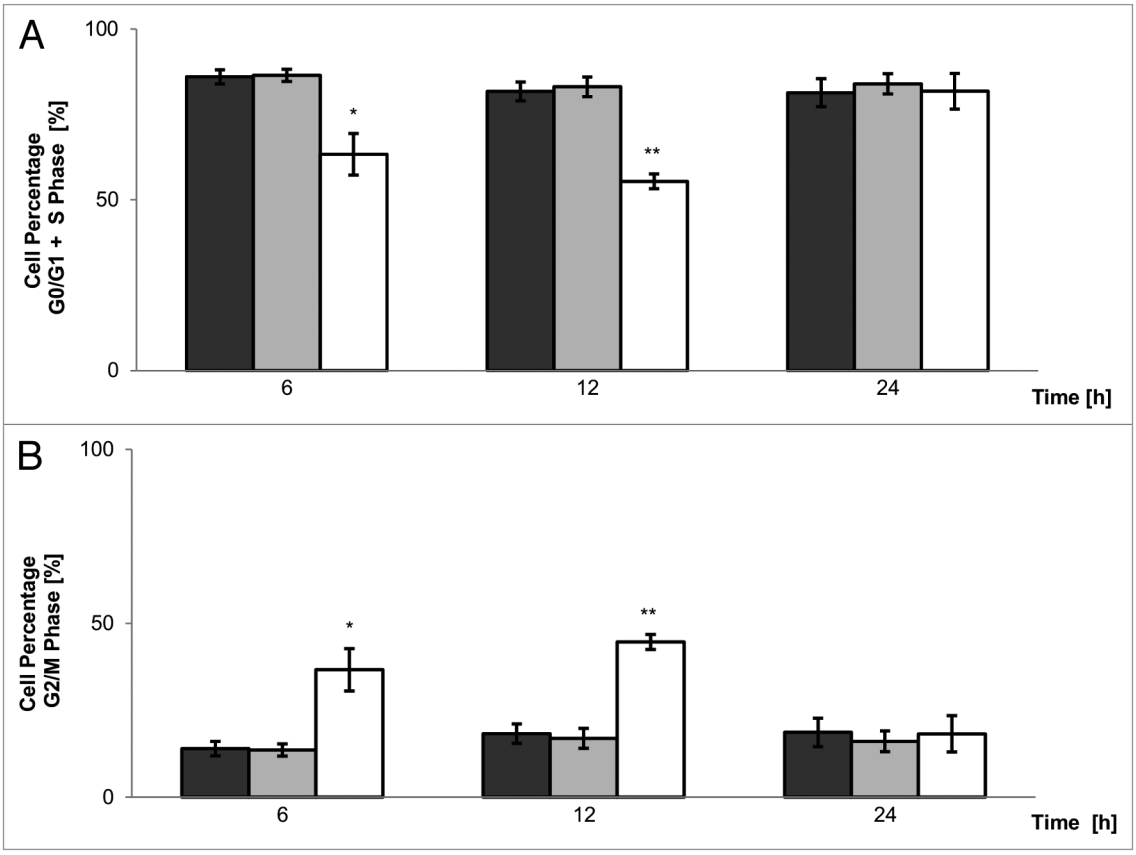

Figure 2. Simulated microgravity causes cells to accumulate at G_2/M phase. Cells were collected after 6, 12, and 24 h for the random positioning machine (RPM) control (dark gray columns), incubator control (light gray columns), and simulated microgravity (SM, white columns). The cell cycle analysis shows that the percentage of cells in G_0/G_1 + S phase (**A**) decreased while the percentage of cells in the G_2/M phase increased (**B**) in SM after 6 and 12 h, returning to control levels after 24 h. Means ± standard deviations were obtained from 3 independent series of experiments. *: $P < 0.05$; **: $P < 0.005$ against each RPM control.

cycle progression, nor the expressions of cyclin B and D when compared with 1 g controls.

Discussion

Loss of muscle mechanical stimulation, both exogenously (gravitational force) and inherently (physical activity), results in muscle atrophy and reduced force generation. Although humoral, nutritional, and physical factors all contribute to the observed loss of muscle regenerative capacity during the loss of gravitational force, here we focus on the mechanical factors impinging on myogenesis, whose absence retards the onset of myogenesis. Astronauts suffer from muscle loss during prolonged spaceflight at a rate of 1% per month despite undertaking active countermeasures such as exercise (aerobic and resistance), muscle training suit (laced with elastic bands) usage, metabolic intervention (pharmacological and nutritional), electrical stimulation, and exposure to artificial gravity through centrifuges and cycling.[1,14] However, none of the aforementioned approaches has been successful in preventing space atrophy. Muscle wasting is not a condition restricted to space travel, but is a broad range clinical scenario with profound health implications exhibited on earth in response to mechanical dysfunction on the neurologic, muscular, and environmental (vocation, injury, disease, and so forth) levels. The severe muscle loss observed with advanced age is unique in that it results from a combination of all of the previously mentioned instigating scenarios. Commencing in mid-adulthood and, if not compensated for by physical activity, a process of overall net muscle loss commences at a rate of around 1% per year.[15] A common denominator in all of these cases is a biologic mechanical dysfunction, adversely influencing downstream cellular mechanotransduction pathways. Gravity, as a mechanical force

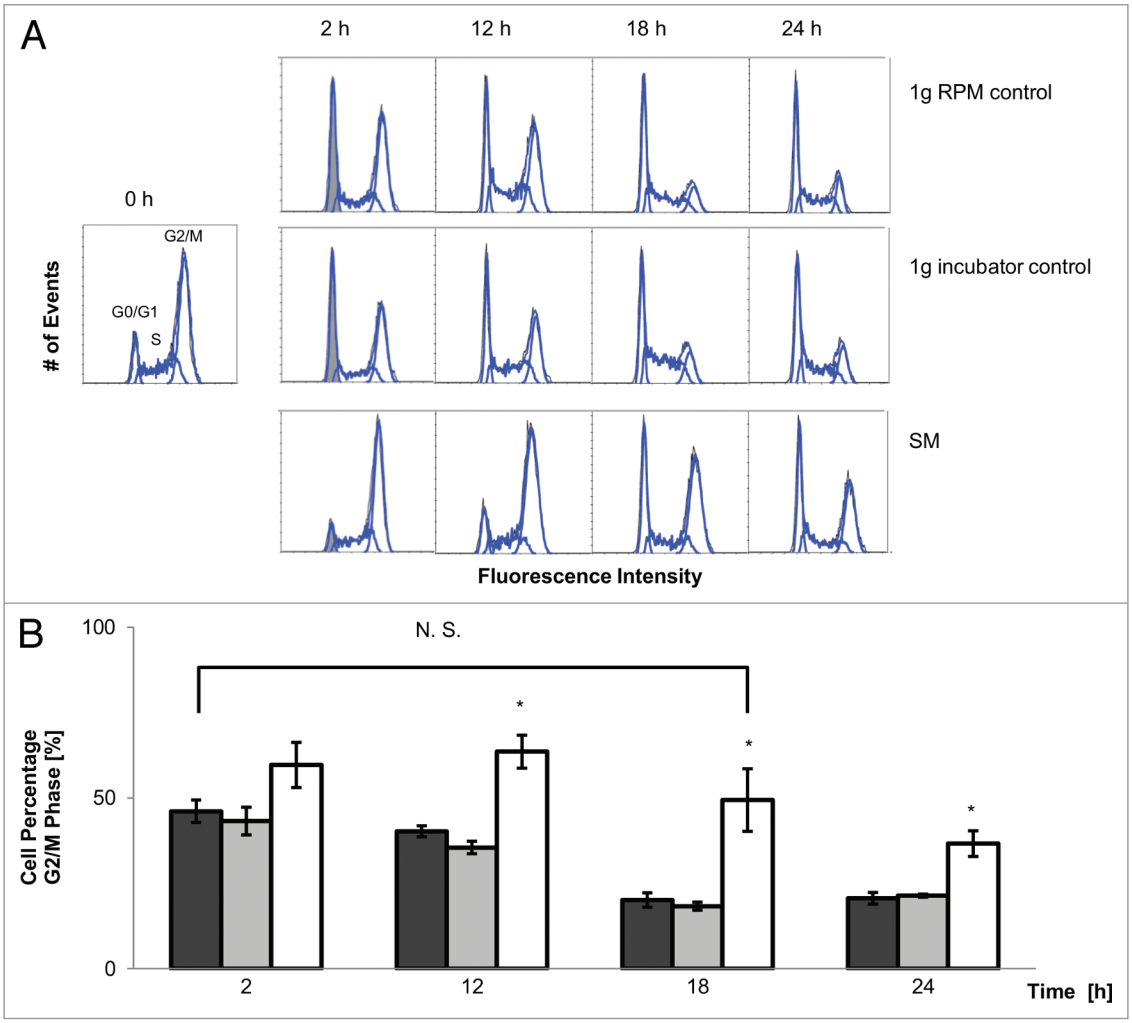

Figure 3. Simulated microgravity delays G_2/M phase exit by 16 h. Representative histograms with the fitted cell cycle model (**A**) are shown for the 0 h start point (middle left) and after 2, 12, 18, and 24 h for the random positioning machine (RPM) control (top), incubator control (middle), and simulated microgravity (SM; bottom). The G_1/G_0 peak is shaded gray to accentuate the difference (between SM and controls) in the rate of cell entry into this phase after 2 h. A summary of the data collected from 3 experiments (**B**) shows the percentage of cells in G_2/M phase over time. Cells were synchronized with hydroxyurea for 3 h, released from the block, and then placed on the RPM 6 h afterwards, when cells were accumulated in the G_2/M phase. Cells were collected after 2, 12, 18, and 24 h for the RPM control (dark gray columns), incubator control (light gray columns), and SM (white columns). Of importance is the similarity of the RPM control at 2 h and SM at 18 h. Means ± standard deviations were obtained from 3 independent series of experiments. NS, not significant; *: $P < 0.05$; **: $P < 0.005$ against each RPM control unless described otherwise.

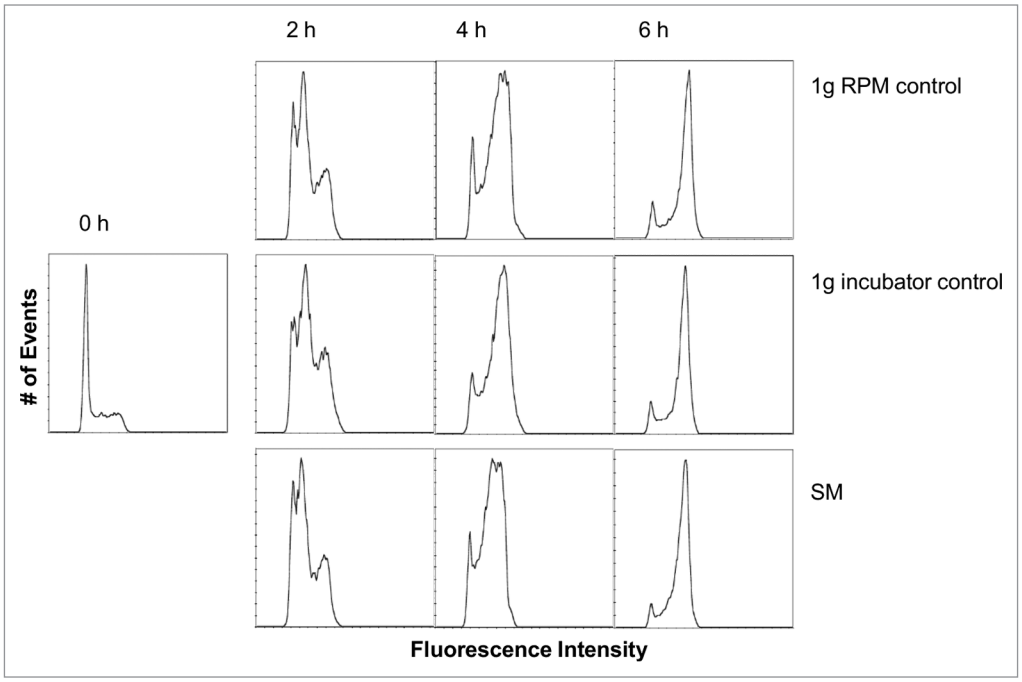

Figure 4. Simulated microgravity does not affect G_0/G_1 and S phase progressions. Representative histograms from 3 independent series of experiments are shown for the 0 h start point (middle left) and after 2, 4, and 6 h for the random positioning machine (RPM) control (top), incubator control (middle), and simulated microgravity (SM, bottom). Cells were synchronized with hydroxyurea for 3 h, released from the block, and then placed immediately on the RPM when cells were accumulated in the G_0/G_1 phase. Cells were collected after 2, 4, and 6 h of cultivation under SM or at 1 g. A summary of the data collected from the 3 experiments is not shown, as the model used to calculate the cell percentages in each phase of the cell cycle could not be fitted to the histograms.

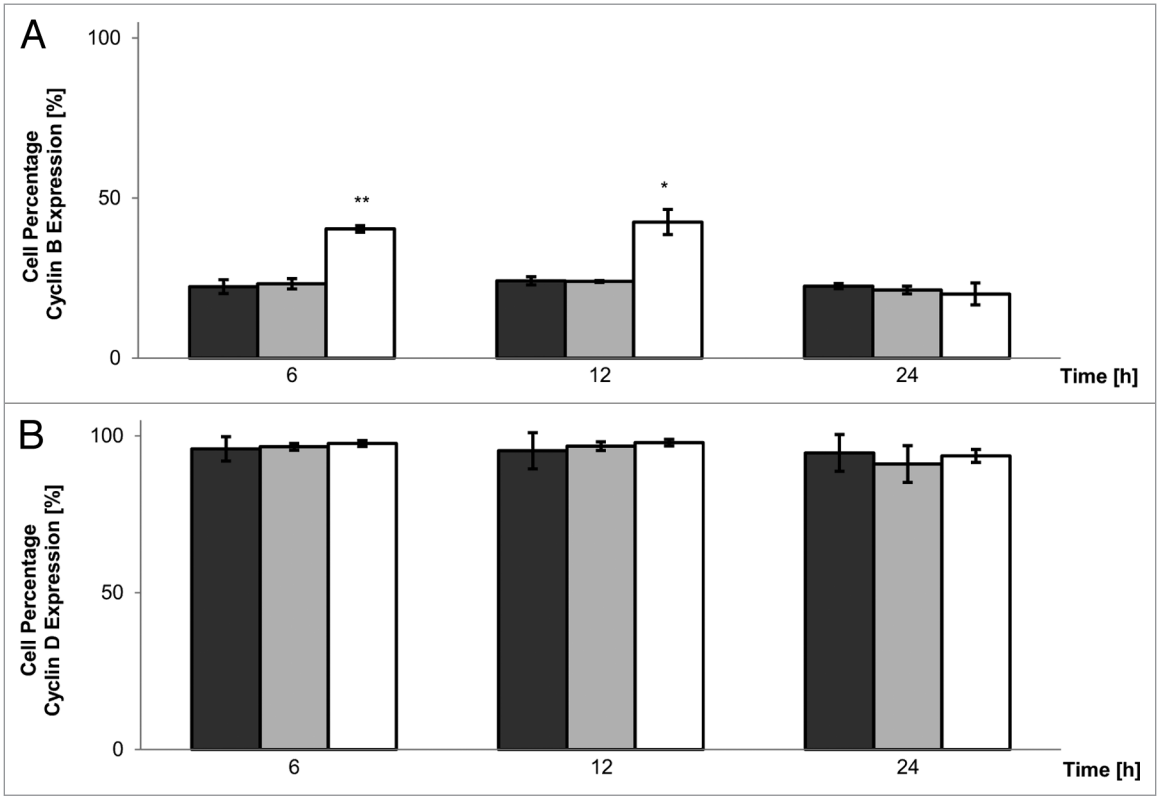

Figure 5. Simulated microgravity enhances cyclin B but not cyclin D expression. Cyclin B (**A**) and cyclin D (**B**) expressions were measured after 6, 12, and 24 h for the random positioning machine (RPM) control (dark gray columns), incubator control (light gray columns), and simulated microgravity (white columns). Means ± standard deviations were obtained from 3 independent series of experiments, *: $P < 0.05$; **: $P < 0.005$ against each RPM control.

per se, is essential for muscle development and maintenance. As we age, the mechanosensitivity of muscle toward gravitational force is reduced, producing similar changes as those experienced by astronauts.[16]

Here we show that SM slows the proliferation of myoblasts by retarding their exit from the G_2/M phase of the cell cycle. As adequate proliferation of the progenitor cell pool is an obligate requirement for an efficacious regenerative response, our observed inhibition of myoblast proliferation under conditions of SM in vitro may help explain the muscle loss suffered by astronauts during spaceflight. Now armed with the information that the G_2/M phase is prolonged in myoblasts cultured under SM, therapeutic options can be developed that would assist to accelerate the G_2/M phase progression under scenarios of gravity withdrawal and, thusly, recuperate cell growth and diminish subsequent muscle loss. Further research is needed to determine whether the observed delay in G_2/M progression is constitutive as long as SM is maintained, or whether myoblasts ultimately adapt and compensate for the loss of mechanical input. The continual loss of muscle mass observed in astronauts during prolonged space flight supports the former mechanism as being most prevalent. This lies in agreement with our previous work, where we cultured myoblasts for 3 d under SM and detected a decrease in their proliferation rate together with a prolongation in the doubling time during the entire period of analysis, therefore the relevance of developing treatments to circumvent the delay in G_2/M phase progression.[4]

The elevated expression of cyclin B we report is consistent with the increased percentage of cells in the G_2/M phase, as cyclin B peaks at mitosis, yet must be degraded in a calcium-dependent manner for progression to anaphase.[11,12] Its upregulation during the G_2/M phase moreover, mirrors our previous observation that calcium-mediated gene expression is mitigated by reduced gravitational force.[4] Specifically, degradation of cyclin B is required for the termination of cytokinesis, explaining its retention during the G_2/M phase.[7] We did not find a difference, however, in cyclin D expression between samples exposed to SM or the 1 g controls. The vast majority of cells, regardless of their cell cycle distribution or gravitational load, were cyclin D-positive. Although, typically considered G_1-specific proteins, the expression of type-D cyclins has been observed in all phases of the cell cycle, and, hence, their loss might better reflect G_1 arrest.[13,17]

Our results corroborate previous reports conducted in true microgravity, where G_2/M phase exit was delayed, slowing proliferation, and moreover, validate our method of simulating microgravity as physiologically relevant. Human lymphocytes (Jurkat) were arrested in the G_2/M phase after 4 h of cultivation during spaceflight.[18] Consistent with our present work, these lymphocytes recovered their normal cycling profiles after 48 h, again indicating that transit through the G_2/M phase is only transiently delayed by gravitational mechanical unloading. In MCF-7 human breast cancer cells flown in space, a delay in mitosis was observed concomitant with abnormalities in microtubule organization.[19,20] The cytoskeleton transmits mechanical forces to subcellular structures involved in dynamic, adaptive, and homeostatic responses to mechanical input. Reducing the gravitational force might therefore disrupt the ability of the cytoskeleton to organize and reorient to mechanical forces, adversely influencing cell behavior. Disorganization of the microtubular network could also contribute to the delay in the G_2/M phase exit reported here. The spindle apparatus is a microtubular-based cellular structure that mediates chromosomal segregation during mitosis. Reduced mechanical input, by compromising microtubular function, might thus impede mitotic chromosomal segregation, complicate cytokinesis, and ultimately delay entry into the G_0/G_1 checkpoint. It remains to be determined whether the microtubular network is similarly compromised by SM in our cell system and will be the focus of our future studies.

We were the first to show that mechanically silencing TRPC1 activity by SM reduces TRPC1 expression, retarding the exit of cells from the G_2/M phase and retaining them therein.[4] Analogously, it has been previously shown that pharmacologically blocking TRPC1-mediated calcium entry downregulates TRPC1 expression and similarly slows proliferation.[21] Further

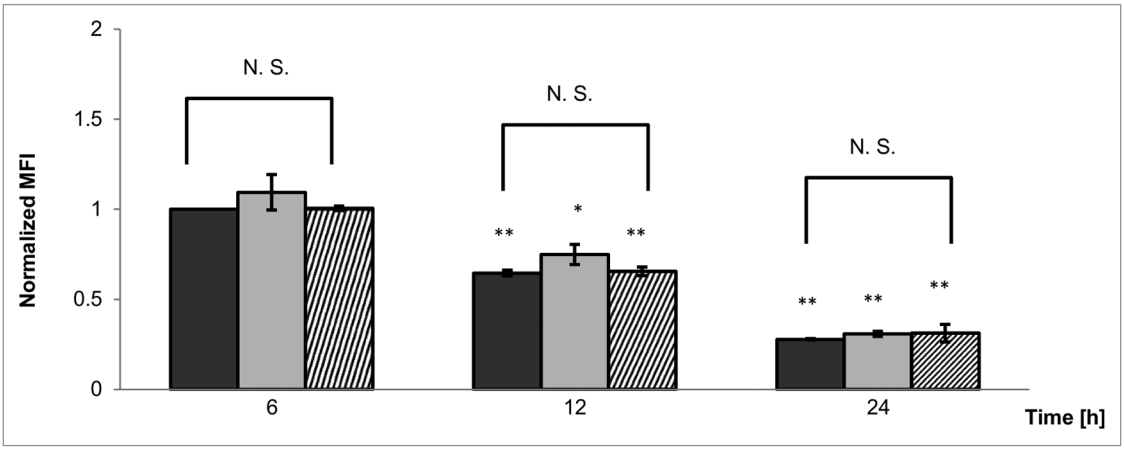

Figure 6. Hypergravity does not affect cell proliferation. Cells were collected after 6, 12, and 24 h for the hyperfuge control (dark gray columns), incubator control (light gray columns), and hypergravity (patterned columns). Cell growth was assessed by collecting CFSE stained cells. The median fluorescence intensities (MFI) were normalized to the hyperfuge control in the first time point (6 h). Means ± standard deviations were obtained from 3 independent series of experiments. NS, not significant; *: $P < 0.05$; **: $P < 0.005$ against the 6 h hyperfuge control unless described as against each hyperfuge control.

paralleling our results, others have shown that silencing TRPC activity either pharmacologically or genetically causes cells to accumulate within the G_2/M phase, and again slows their proliferation.[22,23] Therefore, mechanically, pharmacologically or genetically synchronizing cells within the G_2/M phase is characterized by reduced TRPC channel expression, where, presumably, mechanically gated calcium entry is compromised.[4,21-23] The fact that SM blocks cells in a state when TRPC1 expression is lowest (during G_2/M phase) is intriguing and suggests that TRPC1-mediated calcium influx is able to overcome the effects of microgravity, until entry in G_2/M, when TRPC1 expression is downregulated, at which point the cells are no longer able to progress to the next phase. In the context of our results on myoblasts maintained in SM, TRPC1-mediated calcium entry might still be sufficient to promote the expression of cyclin D and allow progression through G_1, yet be inadequate in G_2/M to promote the degradation of cyclin B and permit G_2/M phase exit (**Fig. 5**). What encompasses the recovery of cells under G_2/M arrest after 16 h in SM is still unresolved, but may entail an accumulation of calcium via other pathways to reach a threshold value of calcium-dependent degradation of cyclin B to permit progression.

Microtubular dynamics is regulated by intracellular calcium, which is altered during weightlessness and by SM.[4,24] Moreover, the activity of mechanically gated TRP channels is influenced by the cytoskeletal organization.[25,26] The loss of gravitational force would hence alter both cytoskeletal organization and TRP-mediated calcium entry in a mutually detrimental manner at the level of the membrane. On the time scale of minutes, cytoskeletal disruptions would adversely influence the gating of mechanically gated channels. On a more protracted time scale, the decrease in the radius of curvature of the cell membrane (cell rounding) due to its detachment from the extracellular environment during blockage before cytokinesis in SM would diminish membrane tension and consequently reduce mechanically mediated calcium entry via stretch-activated cation channels.[4,27]

Calcium influx activates CaM that, in turn, controls cell cycle progression, promoting cell growth and division.[28] In line with our working hypothesis, reduced calcium influx through mechanically gated channels can account for some of the developmental deficits observed in response to mechanical unloading by silencing the permissive influence that activated CaM has over G_2/M phase transition and exit from mitosis. Calcium increments are necessary for general progression through the cell cycle having been correlated with the breakdown of the nuclear envelope, the transition from metaphase to anaphase, as well as during cytokinesis.[29] Moreover, an increase in cytosolic calcium is required for CaM activation, which, in turn, activates store-operated calcium entry through TRPC1 via a mechanism involving CaMKII.[30] Accordingly, a reduction in TRPC1-mediated calcium entry in response to SM, via both stretch-activated and a store-operated

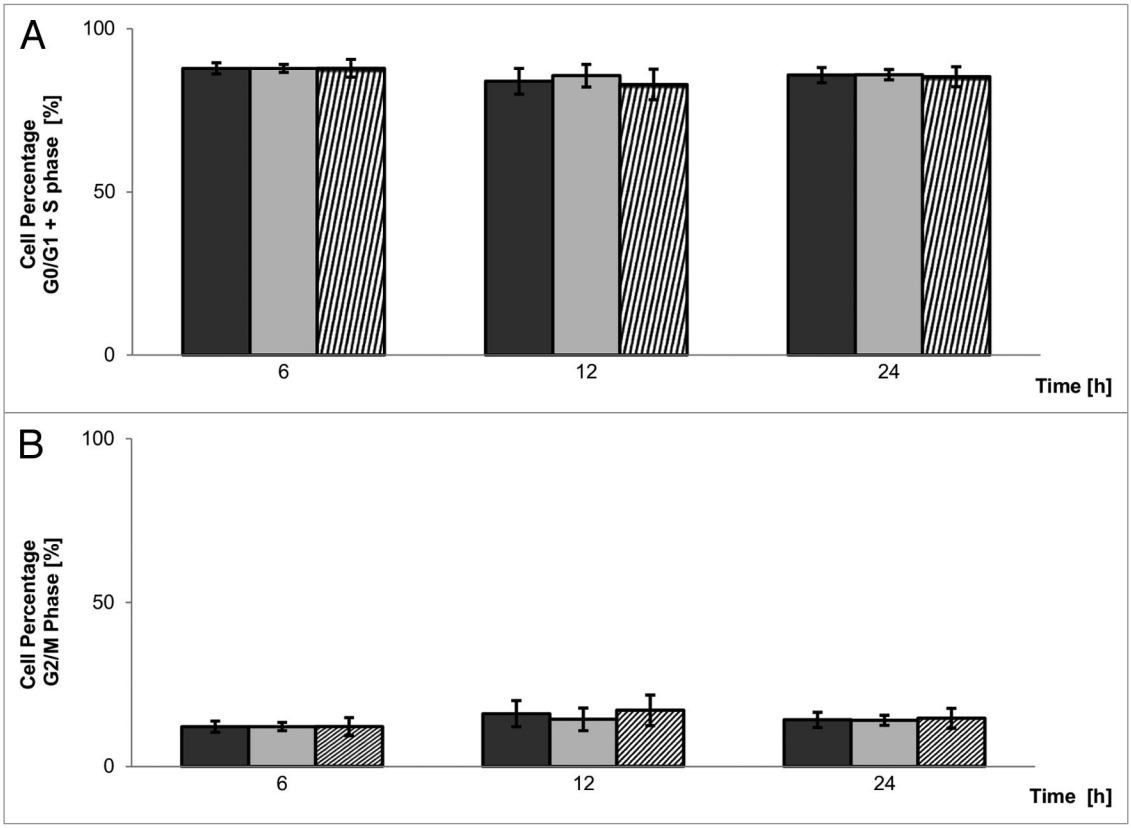

Figure 7. Cell cycle profiles are not disturbed by hypergravity exposure. The cell cycle analysis shows that the percentages of cells in G_0/G_1 + S phase (**A**) and in G_2/M phase (**B**) stay constant throughout all the time points in all the samples. Cells were collected after 6, 12, and 24 h for the hyperfuge control (dark gray columns), incubator control (light gray columns), and hypergravity (patterned columns). Means ± standard deviations were obtained from 3 independent series of experiments, *: $P < 0.05$; **: $P < 0.005$ against each hyperfuge control.

channel gating modalities, would ultimately mitigate CaMKII expression, augmenting cyclin B expression, and explaining the retention of cells within G_2/M phase.[26,31] Conversely, constitutive overexpression of activated CaMKII has been shown to delay mitosis as well as augment cyclin B expression, which, although apparently contradictory to our results, emphasizes the importance for well-regulated calcium influx in a temporally and spatially specific manner, rather than tonic and global changes in transmembrane calcium entry.[32]

An analysis of the effect of gravity over cell homeostasis would be incomplete without an accompanying examination of cell behavior under increased gravitational force. We therefore conducted a series of analyses on cells cultured at 4 g. We did not observe any effect on growth, cell cycle progression, or in the expression of cyclins B or D under conditions of HG. Perhaps, a threshold exists, below which HG is not perceived by cells, and they behave as if experiencing terrestrial gravity. Our results do agree, however, with a recent study where the effects of various levels of HG (5, 10, and 20 g) were analyzed.[33] These authors reported that a 2 h exposure to 5 g produced no noticeable difference in cell expansion relative to 1 g. We were able to extend these results by showing that HG, in accordance with its innocuous effects over cell proliferation, exerted no influence over cell cycle progression. An increase in cell proliferation, however, was detected at 10 g, and was more pronounced at 20 g.[33] As our goal was to develop a potential treatment against muscle atrophy, we did not test such elevated gravitational loads, as they would not be feasible for human-based therapies. In another recent study, CCND1 expression (encoding cyclin D) was analyzed in endothelial cells exposed to different gravitational loads

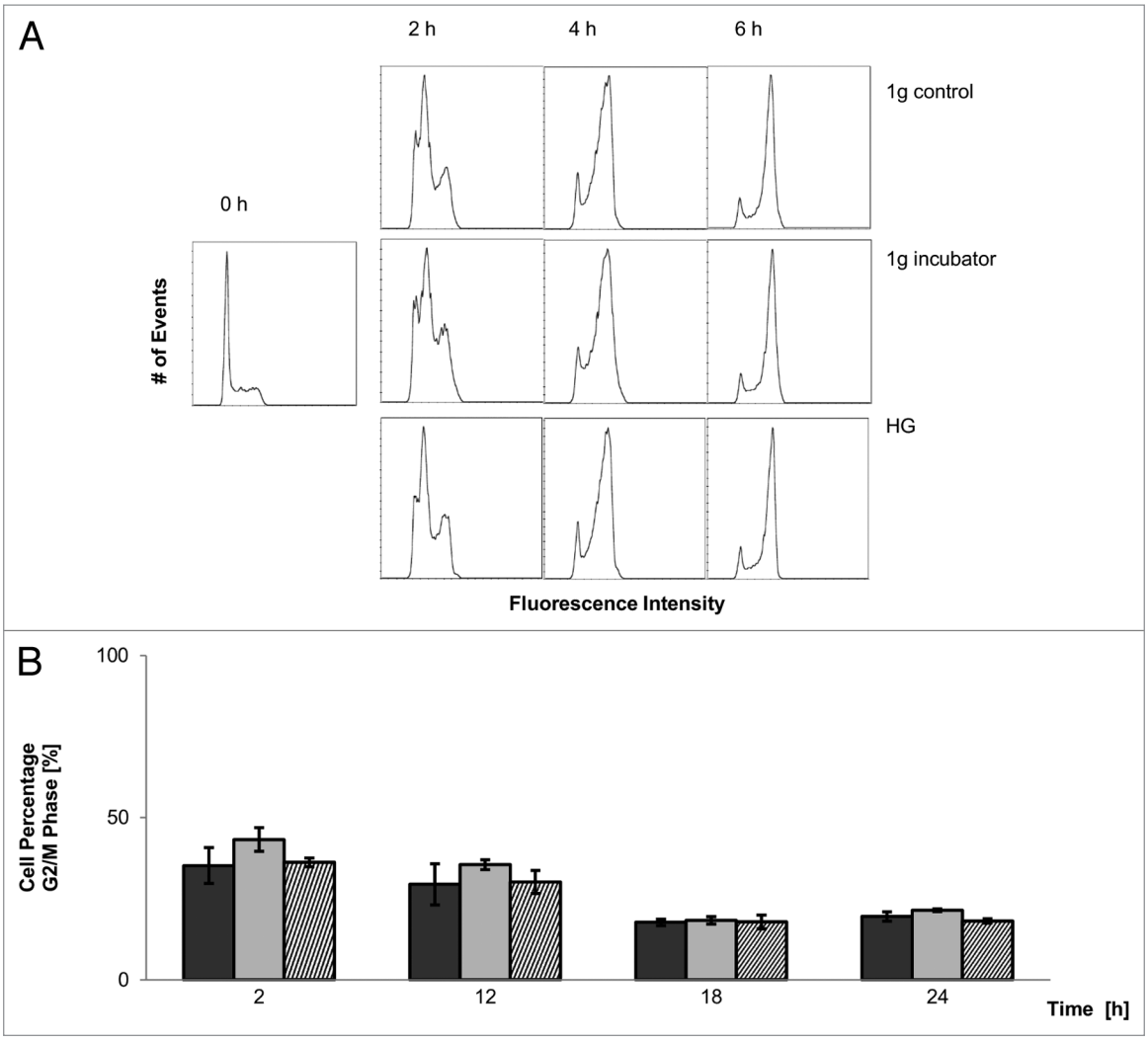

Figure 8. Hypergravity does not affect cell cycle progression. For G_0/G_1 and S phase progressions, cells were synchronized with hydroxyurea for 3 h, released from the block, and then placed immediately on the hyperfuge when cells were accumulated in the G_0/G_1 phase. Cells were collected after 2, 4, and 6 h of growth at 1 or 4 g. Representative histograms (**A**) from 3 independent series of experiments are shown for the 0 h start point (middle left) and after 2, 4, and 6 h for the hyperfuge control (top), incubator control (middle), and hypergravity (HG, bottom). For G_2/M phase progression, cells were synchronized with hydroxyurea for 3 h, released from the block, and then placed on the hyperfuge 6 h afterwards, when cells were accumulated in the G_2/M phase. Cells were collected after 2, 12, 18, and 24 h for the hyperfuge control (dark gray columns), incubator control (light gray columns), and HG (patterned columns). The percentage of cells in G_2/M phase over time is shown (**B**). Means ± standard deviations were obtained from 3 independent series of experiments, *: $P < 0.05$; **: $P < 0.005$ against each hyperfuge control.

during a parabolic flight campaign. In this study, HG (1.8 g) induced a decrease in gene expression, whereas the combination of HG and microgravity during the parabolic flights produced an increase.[34] The authors concluded that cells cultured under HG exhibited expression patterns of slowed cell cycle progression, while the cells exposed to both HG and microgravity showed signs of induced proliferation. Although we did not analyze the combined effects of HG and SM, we did not detect any changes on the myoblasts cultured under HG (4 g). Thus, the previously reported changes may be specific for cell type or are instigated by a lower level of HG or the combination of microgravity and HG. These results suggest that the mechanotransduction apparatus of cells responds selectively to different forms of mechanical input and demonstrates the complexity under which the cell cycle is regulated under distinct mechanical loads. Hence, understanding the underlying molecular mechanisms that control cell cycle progression in response to mechanical stress will ultimately translate into the development of more effective therapies to circumvent disuse muscle atrophies.

Conclusion

We have demonstrated that the retarded cell growth that we previously reported in SM is due to an approximately 16 h delay in the G_2/M phase progression, where cells accumulated specifically between the G_2 checkpoint and the onset of anaphase.[4] Based on our previous work, this accumulation is likely due to a calcium-dependent downregulation of TRPC1 expression, inhibiting CaMKII and slowing cyclin B degradation. Our ongoing research will help us determine the specific factors that cause this delay and will assist in the development of effective countermeasures against muscle atrophy.

Materials and Methods

Cell culture

C2C12 mouse skeletal myoblasts were obtained from ATCC (American Type Culture Collection, http://www.lgcstandards-atcc.org/products/all/CRL-1772.aspx?geo_country=ch) and cultured in growth medium (GM) consisting of Dulbecco modified Eagle medium (Life Technologies, http://www.invitrogen.com/site/us/en/home/support/Product-Technical-Resources/media_formulation.171.html) supplemented with 20% fetal bovine serum (FBS, Life Technologies, http://products.invitrogen.com/ivgn/product/10270106) and 2 mM L-Glutamine (Life Technologies, https://products.invitrogen.com/ivgn/product/25030024?ICID=search-product) in a humidified atmosphere at 37 °C in 7% CO_2. Cells were passed every 2 d and were only used for experimentation before the fifteenth passage. Myoblasts were seeded 15 h before the start of each experiment at 8000 cells/cm^2 in 25 cm^2 flasks (Semadeni, http://eshop.semadeni.com/).

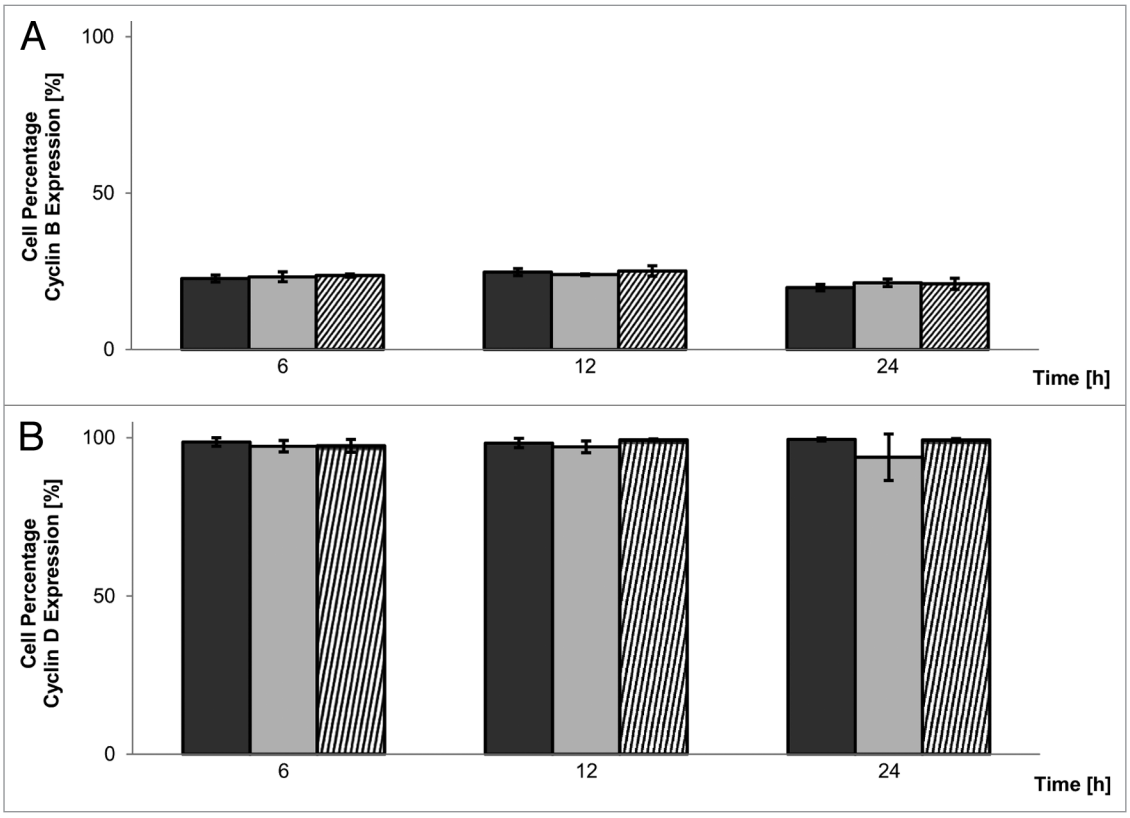

Figure 9. Cyclin B and D expressions remain unchanged during hypergravity exposure. Cyclin B (**A**) and cyclin D (**B**) expressions were measured after 6, 12, and 24 h for the hyperfuge control (dark gray columns), incubator control (light gray columns), and hypergravity (patterned columns). Means ± standard deviations were obtained from 3 independent series of experiments, *: $P < 0.05$; **: $P < 0.005$ against each hyperfuge control.

Random positioning machine (RPM)

The RPM was developed by Dutch Space, formerly Fokker Space; it is in essence a 3D clinostat, in which the gravity vector is continually reoriented, allowing the simulation of microgravity conditions. Samples are fixed near the center of 2 frames that simultaneously rotate independently of the other, one inside the other. The rotation of each frame is random and driven by separate computer-controlled motors. For the experiments described in this report the angular velocity of rotation was 60°/s. The RPM was kept in a temperature-controlled room at 37 °C. SM lasted from 6 to 24 h. Control samples at 1 g were placed on the non-mobile base of the RPM, thereby experiencing the same environment, yet without random rotation, and inside the incubator under normal culture conditions.

Hyperfuge

The hyperfuge (Diport AG) is a centrifuge specially constructed for research under HG conditions, where the speed and the angle of inclination of the cell culture flasks can be regulated to obtain the desired HG force ranging from 2 to 150 g. For the experiments described here, the hyperfuge was set at 4 g, with a speed of 154 rpm and a 14° angle of inclination. The hyperfuge was kept in a temperature-controlled room at 37 °C. HG stimulation lasted from 6 to 24 h. Control samples at 1 g were placed on the non-mobile base of hyperfuge, thereby experiencing the same environment, yet without the centrifugal rotation, and inside the incubator under normal culture conditions.

Cell proliferation

Cell growth was monitored with the CellTrace CFSE Cell Proliferation Kit (Life Technologies, http://products.invitrogen.com/ivgn/product/C34554), according to the manufacturer's instructions. Briefly, cells were stained with 5 μM carboxyfluorescein diacetate, succinimidyl ester (often called CFSE) in phosphate-buffered saline (PBS) + 0.1% bovine serum albumin at 37 °C for 10 min. Cells were plated and collected after 6, 12, and 24 h of growth under different gravitational conditions, and then resuspended in 10% Cell Fix (BD Biosciences, catalog number 340181, http://www.bdbiosciences.com/ptProduct.jsp?prodId=651184&key=cellfix&param=search&mterms=true&from=dTable) for overnight storage at 4 °C before fluorescence activated cell sorting (FACS) analysis. CFSE is a dye that passively diffuses into cells, reacting with intracellular amines and forming fluorescent conjugates. After initial staining of the mother cells, subsequent rounds of cell division dilute the amount of dye inherited by daughter cells by one half with each division.

Cell cycle synchronization and analysis

Cells were synchronized at G_0/G_1 by addition of 1 mM hydroxyurea (Sigma, http://www.sigmaaldrich.com/catalog/product/sigma/h8627?lang=fr&region=CH), an inhibitor of DNA synthesis, for 3 h. Cells were then washed twice with PBS and cultured with fresh GM to resume cycling into S and G_2/M phases. Cells were accumulated within S phase 2 h afterwards, followed by G_2/M phase accumulation 6 h after the release. Cells were fixed at various intervals of growth under different gravitational conditions and stored at –20 °C in 70% ice cold ethanol for at least 12–18 h prior to staining. Cell cycle was analyzed based on DNA content through propidium iodide (PI)/RNase staining (BD Biosciences, catalog number 550825, http://www.bdbiosciences.com/ptProduct.jsp?ccn=550825) by FACS.

Cyclin expression

The samples were fixed after 6, 12, and 24 h of growth under different gravitational conditions with 75% ice-cold ethanol for cyclin B detection and 100% ice-cold methanol for cyclin D, and stored at –20 °C for at least 12–18 h prior to staining. Samples were stained with FITC Cyclin B1 Antibody Reagent Set (BD Biosciences, catalog number 554108, http://www.bdbiosciences.com/ptProduct.jsp?prodId=9845&key=FITC+Cyclin+B1+Antibody+Reagent+Set&param=search&mterms=true&from=dTable) and FITC Mouse Anti-Human Cyclin D1 Antibody Set (BD Biosciences, catalog number 554109, http://www.bdbiosciences.com/external_files/pm/doc/tds/cell_bio/live/web_enabled/1372KK_554109.pdf#search=(FITC Mouse Anti-Human Cyclin D1 Antibody Set)) according to the manufacturer's protocol. In summary, cells were permeabilized with 0.25% Triton X-100 for 5 min on ice, washed, and stained, respectively, with cyclin B or cyclin D antibodies diluted in PBS + 0.1% NaN3 + 1% heat inactivated FBS for 30 min at room temperature in the dark. Samples were then analyzed in PI/RNase solution for simultaneous cell cycle detection by FACS. Staining controls were either singly stained or unstained fixed cells; FITC mouse IgG1 was used as an isotype control to exclude fluorescent background noise.

Flow cytometry

FACS Calibur (BD Biosciences, http://www.bdbiosciences.com/instruments/facscalibur/) was used to analyze cell proliferation, cell cycle, and cyclin expression. A blue laser with excitation at 488 nm was used, with FL1 detector channel for CFSE and FITC, whereas a FL2 channel detector was used for PI staining. Appropriate settings for forward and side scatter gates were applied to examine 20 000 events per sample. CFSE fluorescence intensity, cell cycle profiles, and the percentage of positive cells expressing cyclin B and D were analyzed and quantified with the FlowJo flow cytometry analysis software (Tree Star, Inc., http://www.flowjo.com/). To quantify G_0/G_1, S, and G_2/M populations, the Watson model for cell cycle analysis was used to fit the histograms of gated single cells.[35] For cyclin B and D expression, gates on single cells were set with unstained cells and an isotype control.

Statistics

Data (means ± standard deviations) were obtained from 3 independent series of experiments. Statistical analysis was performed with the R programming software using the Welch 2-sample *t* test against the RPM control (or hyperfuge control where appropriate) at each time point or analysis set unless otherwise stated. Differences were considered statistically significant at $P < 0.05$ (indicated with a single asterisk) or very significant at $P < 0.005$ (indicated with a double asterisk).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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