Loss of Cell-Substrate Adhesion Leads to Periodic Shape Oscillations in Fibroblasts

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We report the phenomenon of periodic shape oscillations occurring in 3T3 fibroblasts merely as a consequence of a loss of cell-substrate adhesion. The oscillatory behavior can last many hours at a constant frequency, and can be switched off and on using chemical agents. This robustness allows for the extraction of quantitative data using single cells. We demonstrate that the frequency of oscillations increases with increasing actomyosin contractility. Both the Myosin Light Chain Kinase as well as the Rho Kinase pathways are shown to operate during this process. Further, we reveal significant similarities between the oscillatory dynamics and the commonly observed phenomenon of blebbing. We show that both these processes cease when extracellular calcium is depleted or when stretch activated calcium channels are blocked. This, along with the fact that these dynamical processes require actomyosin contractility points towards strong similarities in the respective mechanisms. Finally, we speculate on a possible mechanism for the observed dynamical phenomena.

INTRODUCTION

The cell cytoskeleton is a biopolymer gel capable of generating active contractile forces and rapid reorganization [1, 2]. These properties are critical for the ability of these filament networks, especially the actomyosin gel, in controlling cell shape and vital functions like locomotion, cytokinesis, mechanotransduction, etc [1, 2]. The active remodeling of the cytoskeleton during such dynamical processes involves complex signal transduction pathways which include mechano-chemical coupling. Of the myriad of functions the cytoskeleton performs in cells, oscillatory dynamics observed in certain cell types are of particular interest to biologists and physicists alike. Cells like cardiomyocytes and asynchronous insect muscle cells have evolved to generate rhythmic contractile forces [3, 4, 5, 6]. It is also known that certain non-oscillatory cells can be induced to exhibit oscillatory behavior by biochemical intervention. Examples are shape oscillations occurring in lymphoblasts, spreading fibroblasts and in cell fragments, as a result of microtubule depolymerization using drugs [7, 8, 9]. Shape oscillations are also observed in neutrophils and amoeboid cells, usually when exposed to chemoattractants [10, 11] and in the lamellipodia of spreading cells [12, 13]. Recently, spontaneous oscillations of isolated myofibrils lacking regulatory proteins have been observed and theoretically studied [5, 14]. Although oscillatory dynamics in simpler systems like beating flagella are well explained by physical models [6, 15], gaining a theoretical understanding of the dynamics of actomyosin gel is far more complicated and several aspects are still a challenge, despite considerable progress in the field [5, 16, 17, 18, 19].

In this article, we report the observation of oscillatory shape dynamics in freely suspended Swiss-3T3 fibroblasts, without any treatment using drugs. Remarkably, this phenomenon arises merely as a result of loss of cell-substrate adhesion. By maintaining the cells in suspension, we are able to show that the frequency of oscillation can remain constant for several hours allowing quantitative measurements. We show that the frequency is decreased when myosin motor activity is reduced using drugs. Both the Myosin Light Chain Kinase (MLCK) and the Rho kinase pathways play a role in the myosin activation process. Further, we show that a small minimum level of extracellular calcium is essential for oscillations and that this calcium enters the cell via mechanosensitive calcium channels. Later, we compare the oscillatory dynamics with the commonly observed blebbing dynamics and reveal some striking similarities between these two seemingly different phenomena.

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As detachment from the surface is enough to trigger oscillations, we conclude that this dynamics constitute a fundamental response of the cortical actomyosin gel in these cells and may occur under physiological conditions. Finally, we speculate on possible mechanisms for the observed phenomena.

MATERIALS AND METHODS

Swiss-3T3 fibroblasts were obtained from DSMZ Germany and cultured following a standard protocol. The adherent cells, grown to 50% to 80% confluence in plastic cell culture flasks were washed with HBSS devoid of Ca$^{++}$ and Mg$^{++}$ and then treated with a minimal amount of 0.25% Trypsin solution for less than 5 minutes in order to detach them from the cell culture flask. In other cases, mitotic cells were detached by gentle shake-off, without the use of Trypsin. The cells were immediately transferred to a petri dish with a glass coverslip bottom and Iscove (IMDM) cell culture medium with 25 mM HEPES buffer and 10% heat inactivated Fetal Bovine Serum. All cell culture reagents were obtained from Gibco-Invitrogen. Observations were made using a Zeiss Axiovert-135 microscope configured for phase-contrast observation. The temperature was controlled using a home-built temperature controller within ±0.1 ºC.

Hydrophobic surfaces which inhibit cell-substrate adhesion were obtained by coating clean glass coverslips with dimethyldichlorosilane by evaporation and subsequent rinsing using ethanol. In other cases a clean glass coverslip was used to study the effect of cell spreading on oscillatory behavior. Since the cells were held in suspension in most experiments, we maintained a small upward temperature gradient of about 0.1 ºC/mm in the petridish using differential heating in order to avoid convective instabilities. This gradient did not have any observable effect on the oscillations.

The images of the shape changes occurring in the fibroblasts were recorded using a Spot-RT CCD camera (Diagnostic instruments Inc). The 2-dimensional projections were analyzed using a home-developed image analysis program. Typically, the bright edges in the phase contrast images were detected by applying an intensity thresholding. Either the enclosed area or the position of the centroid was used to measure the frequency of oscillations, depending on which of these quantities exhibited clearly detectable oscillations. In cases where oscillations are detected in both these quantities, they have the same frequency. Fluorescence recordings were performed using a high gain EM-CCD camera from Hamamatsu and a 60X, 1.4 NA objective.

The stock solutions of the chemicals were prepared as follows: 0.2 mg/ml Latrunculin-A, 0.37 mM (-)-blebbistatin, and 50 mM ML-7 were prepared in dimethylsulphoxide (DMSO); 100 mM Ethylenediaminetetraacetic acid (EDTA), 1 mM Gadolinium solution using Gadolinium Chloride Hydrate, and 10 mM Y-27632 were prepared in deionized water; 1 mM Lysophosphatidic acid was prepared in PBS buffer (all chemicals are from Sigma). 1 mM Fluo-4 AM in DMSO (Invitrogen, Molecular Probes) was used directly for imaging intracellular free calcium. These stock solutions were added to the culture medium to obtain the desired final concentrations. For calcium imaging, 1 µl Fluo-4 was added to adherent cells in a culture flask containing 2 ml IMDM medium and 1% serum, and left at room temperature for 10 to 20 minutes. The excess dye was then washed off and the cells were incubated for an hour before use.

Specifically coated beads were prepared by rinsing 4.5 µm Dynabeads M-450 Epoxy (Dynal) in PBS three times and suspending them in a solution of Fibronectin (0.1 %, Sigma, further diluted by 100 times in PBS) for 1 hour. The suspension was maintained in a vertical rotator to prevent clumping of the beads and later added to the cells in culture.

RESULTS

Shape oscillations in cells: When the cells are maintained in suspension using hydrophobically treated glass substrates, about 40% to 60% of them show continuous shape alterations. Most of these cells exhibit an oscillatory behavior as shown in Fig. 1. The periodic behavior can be much
FIG. 1: Spontaneously occurring shape oscillations in non-adherent 3T3 fibroblasts. Image sequences corresponds to one oscillation period of a small cell at 3 s/frame (above), and of a larger one at 5 s/frame (below). The smaller cell exhibits an elliptic-like mode, whereas the bigger one shows a more complex shape dynamics, although also periodic. Also see supplementary movies Movie-1 and Movie-2.

FIG. 2: (a) Oscillations in the projected area of the 3T3 fibroblast shown in the lower sequence of Fig. 1. The cell was maintained at 35 °C. The oscillations lasted many hours without any significant change in frequency. (b) Power spectrum of the data in (a) showing peaks at the main frequency of 0.028 Hz and its higher harmonics.

better perceived from the supplementary movies Movie-1 and Movie-2 (available at [21]). Image analysis of the shape reveals a highly periodic dynamics as shown in Fig. 2. On hydrophobic surfaces constant frequency oscillations can then be observed for several hours. When oscillating cells are left at room temperature overnight and heated to the normal working temperature (35 °C) the next day, the oscillations resume with almost the same frequency as before. This shows that the oscillatory behavior is a very robust feature of these cells. The period of oscillations, however, depends on temperature as shown in Fig. 3. It may be noted that all metabolic processes, including motor protein activity and filament dynamics, are strongly temperature dependent.

Different dynamical behaviors can be observed among active cells, in general depending on the cell size. (i) Small cells tend to show very periodic behavior. The oscillations in them often show an elliptic-like mode with the cell tending to oscillate along a preferred direction as shown in Fig. 1 (upper sequence). (ii) Larger cells often show very periodic dynamics but the oscillation modes are more complex as is the case in Fig. 1 (lower sequence). No clearly defined symmetries could be observed in them. Image analysis shows that in some cases the shape change appears as a propagating cortical wave. (iii) Among larger cells, a few tend to show highly dynamical behavior but without any well defined periodicity in any of the analyzed quantities (see supplementary movie Movie-3 [21]). In a population of oscillating cells, the period ranges from about 25 s to 45 s without any clear dependence on cell size. The cell size varies only by about a factor of two making it difficult to quantify these tendencies in behavior with size. Determination of volume of the oscillating cells is also difficult due to the fact that the observed shapes, in general, do not have any symmetry properties that can be exploited to estimate the volume. With this in mind,
no clear indication of any volume change could be observed in small cells with less complex shapes.

**Effect of cell-substrate adhesion:** Since the oscillations are observed in cells which are detached from the substrate, we explored the role of adhesion on the oscillatory process and find the following. (i) Cells which are not attached to the hydrophobic substrates (free floating) and those which have point-like contacts behave identically as far as the oscillatory dynamics is concerned. The absence or existence of adhesion is easily verified by observing the diffusive motion of the cells and/or by applying a small flow disturbance to the culture medium. (ii) When cells are seeded on clean, untreated glass substrates (a non-specific substrate), they attach and eventually spread. The oscillations are observed before the cells attach and after attachment. As cell spreading proceeds the oscillations become progressively weaker and eventually dies out. In cases where the cell subsequently detaches from the surface on its own, the oscillations resume with almost the same frequency as before. (iii) When an active cell is observed to drift and make contact with another cell, forming an adhesion patch of about 5 µm, the dynamics continues without any observable change. (iv) Non-specific adhesions are different from specific adhesions to adhesion promoting proteins as far as signaling and cytoskeletal organization are concerned. For this reason we used 4.5 micron epoxy beads coated with Fibronectin and let them attach to the cells. The adhesion is verified by pulling on beads stuck to partly spread cells using a home built magnetic tweezer. When these cells are observed in suspension one to three beads are seen firmly sticking to most of the cells. These cells exhibit oscillations with almost the same probability as control cells.

It may be assumed that detachment of the cell-substrate contacts triggers signal transduction pathways which lead to oscillations. However, the above mentioned experiments clearly show that having no cell-substrate contact or having small adhesive contacts to specific or non-specific substrates do not affect the observed dynamics. Importantly, whether oscillations are observed or not does depend on the extent of adhesion suggesting mechanical constraints may impede oscillations (see point (ii) mentioned above).

For a given cell, the period of oscillation can remain constant for hours. In what follows, we take advantage of this fact to investigate the response of individual cells to biochemical perturbations using drugs and other chemical agents.

**Effect of actomyosin contractility on oscillations:** We observe that depolymerization of actin filaments using Latrunculin-A or deactivating myosin using blebbistatin kills the above described dynamical behavior. In order to quantify how the activity of cortical actomyosin gel influences the dynamics we exposed the cells to increasing concentration of blebbistatin, a highly specific inhibitor of myosin II. We observe a clear dependence of the oscillation period

![FIG. 3: Variation in oscillation frequency as a function of temperature obtained using a single cell.](image-url)
on the concentration of the drug as shown in Fig. 4. This implies a direct relation between the activity of myosin motors and the frequency of oscillations. At drug concentrations of about 5 µM the oscillations are observed only intermittently. At still higher concentrations the shape dynamics ceases completely.

In order to perform a complementary experiment to verify the effect of contractility on the period, we exposed normal cells to increasing amounts of serum. Serum is known to increase contractility in fibroblasts in a linear fashion with concentration [24], due to increased myosin phosphorylation [25, 26]. Indeed, we observe a decrease in oscillation period with increasing serum concentration as shown in Fig. 5.

These experiments show that the frequency of oscillations increases with increasing actomyosin contractility. Remarkably, there is a clear transition from erratic shape dynamics to periodic
oscillations as the contractility is increased as shown in Fig. 5\(^{\text{inset}}\). Erratic oscillations are also observed at high blebbistatin concentrations (about 5 \(\mu\)M), suggesting a dynamic transition from erratic to regular oscillations as the myosin activity or contractility of the cell is increased gradually.

**Myosin activation pathways:** We also performed experiments aimed at understanding the myosin activation mechanisms involved in the oscillation process. Experiments using drugs to block the myosin light chain kinase (MLCK) or the Rho kinase pathway reveals the following. (i) When 25 \(\mu\)M Y-27632, a Rho kinase inhibitor \(^{27}\), is added to oscillating cells, all the observed cells (> 100) stopped oscillating well within a minute, the time taken for the drug concentration to homogenize. (ii) Most cells (more than 90%) suspended in medium without any serum do not show any shape dynamics. When 50 \(\mu\)M lisophosphatidic acid (LPA) is added more than 90% of the cells become highly dynamic with many showing oscillatory behavior. LPA activates contractility via the Rho pathway \(^{28, 29}\). (iii) When exposed to 25 \(\mu\)M ML-7 to block MLCK \(^{30}\), in presence of 1% serum, all the oscillating cells (> 100) stopped oscillating in about 5 to 10 minutes. The time taken appears to depend on the amount of serum. When 25 \(\mu\)M ML-7 was added to cells in medium without serum and with 50 \(\mu\)M LPA, the action of the drug was dramatically faster. Almost all cells (> 95%) stopped oscillating within the first minute and a few that continued stopped in 5 minutes.

The above experiments clearly show that both the Rho kinase as well as the MLCK pathways are necessary for the oscillatory dynamics. Further, it shows that oscillations can be triggered in these cells purely by increasing the Rho kinase activity using LPA.

**Effect of extracellular calcium on oscillations:** It is known that transient variations in free calcium ion concentration inside the cell can lead to a modulation of the mechanical properties of the cortical actin gel \(^{1}\). Such Ca\(^{++}\) transients could be triggered as a result of small influxes of extracellular calcium. In our experiments, when the external Ca\(^{++}\) concentration was gradually depleted from its normal concentration of about 1–2 mM using EDTA there was no substantial change in frequency as shown in Fig. 6. At an EDTA concentration of 4 mM or above, sufficient to remove all of the extracellular Ca\(^{++}\), the oscillations ceased for all the observed cells. When Ca\(^{++}\) was subsequently replenished using CaCl\(_2\), the oscillations resumed, clearly showing that the oscillations can be switched off and on by altering extracellular free Ca\(^{++}\) concentration in a narrow range.

![Graph](https://via.placeholder.com/150)

**FIG. 6:** Extracellular calcium concentration does not affect the period in a significant way (different symbols are for different cells). Above 4 mM EDTA, the oscillations ceased. Oscillations could be restarted in the same cells by addition of CaCl\(_2\) to the medium.

In order to explore the mode of action of extracellular Ca\(^{++}\) we exposed oscillating cells to Gadolinium ions, a potent blocker of mechanically activated Ca\(^{++}\) channels on the plasma membrane \(^{31, 32}\). Upon exposure to this agent the oscillations stopped abruptly in all cells, even in presence of normal concentration of extracellular calcium. This experiment, along with EDTA experiments, shows that Ca\(^{++}\) influx via mechanosensitive ion channels is vital to the oscillatory mechanism.
**Intracellular calcium variations:** When the cells are loaded with Fluo-4 AM dye and observed under a fluorescence microscope continuous variations in the fluorescence intensity can be observed in dynamic cells. In some cases the variations appears to be roughly periodic. Long term observation is not possible due to photobleaching of the dye and the resulting toxicity affecting the dynamics. A typical example is shown in Fig. 7. Note that the longer period of oscillation (about 100 s) in this case is due to the lower temperature at the cell resulting from the use of an oil immersion objective. Although this suggests an oscillatory variation of free calcium in the oscillating cells, this result should be treated a preliminary for the following reasons. (i) The cytoplasm inside the oscillating cells flows in correlation with the shape changes. This flow carries with it fluorescently labeled regions (like calcium rich areas or calcium stores inside the cell). This problem is greatly reduced by reducing the quantity of dye used as well as the dye loading time. (ii) The cells are very sensitive to the excitation frequency of about 488 nm once they are loaded with the dye. Under strong illumination the cells stop oscillating or blebbing within a few seconds (< 5 s). Under weak illumination, cells can be observed for a few minutes. (iii) Even though Fluo-4 is widely accepted as an efficient indicator of free calcium in cells, a recent study finds that the dye, when exposed to the excitation light, can in itself cause calcium transients [33].

**Bleb dynamics in relation to oscillations:** It is observed that about 50% of the oscillating cells exhibit moderate blebbing or membrane bulge formation while showing shape oscillations. About a few percent can exhibit severe blebbing or blebbing without oscillations (see supplementary movie Movie-5 [21]). Often a cell oscillates for a while without any signs of membrane blebs or bulge formation and then begins to exhibit small localized bleb formation which correlates with the oscillations (see supplementary movie Movie-6 [21]). Detailed experiments on cell oscillations and blebbing demonstrate a remarkable set of similarities between these two processes. (i) They are both dynamical processes requiring acto-myosin contractility as shown by treatments using blebbistatin to inhibit myosin or with Latrunculin-A to disrupt actin filaments. In suspension, both these shape dynamics can occur continuously for several hours without any significant change in their rates. (ii) Some cells show both periodic oscillations and blebbing simultaneously. In
FIG. 8: (a) Sequence of images showing blebbing dynamics in a cell which also shows shape oscillations. In this particular case an untreated glass substrate was employed, and the cell eventually attaches and spreads on the glass. Initially, blebbing appears to be roughly periodic with maxima at frames indicated by the arrows. (b) A rough measure of blebbing is obtained by detecting the envelope which includes all the blebs (outer boundary). The cortical region can also be detected due to the higher intensity of this region in phase contrast images (inner boundary). (c) Oscillations of the area of the envelope of blebs (squares) and area of cortex (circles). The centroid of the detected cortex also oscillates. The plots show that shape oscillations and blebbing can coexist and blebbing can be roughly periodic in some cases.

rare cases, the extent of blebbing appears to be roughly periodic with a periodicity comparable to that of the oscillations as shown in Fig. 8 (also see supplementary movie Movie-7 [21]). (iii) More remarkably, a cell can spontaneously get into one of these two modes or even switch from one to the other as a function of time. (iv) Removal of extracellular calcium using EDTA or exposure to the Ca$^{++}$ channel blocker gadolinium ions immediately stops blebbing as well as oscillations. Introducing Ca$^{++}$ after EDTA treatment reinitiates blebbing or oscillations in the same cells. (v) All the drug and LPA experiments mentioned earlier give the same results for both the blebbing as well as the oscillatory dynamics.

The oscillatory shape dynamics and the blebbing dynamics are separated by time and length scales. The typical time scale for bleb growth and disappearance, which is about 10 s, is shorter compared to 40 s for oscillations at the same temperature. Similarly, blebbing occurs with a typical separation between blebs of a few microns, about 5–10 times shorter than the cell size. Blebbing dynamics shows a wide distribution of these time and length scales while the oscillations are periodic and global. Further, membrane bulge formation, which characterizes blebbing, occurs only in a subset of oscillating cells. However, despite these differences, the above listed experiments suggest that the underlying mechanisms responsible for cell oscillations and cell blebbing dynamics are essentially the same. More specifically, both dynamical behaviors arise from the inherent contractile nature of the actomyosin gel and involve Ca$^{++}$ signaling via stretch activation of calcium channels. This point will be further elaborated in the following section.

DISCUSSION

Oscillatory shape dynamics can be induced in a variety of otherwise non-oscillatory cells, usually when they are exposed to toxins that specifically depolymerize microtubules. Bornens et al. [8] observed that lymphoblasts with disrupted microtubules exhibit an actomyosin constriction ring which propagates back and forth with a constant velocity of about 0.1 μms$^{-1}$. Subsequently,
Pletjushkina et al. [8] reported transient periodic cortical oscillations occurring in spreading fibroblasts when microtubules are disrupted. Treated cells also exhibited strong blebbing. Further, they demonstrated a correlation between shape oscillations and free Ca^{2+} concentration inside the cell. Calcium influx could also be imaged in large blebs. More recently, Paluch et al. [9] investigated oscillatory dynamics in cell fragments occurring when microtubules are disrupted. It is shown that the actin cortex in fragments can break as a result of excess tension. This leads to an oscillatory dynamics which involves the repeated formation of a bare membrane bulge and a contractile ring which propagates back and forth in correlation with the oscillations.

Unlike the above examples, the oscillatory dynamics reported here do not involve any biochemical treatments. Non-adherent cells are observed to oscillate spontaneously. Since myosin-II and actin are necessary for this dynamics, it points to an inherent instability of the actin cortex in these cells when the contractile cortex is unconstrained by cell-substrate adhesion. Moreover, the oscillations are very periodic and can be sustained for many hours. The experiments show that myosin phosphorylation via the calcium dependent MLCK pathway as well as the calcium independent Rho kinase pathway are involved and necessary. The oscillatory dynamics is also coupled to extracellular calcium, which enters the cell via stretch activated calcium channels. The oscillation frequency is independent of the external calcium concentration except when the external calcium is almost completely depleted. But there is a clear increase(decrease) in frequency when myosin activity or cell contractility is increased(decreased) gradually. While some cells show moderate to severe blebbing, these cells can also oscillate without any observable bleb formation or membrane bulge formation. This suggests that in such cells the cortex and the membrane-cortex adhesion remain mostly intact during oscillations.

Based on these observations, we propose a possible mechanisms for oscillations of the actomyosin cortex which involve a coupling to stretch activated calcium channels on the outer membrane. Due to the presence of myosin motors, the cortical gel is contractile and generates lateral tension. In a general case, this tension is anisotropic either due to a non-uniform distribution of myosin or due to anisotropies in the gel structure. This leads to an anisotropic shape change of the cortex. If the shape change is significant, it can lead to a stretching of the membrane due to a change in the hydrostatic pressure inside the cell. The cytoplasmic streaming which is observed suggests such gradients in pressure (Movie-8 [21]). The tension thus generated opens tension sensitive calcium channels causing an influx of calcium ions into the cell. Tension on actin filaments may also play a role in activating gadolinium-sensitive calcium channels as has been reported for fibroblasts [34]. The free calcium ion concentration inside a cell is about 10^{-4} mM is far lower than that of its extracellular value of 1–2 mM [1]. The entry of calcium from outside can thus lead to a significant increase in the free calcium ion concentration inside the cell. This calcium influx may also trigger a calcium-induced calcium release from internal calcium reserves. Such a process has been observed in fibroblasts and other non-muscle cells in response to mechanical stretch [34, 35], although this may depend on cell type [36, 37]. Subsequently, the calcium concentration is brought back to the rest value by absorption of free calcium and by the action of calcium pumps [1]. The initial rapid elevation of free calcium has mainly two effects on the cortical actomyosin gel. Large increases in free calcium concentration can lead to a partial solation or weakening of the gel due to gelsolin mediated severing of actin filaments and can alter the actin polymerization rates [1, 2, 38, 39]. Calcium also affects actin crosslinkers like α-actinin and other actin binding proteins leading to a weakening of the gel at higher concentrations (∼ 10^{-3} mM, ref. [40], and references therein). The extent of solation depends on the calcium concentration. In-vitro studies show that a low degree of solation can increase the contraction rates and strong solation can make the gel non-contractile [38]. At low concentrations, calcium also causes myosin phosphorylation via the calmodulin-MLCK pathway, by which myosin is assembled into contractile filaments [1, 2, 33, 41, 42]. This enhances the contractility of the cortex. These two processes—the weakening of the gel and increased contractility—may be separated in time. At high calcium levels the gel is partially solated and as the calcium is reabsorbed into the stores, actin polymerizes, myosin filaments bind and the cortex become contractile again. The whole cycle is then repeated. This scenario, where repeated contractions occur with calcium playing a feedback role is schematically represented in Fig. 9.
It is not clear from the present experiments as to what extent the actin gel structure is altered by the increase in calcium levels. If severe calcium mediated disruption of the cortex occurs, it will have the effect of increasing the time delay between calcium influx and the initiation of contractile response, as the cortex has to reform before contraction can take effect. The role of calcium in effecting myosin phosphorylation is more clear as shown by experiments using drugs that block the MLCK pathway. However, experiments show that the frequency of oscillations do not depend on the extracellular calcium levels even when external calcium levels are reduced using EDTA. This may be understood if a small influx of extracellular calcium is enough to trigger a much larger calcium-induced calcium release from internal stores.

It is observed that cells which have stopped oscillating after external calcium is completely chelated using EDTA can restart oscillations if calcium is reintroduced in the form of CaCl$_2$. How this calcium enters the cell and triggers oscillations in a non-oscillating cell is not clear. However, it may be noted that calcium channels on the membrane can open stochastically and generate “calcium puffs” [43]. Such stochastic Ca$^{++}$ puffs may aid in “kick starting” oscillations by inducing contraction when Ca$^{++}$ is added from outside.

Experiments clearly demonstrate that the frequency of oscillation increases with increasing myosin activity. The maximum observed variation is about 30% in the blebbistatin experiment. This may be understood as a direct effect of the rate of contraction of the gel, which is expected to increase with increasing number of active myosin motors as observed in in-vitro gels [38]. The fact that the oscillation ceases at a finite frequency in the blebbistatin and serum experiments indicates the existence of a threshold in myosin activity below which an oscillatory instability cannot occur. Moreover, the observation that myosin phosphorylation via both Rho kinase as well as the MLCK pathways are necessary further suggests that a high level of phosphorelated myosin is necessary for this dynamics. Indeed, crosslinked in-vitro gels too exhibit such a threshold as a function of myosin-II concentration [38]. In earlier examples of oscillations induced by microtubule depolymerization in spreading fibroblasts, the Rho kinase activation was proposed as the triggering mechanism for oscillations [8]. This enhanced contractility may have been necessary as these cells are attached and spread on a substrate and hence the cell cortex is partly constrained and under tension. In our case, we observe that the oscillations become progressively weaker in amplitude.
and die out completely as cell spreading proceeds, supporting this hypothesis. Thus, detachment of cells from the substrate may have the effect of lowering this contractility threshold.

In Paluch et al. [9], observations made on cell fragments clearly show breakage of the actin cortex indicating large tension. The cortical breakage is accompanied by a membrane bulge formation. This may be because the bare membrane is unable to sustain the excess hydrostatic pressure generated due to contraction. In the present case, cells showing periodic oscillations often show no membrane bulge formation or blebbing. This is a strong indication that the actin cortex as well as the membrane-cortex adhesion are intact during oscillations. Indeed, in some cases, a cell can oscillate without any membrane bulge formation for tens of minutes (observation time) and then suddenly developed repeated bulge formation in correlation with oscillations. An example is shown in Movie 5. In such cases either the cortex has become locally weak and tend to break during contraction or the membrane-cytoskeletal linkages become locally weak resulting in membrane detachment and bleb formation.

Unlike shape oscillations, blebbing dynamics has been investigated in detail (see, for example, C. Cunningham [44]) and mechanisms have been proposed recently [45, 46]. It is shown that repeated local contractions of the actomyosin complex occurs during bleb formation. According to Charras et al. [45], the contractions generate a pressure which is diffusive due to the resistance to flow between the cytoplasm and the cortical gel. This produces local maxima in pressure which causes the membrane to detach and form blebs, at a constant cell volume. As mentioned earlier, in rare cases (a few percent), cells can be observed to switch between seemingly random blebbing dynamics to regular oscillatory dynamics as a function of time in the range of tens of minutes. Moreover, experiments show that actomyosin contractility and similar calcium signaling are involved in both these dynamical processes.

Why do some cells show periodic and global shape oscillations (Movie-1) while some others show more complex contractile behavior (Movie-3) and some show blebbing (Movie-5)? A possible answer to this question may lie in the viscoelastic and contractile nature of the actomyosin gel. Stresses generated locally in such a gel will typically relax over a certain length and time scale which depends on the extent of crosslinking and hence the elasticity of the gel. If the gel is highly crosslinked (more elastic), for example, stresses may propagate distances comparable to the cell size. Therefore, any contractile stresses are felt all over the cell. This could lead to correlated shape alterations as is observed in periodically oscillating cells. If on the other hand, the gel is elastically weak (more viscous than elastic) the stresses die out very quickly, over distances considerably smaller than the cell size. Volume elements of the gel which are separated by distances which are bigger than this length scale will not feel each other’s mechanical state. Thus the cell could sustain several local contractions or relaxations which are largely uncorrelated. Such a transformation from global contraction to local small-scale contractions is observed in in-vitro gels as the extent of crosslinking is reduced [38]. Local contractions could then lead to blebbing due to local build up of pressure and generate local membrane tension [13]. The viscoelastic properties of the gel may vary from cell to cell or during a cell cycle giving rise to a range of dynamics in a given cell population. The gel properties may also be modulated over time either due to effects of calcium or due to other complex biochemical events and the cell can switch from one mode of dynamics to another. The timescale for bleb formation and retraction will, in general, depend on the bleb size and the time taken for a fresh cortex to form below the bare membrane and induce contraction [13]. In fact, this is akin to the shape dynamics in cell fragments where cortical breakage and membrane bulge formation occurs [6]. In periodically oscillating cells no such membrane bulge formation is evident and this may explain why the oscillations become remarkably regular.

Finally, it is interesting to note that periodic dynamics and propagating waves are observed in the lamellipodea of a wide variety of spreading or locomoting cells [12, 13, 47, 48], and in cells recovering from actin depolymerization [49]. The wave-like cortex dynamics observed in some of the oscillating cells (see supplementary movie Movie-2) is reminiscent of the lamellipodial waves observed in some of these cases [13, 48].

**Conclusion:** We show that fibroblasts can exhibit sustained, remarkably periodic, shape oscillations when maintained in suspension, without any treatment with drugs. Even cells removed from the culture by gentle shaking show sustained oscillatory behavior. These cells are, in general,
close to the mitotic state and hence loosely adherent. Thus, the observed dynamics is fundamental to fibroblast mechanics and may well be relevant under physiological conditions. The implications of this phenomenon to cytokinesis [50], cell locomotion [11, 51], or during embryogenesis when the cells are loosely adherent will be of interest for future research. The robustness of the oscillations and the fact that it can be sustained for several hours makes it an exceptional and relatively simple model system for experiments aimed at understanding the dynamical properties of cortical actin gel. The results presented here are also expected to motivate future theoretical modeling based on the mechanics of contractile actomyosin gels.

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