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Photoactivatable surfaces resolve the impact of gravity vector on collective cell migratory characteristics

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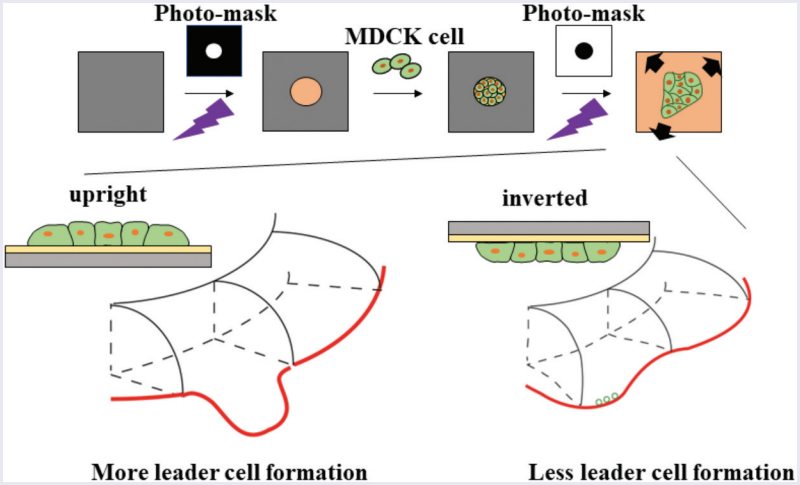
ABSTRACT

Despite considerable interest in the impact of space travel on human health, the influence of the gravity vector on collective cell migration remains unclear. This is primarily because of the difficulty in inducing collective migration, where cell clusters appear in an inverted position against gravity, without cellular damage. In this study, photoactivatable surfaces were used to overcome this challenge. Photoactivatable surfaces enable the formation of geometry-controlled cellular clusters and the remote induction of cellular migration via photoirradiation, thereby maintaining the cells in the inverted position. Substrate inversion preserved the circularity of cellular clusters compared to cells in the normal upright position, with less leader cell appearance. Furthermore, the inversion of cells against the gravity vector resulted in the remodeling of the cytoskeletal system via the strengthening of external actin bundles. Within the 3D cluster architecture, enhanced accumulation of active myosin was observed in the upper cell-cell junction, with a flattened apical surface. Depending on the gravity vector, attenuating actomyosin activity correlates with an increase in the number of leader cells, indicating the importance of cell contractility in collective migration phenotypes and cytoskeletal remodeling.



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1. Introduction

Since the introduction of space travel in the early 1960s, scientists have been interested in studying the effects of gravity on mammalian cells [1]. Additionally, with more private companies currently supporting space travel, the possibility for common individuals to explore space has increased. Various studies have attempted to understand biological responses to changes in the magnitude of gravity using mammalian cells or animal models [2]. For instance, the impacts of microgravity (expressed as μG, which is lesser than the Earth's gravity G, such as the moon's gravity, which is equal to 0.16 G) and hypergravity (higher than the Earth's gravity G, such

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as Jupiter's gravity, which is equal to 2.4 G) have been examined. Because gravity is a force, it has both magnitude and direction. The direction of gravity, or the gravity vector, substantially influences the whole body through its effect on a specific organ called the vestibular system. This organ, which is localized in the inner ear, perceives changes in the gravity vector and maintains the body's equilibrium; consequently, any abnormality in the vestibular system alters the physiological conditions of the whole body with the alteration in the gravity direction [3]. Nevertheless, the effects of the gravity vector at the cellular and molecular levels remain unclear. One early observation associated with the gravity vector is the translocation of the nucleus, which is the largest organelle inside cells. In the case of single cells, the inversion of substrates resulted in translocation of the nucleus near the apical surface of the cells, with strengthening and bundling of actin network at the cellular periphery [4]. Later, a detailed study was conducted to understand the relationship between nuclear translocation induced by gravity vectors and cytoskeletal systems [5]. In this study, increased expression of actin and vimentin was associated with the inversion of single cells against the gravity vector for 24 h and 72 h, respectively. Furthermore, actin stress fibers were redistributed around the nucleus; however, vimentin distribution remained unaltered. Blocking of actin polymerization resulted in the loss of longitudinal translocation of the nucleus. Therefore, remodeling of cytoskeletal systems induced by the cell inversion against the gravity vector is necessary for safe nuclear translocation. Contrary to a previous report, another report observed cytoskeletal remodeling within a faster time frame (10 min) for single cells; this report revealed a transient reduction in actin stress fiber formation with a considerable increase in lamellipodia formation, followed by the complete loss of such changes within one or two hours [6]. Therefore, the cellular response to gravity is time-dependent; the transient effect shows lower actin stress fibers, whereas the adaptive effect shows higher actin stress fibers.

Another critical biological function affected by gravity is cell migration. Bovine aortic endothelial cells (BAECs) exhibit upregulated motility when they are sparsely cultured and inverted against gravity, whereas confluent cells exhibit delayed motility when the wound scratch assay is performed [6]. These discrepancies in migratory behavior can be attributed not only to the collective or single-cell motility characteristics but also to the essential methodological limitations of the conventional wound healing assays [7]. For example, the damage and displacement of external cells are associated with the release of inflammatory cytokines and proteins that might influence the migration behavior, cellular proliferation ratio owing to prolonged confluency, and remodeling of extracellular matrix (ECM). Thereby, conventional methods are incapable of inducing the migration of cells while in the inverted position and do not account for the transient changes associated with gravity in an early time frame.

In this context, photoactivatable surfaces offer an ideal platform for analyzing the impact of gravity vectors on collective cell migration. These surfaces were initially functionalized with photocleavable poly (ethylene glycol) (PCP), which made them unfavorable for protein adsorption and cell adhesion; upon ultraviolet irradiation, the PCP group is cleaved, and the surface becomes suitable for cell adhesion [8]. Photoactivatable surfaces have several advantages over other techniques; for instance, using photoactivatable surfaces, well-controlled cellular confinements with any required geometries can be attained [9]. Moreover, undamaged cellular frontiers are readily maintained during the induction of migration [10]. This technique is versatile and suitable for diverse surfaces such as glass surfaces [11], nanopatterned surfaces [12], and polymeric hydrogels [13]. Therefore, photoactivatable surfaces have features suitable for analyzing the effects of gravity vectors on collective cell migration in a non-invasive and cell-friendly manner via remote control of cell migration, even when the cells are in the inverted position.

Utilizing these beneficial features, we implemented photoactivatable surfaces to evaluate alterations in the cluster expansion behavior in response to changes in the gravity vector. Specifically, we discuss the differences in migration behaviors in upright and inverted configurations. Inversion of substrates substantially altered the cluster geometry and the appearance of leader cells, along with remodeling of the actin cytoskeleton. Pharmacological manipulation of myosin activity implied that cellular contractility played an important role in the acquisition of the migratory phenotype.

2. Material and methods

2.1 Preparation of gold substrates and photoirradiation conditions

Gold substrates were prepared by consecutively depositing a 5-nm titanium layer and a 20-nm gold layer onto a glass slide (0.25 mm thick, Matsunami, Osaka, Japan) under vacuum using an e-beam evaporator. A UV-ozone cleaner (UV253; Filgen, Nagoya, Japan) was used to clean the gold substrates. PCP disulfide (PCP-ds), hexane(ethylene glycol) disulfide (EG_6-ds), and cyclic RGD disulfide (cRGD-ds) were prepared as previously described [14–16]. The cleaned substrates were then incubated overnight at 25°C with a mixed solution of disulfides in a specified ratio (9:0.9999:0.0001) of PCP-ds, EG_6-ds, and cRGD-ds

respectively; the total concentration of the ligands was 10 μM. The gold substrates were washed with methanol and sterilized by incubation with 70% alcohol for 5 min. Photoirradiation was performed as previously described [15], using an energy of 60 J/cm^2. The size of the photomask allows a pattern of circular clusters with a diameter of 130 μm.

2.2 Cell culture, patterning, and immunostaining

Madin-Darby canine kidney cells (MDCK; RCB0995, RIKEN cell bank) were cultured in Eagle's minimal essential medium (MEM; Sigma, St. Louis, Missouri, U.S.A) containing 10% heat-inactivated fetal bovine serum (FBS; EU origin, Biowest, Nuallie, France),100 units/mL penicillin and 100 mg/mL streptomycin (Nacalai, Japan), 1% MEM-nonessential amino acids (Nacalai, Japan), 1% sodium pyruvate (Nacalai, Japan), and 1% L-glutamine (Nacalai, Japan) at 37°C in a humidified atmosphere containing 5% CO_2 at 75% confluency of cell subculture, and cells were collected by trypsin/ethylenediaminetetraacetic acid (EDTA) (Wako, Japan) to be seeded on gold surfaces after the first photoirradiation. Cell seeding occurred first in FBS free medium for 1–2 h, and then the medium was changed into a complete medium for the rest of the experiments. Cells were cultured for 5–10 h after seeding, and the inverted substrates were fixed in a custom-made glass device at an inverted position after 1–2 h. The second irradiation was performed using a photomask 12 h after the initial cell seeding. Time lapse imaging was performed using controlled chamber (at 37°C and 5% CO_2) and Axiovert 200 Zeiss microscope (Oberkochen, Germany). All systems were controlled using Metamorph software (Molecular Devices, Sunnyvale, California, U.S.A); captured images were processed using Fiji (Image J, U.S.A). Cluster circularity and expansion rates were calculated using Fiji by manually outlining the migrating clusters at different time points after the induction of migration. Cluster circularity is calculated as 4π area/perimeter^2. FluoSpheres® Carboxylate 1 μm red (580/605) (Life Technologies, Eugene, Oregon, U.S.A) were used to examine the homogenous mixing of medium using the above experimental conditions. Blebbistatin (Wako, Osaka, Japan) was used at a concentration of 20 μM to manipulate the external bundles, and was added directly before the second irradiation. For immunofluorescence (IF) staining, cells were fixed with 4% paraformaldehyde (Nacalai, Japan) for 15 min, quenched with 5% glycine (Wako, Japan) in PBS for 5 min, permeabilized with 0.5% Triton X-100 for 5 min, and blocked with bovine serum albumin (BSA, Wako, Japan) for 30 min. Cells were then incubated with Alexa Fluor™ 555 Phalloidin (ThermoFisher scientific, U.S.A) for 1 h. Confocal images were obtained under the Olympus microscope (IX81-PAFM, Olympus, Tokyo, Japan) by using a disk-scan unit (CSU-10, Yokogawa, Tokyo, Japan) and Andro CCD camera (SONA 4BV6U, UK). Peripheral actin bundles were quantified using Fiji by hand selection of the external bundle of the whole cluster and calculating the mean fluorescence intensity within this bundle's area. The area between the clusters was kept constant. Orthogonal (side) views of confined clusters were obtained from the Z-stack series using Fiji for cells cultured in inverted/upright positions for nearly 15 h with and without blebbistatin treatments. To detect active myosin, phosphorylation of the serine 19 residue of myosin II regulatory chain (pMLC) was examined by IF staining. Mouse monoclonal p-myosin light chain (S19) antibody (1:500; Cell Signaling Technology, Danvers, Massachusetts, U.S.A) and anti-mouse IgG Alexa Fluor 488 (1:1000) (Life Technologies, Eugene, Oregon, U.S.A) were used. The intensity of p-MLC was quantified using Fiji through the line scan option as previously reported [17]. The upper junctional region was chosen to be 4–5 μm above the peripheral actomyosin bundles from Z-stack files, and the average cell number within the evaluated clusters was 55 ± 6 cells per cluster, which avoided higher/smaller cell density because it might cause false negative/positive pMLC intensity calculations. The statistical analyses were performed for the presented data using student *t*-test.

3. Results and discussion

3.1 Strategy for the utilization photoactivatable substrates for analysis of cell migration in inverted and upright positions

Photoactivatable substrates composed of self-assembled monolayers (SAMs) were prepared by mixing three disulfides bearing a cRGD peptide, the cell-repellent EG_6, and photocleavable PEG. Their chemical structures and our strategy to use photoactivatable substrates are shown in Figure 1(a,b), respectively. cRGD ligands become accessible to cells only when bulky PEG brushes are cleaved by photoirradiation. Furthermore, the inhibition of nonspecific adsorption of serum-derived proteins and minimal ECM remodeling were confirmed by the presence of the EG_6 ligand [17], consequently minimizing changes in the chemical composition of the surface during the experimental procedure. After the first photoirradiation of the substrate with a circular photomask, the MDCK cells were allowed to attach and fill in the irradiated region for several hours. Then, the substrates were inverted upside down, fixed to a homemade glass device, and left for an additional 1–2 h before migration induction. The glass device raised the gold surface from the glass-bottom dish to prevent compression of

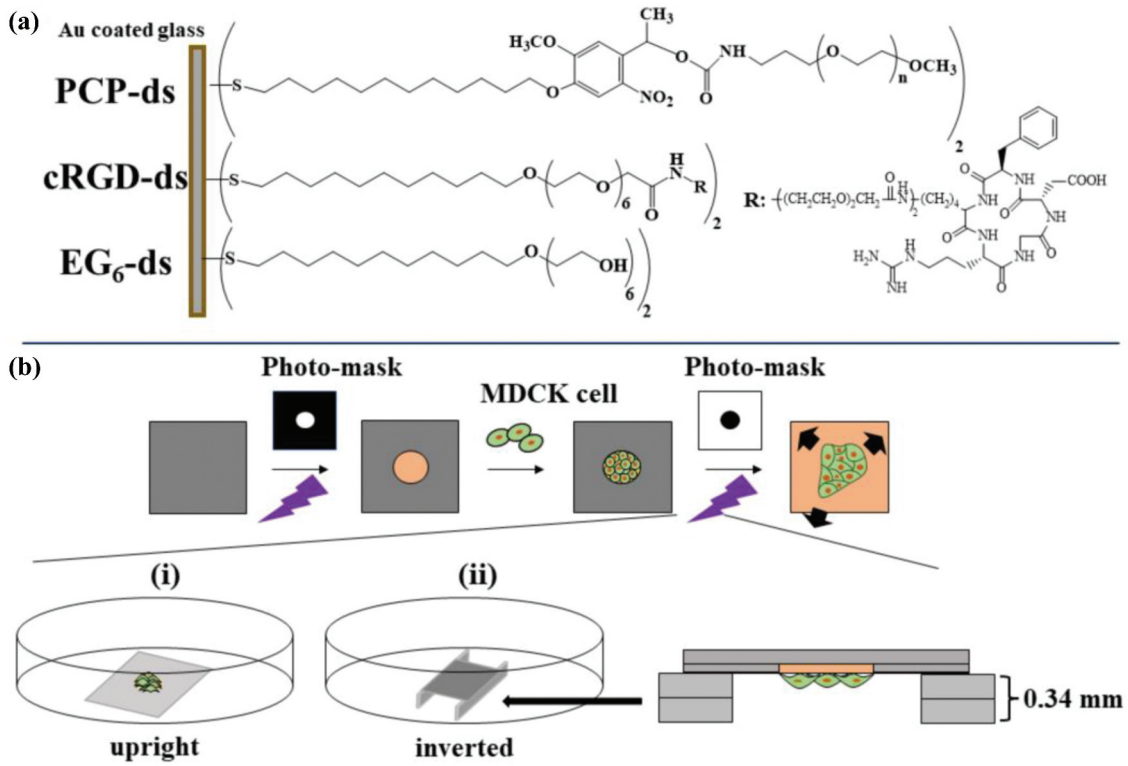

Figure 1. (a) The chemical structures of the PCP, cRGD, and EG_6 used for gold functionalization. (b) Schematic description of the use of photoactivatable substrate for examining the effect of gravity vector on cell migration for (i) upright and (ii) inverted and the use of a custom-made glass device to avoid the compression of cells.

the cells, so the effect of the gravity vector was solely examined to avoid any cellular mechanical compression (Figure 1(b)). To confirm homogenous mixing and cyclization of the medium underneath the gold substrates due to the space offered by the glass device during experiments, we examined the diffusion of fluorescent beads added at the side of the plates (Figure supporting 1A) by time-lapse imaging. The time necessary for beads to be distributed from the side of the plate to below the inverted gold surfaces (1.5 cm) was nearly the same as that to above the upright gold surfaces (Figure supporting 1B, C). This confirms the medium exchange and mixing occur from the outside to inside of the glass chamber below the inverted gold substrates at the same rate as in the upright substrates. For migration induction, the surrounding regions were irradiated by keeping the substrate on the glass device through another photomask, and the migration behaviors of cell clusters were monitored in the upside-down configuration (Figure 1b(ii)). The results were compared with migration behavior in the normal upright configuration (Figure 1b(i)).

3.2 Cluster circularity and leader cell formation are altered by gravity vector on cRGD gold surface

Figure 2(a) shows the phase-contrast images of cell clusters cultured in inverted and upright positions at different time points after the induction of migration. First, the expansion rate of the cell cluster was quantitatively evaluated by tracking the change in the cell cluster area over 14 h (Figure 2(b)). Four hours after induction of migration, the difference in the expansion rate became significantly different (Supporting movie 1). We then examined two possibilities that could be associated with the delay in the expansion. The first is the alteration in cellular migration velocity and the second is the alteration in the proliferation ratio. To address the first possibility, we examined the change in migration velocity of single cells. This was done by photoirradiation of the whole substrate without any photomask, followed by sparse seeding of the cells, and evaluation of cell migration velocity was carried out for 10 h. We did not observe any significant differences in single-cell migration velocity (Figure supporting 2A). Under our experimental conditions, the decrease in the expansion rate associated with the inversion of the substrate against the gravity vector was not due to a reduction in the intrinsic migration velocity of the single cells. However, there might be a difference between the intrinsic migration velocity for single cells compared to cells in a group, especially when there are significant alterations in the biological functions of cells in groups compared to single cells, such as cellular adhesion, migration persistence [18], and drug sensitivity [19,20]. In contrast, Ju et al. recently reported that upon inversion against gravity, single cells showed enhanced motility [6], which could be due to the intrinsic difference between

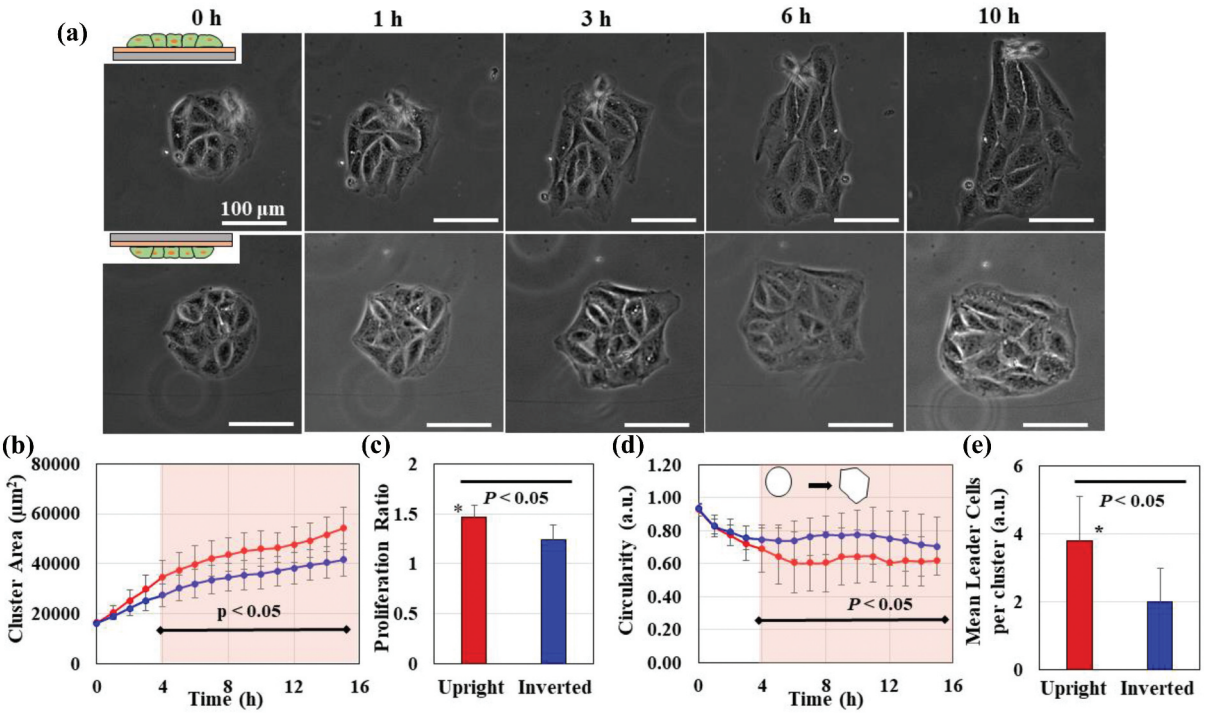

Figure 2. (a) Phase contrast images for the cluster expansion of cells on the photoactivable surfaces after the second irradiation at different time point. (b) The average expansion rate clusters in inverted and upright positions as determined by the change in the cluster area with time (N = 10–11 clusters). (c) Average calculated proliferation ratio for cellular clusters in upright and inverted positions showing a decrease in cellular proliferation in the inverted position (N = 10–11 clusters). (d) The average change in the calculated cluster circularity at different times (N = 10–11 clusters). (e) The mean calculated average of leader cells emerging from clusters in upright and inverted positions (N = 10–11 clusters).

the various cell lines or the difference between the substrates used for cell culture. Next, we examined whether the proliferation ratio was affected by the gravity vector, and the proliferation ratio was calculated by counting the number of cells just before the cluster expansion (0 h) and at 14 h after expansion. We observed a decrease in proliferation ratio for cellular clusters cultured in the inverted position compared to the upright position (Figure 1(c)), and this decrease was observed for single cells as well (Figure supporting 2B). Thus, the decrease in the cluster expansion for cells in the inverted position is not due to the change in the intrinsic cellular migration velocity of single cells but might be due to the decrease in the proliferation ratio between cells in inverted and upright positions. It was previously reported that the inversion of osteoblasts was associated with a decrease in proliferation rate up to 50%, even though it was discussing the sparsely seeded cells [21], which is suggested to be due to the increase in the lag-phase duration on which the cells are able to perform all vital processes without division. An extended lag-phase of ROS 17/2.8 (rat osteosarcoma cells) was also exhibited during the early seeding in the inverted position, even though the overall proliferation rate over a longer period (4 days) did not change [4].

Next, we evaluated the change in circularity of clusters during migration with time (Figure 2(d)). Circularity evaluates the symmetry break in the circular cluster; therefore, it indicates the asymmetric emergence of actively migrating cells. In both cases, the cluster circularity gradually decreased; however, the changes were significantly larger for the upright than for the inverted ones. In other words, the inversion of the substrate and the induction of cell migration within this position hinders protrusion emergence and maintains circular geometry compared to cells in the upright position. Furthermore, when we calculated the number of leader cells appearing after 5 h of migration, the orientation of the substrate against the gravity vector (inverted position) was associated with a further decrease in the number of leader cells (Figure 2(e)). Leader cells provide the required guidance and control over the speed and directionality of collective migration [22]. This result indicates a correlation between the inversion of cells against the gravity vector and migratory characteristics such as leader cell formation. By summarizing the above results, we can conclude that the characteristics of MDCK cluster expansion behaviors in the inverted position are as follows: 1) a decrease in leader cell formation and 2) maintenance of the circularity of migrating clusters. It is noteworthy that these findings became apparent because our photoactivation surfaces are useful for precisely controlling the initial cell cluster geometry. Moreover, it is also critical to analyze

leader cell formation from light-mediated remote control of surface cell adhesiveness without time delay and physical perturbation, which is inevitable with conventional cell migration assay platforms.

3.3 Alteration in cytoskeleton structure in response to the gravity vector

Given that the difference in the sensitivity of cluster circularity and leader cell formation was observed upon inversion against gravity, we further investigated the effect of the gravity vector on cytoskeletal rearrangements for cells in clusters. We focused on the actin cytoskeleton for three major reasons: 1) The integrity of the peripheral actomyosin bundle is essential for the appearance of the leader cells [23,24]. 2) The organization and dynamics of actomyosin bundles play a significant role in cluster polarization, which is sensitive to local curvatures [25]. 3) Actin cytoskeletal remodeling and redistribution are observed in single cells upon their inversion against gravity [5]. To examine peripheral actomyosin bundles, we fluorescently stained actin in a geometrically confined cluster without inducing their expansion. Figure 3(a) shows the fluorescence staining of actin in the external bundles surrounding the clusters in upright and inverted positions after 18 h of culture. We observed that actin bundles in the periphery of the cluster were significantly stronger for the clusters in the inverted position than for those in the upright position (Figure 3(b)). Based on previous reports, the number of leader cells increases by manipulating the curvature of cluster edges [26] and surface chemical cues [17] via weakening of actin contractile bundles at the edges of cell clusters. Therefore, it is suggested that the less frequent observation of leader cells in the inverted configuration is mediated by similar mechanisms.

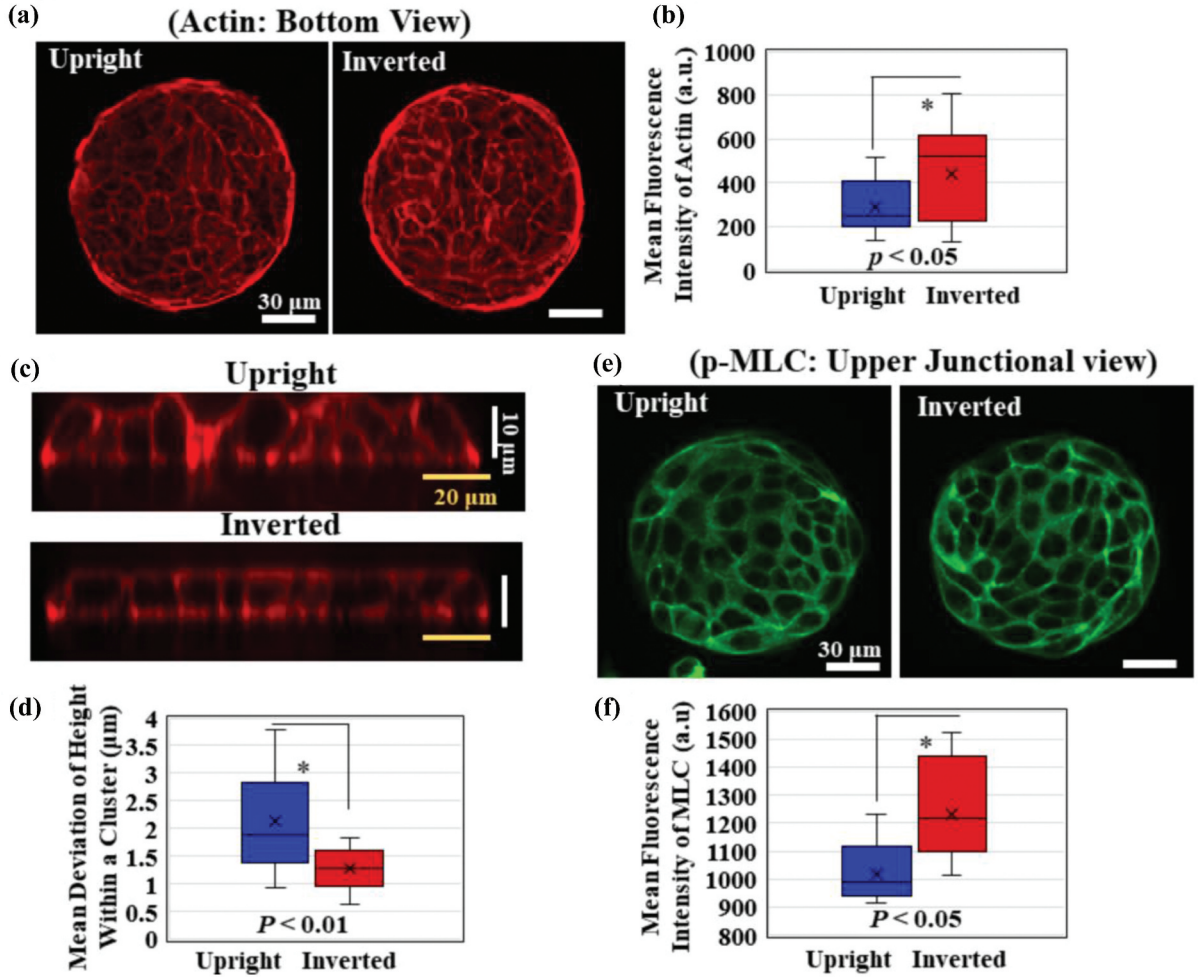

Figure 3. (a) Immunofluorescence (IF) images showing the actin (red) within the cellular clusters without release in the upright and inverted positions. (b) The calculated mean fluorescence intensity of peripheral actin bundles showing stronger actin bundles for cells in inverted position (N = 12–15 clusters). (c) Side view of cellular cytoskeletal actin (red) for clusters in upright and inverted positions using Z-stack images. (d) Roughness of cluster apical surfaces as calculated from the standard deviations between the cell's height within a cluster (N = 14–18 clusters). (e) IF images of p-MLC staining in the upper cell-cell junction. (f) The calculated mean fluorescence intensity of p-MLC distributed in the apical surface of cell-cell junction (N = 4–7 clusters with total measured cells = 199–406).

Next, we examined the shapes of the cell clusters by creating an orthogonal view of the confined clusters using confocal Z-stack photos. From the side view of multi-cell clusters, we observed differences in the 3D cluster architecture in the upright position compared to the inverted position (Figure 3(c)). Most clusters cultured in the upright position had top surfaces with disheveled appearances within the cluster. Additionally, many cells in an upright position have barrel- or pot-like shapes. In contrast, cells cultured in the inverted position had more cubical or trapezoidal morphologies with strictly and neatly extended surfaces. To quantitatively evaluate such changes in the cluster architectures, we calculated the average cluster roughness by calculating the deviation between the heights of the cells within the cluster. The results showed that clusters in inverted positions had smoother apical surfaces than those in upright positions (Figure 3(d)). A recent paper has discussed that the flattening of cellular apical surfaces is solely dependent on the maturation of cell-cell cohesion, in contrast to immature disorganized apical surfaces resulting from the balance between cell-cell cohesion and cell-substrate adhesion [27]. Considering this, we could expect that the 3D architecture of clusters cultured against gravity experienced a higher level of intercellular tension compared to those in the upright position. This increased tension could cause the apical surface to flatten compared with the clusters in the upright position.

Myosin is the main protein responsible for cellular force production and is activated through the phosphorylation of its light chain, which indicates higher myosin activity [28]. The localization of actomyosin structures determine their contraction outcomes [29]; for instance, the higher accumulation of actomyosin in apical surface relative basal region involves in the 3D architecture rearrangement and invaginations of intestinal epithelium [30]. Therefore, we checked the distribution of the phosphorylated myosin light chain in the upper junctional region surfaces within the clusters. As expected, a higher localization of pMLC was found in the upper intercellular junctions within the clusters cultured in inverted positions compared to upright ones (Figure 3(e,f)). This active myosin causes the sliding of actin filaments connecting the intercellular junctions, which might result in smoothening of the cellular apical surfaces. These observed alterations in the spatial distribution and activity of actomyosin cytoskeletal systems are correlated to inversion against gravity, presumably due to the adaptation mechanism for cellular clusters against the gravity vector. Previously, Zang et al. [5] reported that single cells in the inverted configuration responded to the gravity vector through alteration in actin stress fiber expression and redistribution. Actin filaments act as tension-resistant filaments that is necessary to adapt nuclear translocation in coordination with other cytoskeletal systems [5]. This cytoskeletal network remodeling is necessary for the single cells to adapt against the gravity vector. On the other hand, our experiments monitored cells in a group in which the actin cytoskeleton not only connects the cells to substrates in the form of stress fibers but also maintains the mechanical balance through the distribution of force within cells through the cortical actin and stress fibers associated with cell cohesion proteins [31,32]. Therefore, in our case, the clusters as a whole are expected to respond to such changes. These responses include the strengthening of actin localization in the peripheral bundles and higher intercellular tension that causes flattening of the cluster apical surfaces.

3.4 Migratory characteristics and cluster morphological changes are correlated with actomyosin activity

Since we observed stronger peripheral actin bundles, altered cell cluster morphology, and accumulation of myosin in the upper cellular junctions in cells cultured in the inverted position, we next tried to weaken myosin activity using blebbistatin. Here, we chose a relatively moderate concentration (20 μM) to slightly decrease myosin contractility using blebbistatin, but did not disrupt it completely. This treatment allows the release of the actin that is assembled in the peripheral actomyosin bundles and makes it available for actin polymerization and lamellipodia extension, thereby driving cellular motility [33]. In addition, blebbistatin interferes with the activity of myosin, which we suggest causes flattening of cell apical surfaces [5]. To confirm the effect of blebbistatin treatment on peripheral actin bundle localization, we first performed an experiment against the confined clusters in the inverted and upright positions. Upon staining with actin, we observed a weakening of the peripheral actin external bundles (Figure 4(a)). When we quantified the external actin bundle fluorescence intensity upon treatment with blebbistatin, significant weakening was observed compared to the clusters without blebbistatin (Figure 4(b)). When we looked at the shape of the 3D cluster architecture from an orthogonal view, we observed the loss of coordinated cubic-like cellular structures with smooth apical surfaces (Figure 4(c)). A similar result was also reported in a more complex cellular sheet called the organ of Corti, (it is composed of an arranged mosaic line of outer hair cells followed by several lines of other cells

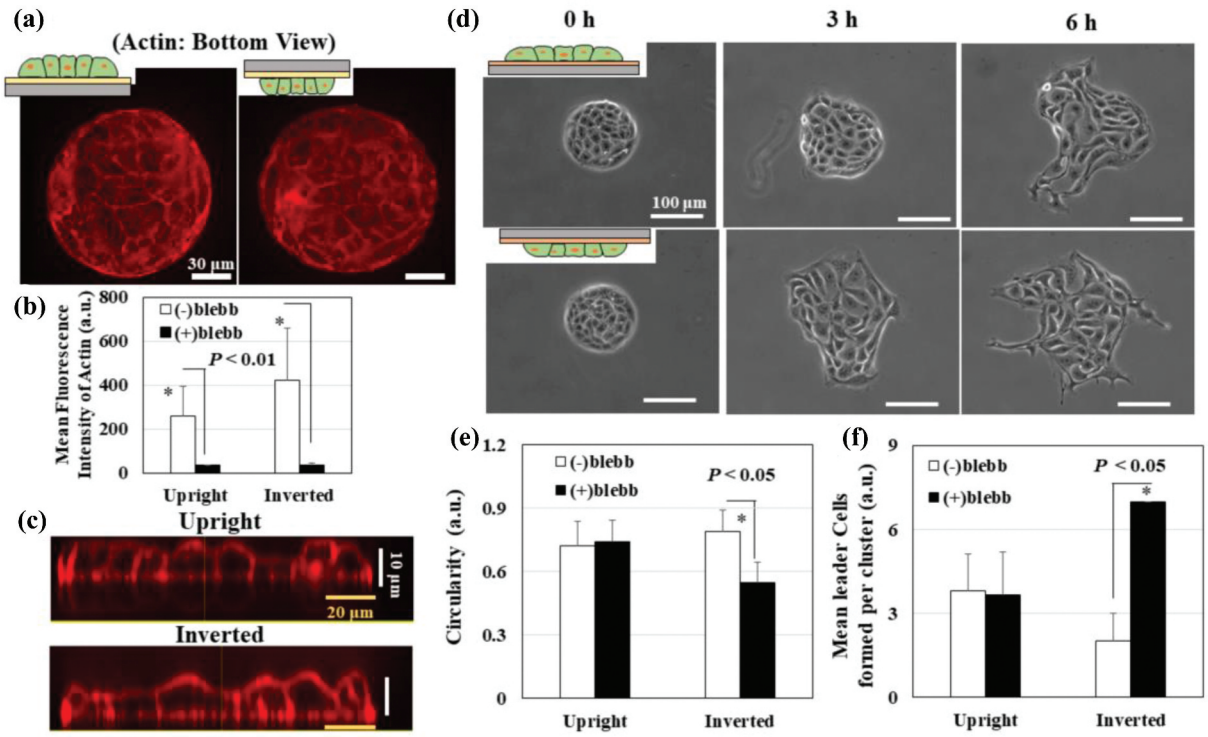

Figure 4. (a) Actin immunofluorescence staining of confined clusters after treatment with blebbistatin (b) Mean fluorescence intensity for the circumferential actin belt with and without blebbistatin treatment (c) Side views of confined clusters after blebbistatin treatment. (d) Phase contrast images showing the cluster expansion after blebbistatin treatment at different time point. (e) Mean calculated circularity of clusters with and without blebbistatin treatment after 5 h of cell migration (N = 3–24 clusters). (f) Mean leader cells calculated with and without blebbistatin treatment after 5 h of cell migration (N = 3–5 clusters).

as epithelial cells), in which the outer hair cells lose their native extended apical surface upon blocking myosin activity [34]. This suggests that the cluster rearrangement correlated with the inversion of cells against gravity is mainly due to the activity of myosin II in the upper junctional region. Next, we examined the migration of upright and inverted clusters. Figure 4(d) shows the phase-contrast images at several time points during migration in the presence of blebbistatin. The circularity of migrating clusters dramatically decreased in clusters cultured in inverted positions. At the same time, there was no significant decrease in the circularity of cells in the upright position upon treatment with blebbistatin at the same time point (5 h) (Figure 4(e)). When we compared the leader cell formation at 5 h of migration induction for the circular clusters with and without blebbistatin (supporting movie 2), we observed the same trend: the cellular clusters in inverted positions showed increased leader cell formation, whereas those in the upright position remained nearly the same as the blebbistatin treatment (Figure 4(f)). We expected that attenuating peripheral actomyosin bundles would result in the release of spatially restricted actin. Thus, free actin moieties would be utilized in lamellipodia extension and the appearance of leader cells, in a similar trend to the upright position; however, surprisingly, it showed a higher emergence of leader cells in the inverted positions than in the upright positions. A previous study discussed the enhanced activity of Arp2/3 upon inverting single cells against the gravity vector [6], even though it will not contribute to actin network branching and cell protrusions due to poor actin resources in the vicinity of the strong peripheral actomyosin bundles [33]. It is expected that the blebbistatin treatment released the actin molecules from the bundles and makes it available for actin polymerization and lamellipodia extension together with highly active Arp2/3, thereby stronger lamellipodial extension and leader cell formation were observed in the inverted position. The detailed molecular mechanisms behind this alteration in migratory phenotypes between upright and inverted would to be further explored in the future.

Finally, as described in Figure 5, we have shown that the gravity vector alters the cellular reorganization and actomyosin activity, these cytoskeletal remodeling include the formation of stronger peripheral actin bundles and flattened apical cluster surfaces. This cytoskeletal alteration has been associated with a delay in the emergence of leader cells. Therefore, manipulating actomyosin activity enhanced the formation of leader cells. This emphasized the significant role of cell contractility in the collective migration phenotypes and cytoskeletal remodeling depending on the gravity vector.

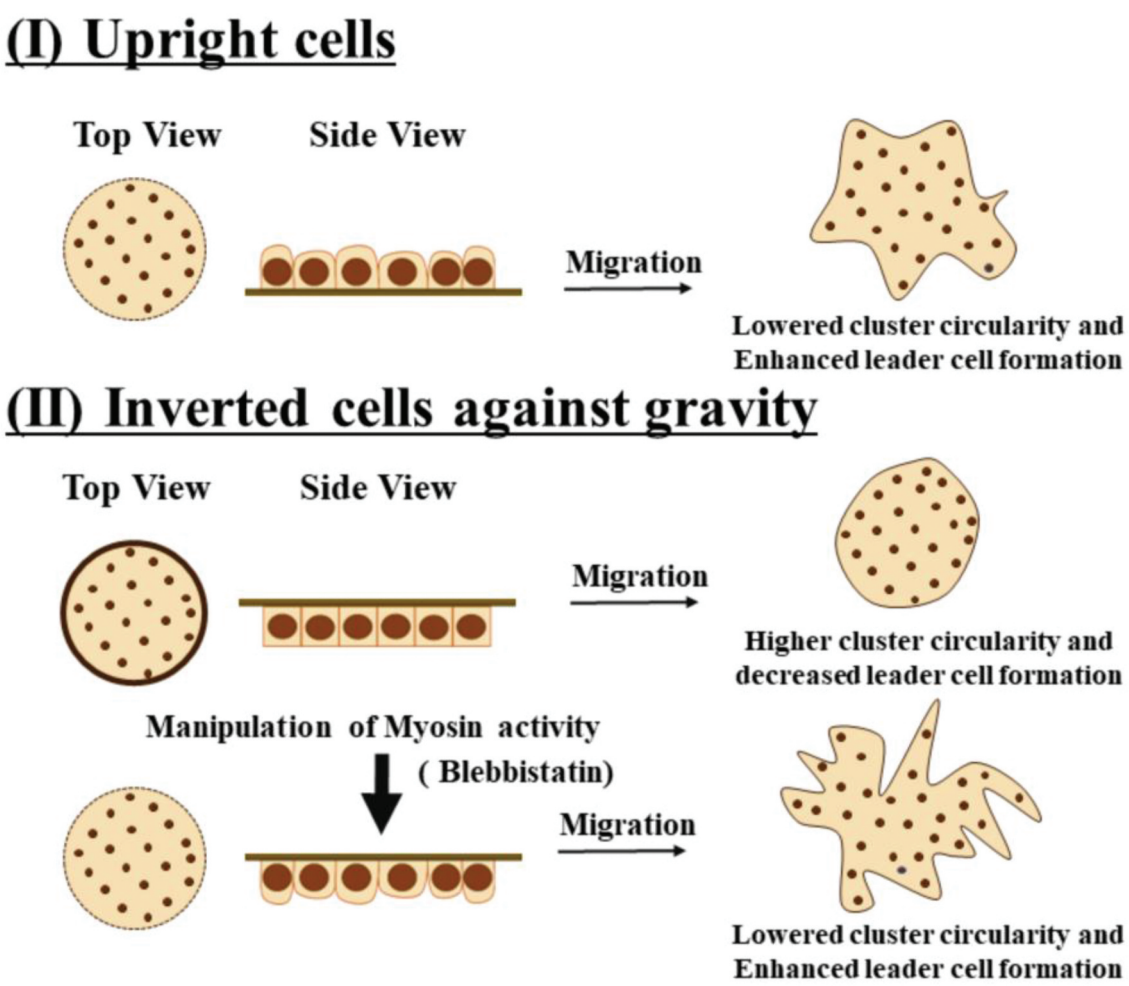

Figure 5. A scheme describing the observed cytoskeletal remodeling in cells cultured in upright positions compared to those cultured in inverted positions and the associated collective migratory phenotype; blocking of myosin contractility for cells cultured in inverted position abolishes the cytoskeletal remodeling and restores the highly motile phenotype.

Conclusions

Photoactivatable substrates were used to examine the effect of gravity vectors on the migration characteristics of MDCK cells. Compared to the conventional scratch wound healing assay or polydimethylsiloxane (PDMS) stencil method, where physical contact with the substrate surface is indispensable, photoactivatable substrates are more advantageous because they enable in situ and remote induction of migration by light from geometrically controlled clusters even in the upside-down configuration. The inversion of the substrate was associated with maintained cell cluster circularity compared to cells in the upright position, with less leader cell appearance. The decrease in expansion rate could be considered non-significant by the observed decrease in cell number and proliferation rate. Furthermore, the inversion of cellular clusters is associated with the reinforcement of peripheral actin bundles and the flattening of cluster apical surfaces. Manipulation of actomyosin activity using blebbistatin caused a significant increase in leader cell formation for cells in the inverted position and loss of flattened clusters. Therefore, gravity has shown a potential effect on migratory characteristics and leader cell formation in MDCK cells cultured on a cRGD-coated gold surface.

Disclosure statement

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