1. Introduction

Cell migration is a critical process both in physiological and pathological conditions involving immune response (Renkawitz and Sixt, 2010; Miyoshi et al., 2013), embryonic development (Bischoff and Cseresnyes, 2009; Scarpa and Mayor, 2016), the formation, maintenance, and regeneration of tissues (Lutolf and Hubbell, 2005), and cancer metastasis (Stoletov et al., 2010; Lu et al., 2012). Migrating cells in vivo encounter a complex extracellular environment and change their migratory behavior in response to extracellular environmental factors (Friedl and Wolf, 2010). Thus, it is important to identify the effects of extracellular environmental factors on cell migration to understand the mechanism of cell migration and how the migrating cells achieve their functions in complex environments in vivo. Furthermore, it is important from the biomedical engineering point of view to develop a strategy for the external control of cell migration.

Various extracellular environmental factors affect cell migration. In vitro assay with a simple synthetic extracellular
environment is useful to identify the effects of the huge numbers of the extracellular environmental factors. The effects of chemoattractants have long been studied and are well understood with the in vitro assays (Wu, 2005; Wang, 2009). In addition, a growing number of studies have focused on the chemical and physical properties of the insoluble extracellular matrix (ECM) (Ventre et al., 2012; Miyoshi and Adachi, 2014), such as the composition of the ECM protein, nano-/micro-topography of the ECM, and the stiffness of the ECM. Several experimental models of the extracellular environment have been developed to help understand the mechanism by which cells sense these factors in the ECM and change their migratory behavior accordingly.

For example, studies using a two-dimensional flat surface with chemically modified asymmetric cell adhesive and repulsive patterns (Jiang et al., 2005; Rolli et al., 2012), and the use of photolithographic microelasticity patterns on the flat surface of cell culture substrates (Kawano and Kidoaki, 2011; Ueki and Kidoaki, 2015), clearly demonstrated the mechanisms underlying guided cell migration. The effects of topographical features on cell migration have been extensively studied using cell culture substrates decorated with arrays of dots, pits, and grooves ranging from the sub-micrometer to micrometer scale (Bettinger et al., 2009; Miyoshi and Adachi, 2014). For cells interacting with these topographical substrates, the nano-/micro-topography on the substrate works as a confinement on the ventral cell surface but provide no confinement on the dorsal cell surface. Thus, surfaces with nano-/micro-dots, pits, and grooves are 2.5 dimensional environments. An unbranched microchannel (Mahmud et al., 2009; Wilson et al., 2013) can mimic a feature of the three dimensional environment, but it cannot be a model of the porous structures with “branches” often seen in in vivo extracellular environments.

In this study, we focused on the effect of a branched opening in a porous extracellular microstructure on cell migration. Porous structure with branching is typically seen in randomly organized collagen fibers in connective tissues such as epidermis, the provisional matrix after wounding and in loose primordial connective tissue (Wolf and Friedl, 2011). A feature size of spacing between the ECM fibers in the connective tissues is micrometer order (Stoitzner et al., 2002; Friedl et al., 2007). The branched opening in micrometer order is assumed to affect the shape of the cell–ECM interface, and then lead to remodeling of the actin cytoskeleton to maintain the mechanical homeostasis between the cell and ECM (Schwartz and Chen, 2013). We designed intersecting grooves with sizes of micrometer order, aiming at identifying the effect of the structural feature of the branched opening in micrometer order on the cellular organization of the actin cytoskeletal system including phosphorylated myosin, and cell migration. Fibroblasts are one type of cell inhabiting such extracellular environments (Friedl and Wolf, 2010), and thus Swiss3T3 fibroblasts were used as a model cell type for the analysis.

2. Materials and Methods

2.1 Cell Culture Substrate with Microgrooves

We fabricated a polydimethylsiloxane (PDMS, Sylgard 184 Silicone Elastomer, Dow Corning) cell culture substrate with two types of branched grooved structures. One was a lattice groove 5 μm wide, 13 μm deep, with grooves spaced 5 - 10 μm apart (Fig. 1a) and the other was a comb-like pattern with a major groove and branched sub-grooves of various sizes (Fig. 1b and c).

The PDMS substrates were made by replication of a mold with epoxy resin ridges (SU-8, MicroChem) on a silicon wafer. The finishing state of the SU-8 mold was inspected with a scanning electron microscope (JSM6330F, JEOL). The PDMS substrates were treated with oxygen plasma for 1 min to increase the hydrophilicity of the surface and then coated with fibronectin (20 μg/ml, BD Biosciences) for 1 hour.

2.2 Cells

A fibroblast cell line (Swiss3T3 Albino) was obtained from RIKEN BRC and cultured in MEMα (Gibco) containing fetal bovine serum (Sigma) and penicillin-streptomycin (100 units/ml, penicillin, and 100 μg/mL, Sigma).

2.3 Live Cell Microscopy

Fibroblasts were seeded on the lattice-type grooved PDMS and observed every 1 min using a 20×, 0.5NA Plan objective lens and a microscope (Ti-E, Nikon) equipped with a digital camera (DS-Fi2-U3, Nikon) at 37°C.
2.3 Fluorescent Staining

Fluorescent staining was performed by fixing the cells in 4% paraformaldehyde for 15 min. The cells were then washed and permeabilized with 0.2% Triton-X. For actin staining, the cells were washed several times and then incubated with Alexa Fluor 488 phalloidin (diluted 1:40, Invitrogen) for 1 hour. Otherwise, for actin and phospho-myosin light chain double staining, the cells were washed several times, blocked with 0.2% gelatin in PBS, then incubated with the first antibody (Phospho-Myosin Light Chain 2 (Ser19) Mouse mAb, diluted 1:400, Cell Signaling) for 1 hour, and then with Alexa 546-labeled anti-mouse IgG (diluted 1:400, Invitrogen) for 1 hour. For filamentous actin staining, the fixed and immunofluorescent-stained cells were incubated with Alexa Fluor 488 phalloidin (diluted 1:40) for 1 hour. Fixation and staining were conducted at room temperature.

2.4 Fluorescence Microscopy and Image Processing

A series of optical slice fluorescence images were acquired at a z interval of 0.41 μm using a confocal laser scanning microscope (FV1000, Olympus) with a SApo 60× silicone oil immersion objective lens (NA 1.3, Olympus). The slice images were projected along the z direction using the maximum intensity projection method. Cell outlines were detected based on the binarized image of the projected image of actin. ImageJ was used to process the series of images. For the analysis of the actin distribution in the in-depth direction, top face of the grooved structure was judged by sight and defined as z = 0.

3. Results

3.1 Fibroblast migration in a lattice-type groove

The migratory behavior of fibroblasts penetrating a lattice-type groove was investigated to better understand fibroblast migratory behavior in a three-dimensional environment. A lattice-type groove 5 μm wide, 13 μm deep, and spaced 10 μm apart, shown in Fig. 1a, was used for the analysis. We observed 8 cells. The fibroblasts penetrated into a concave rail consisting of the intersecting lattice groove. A representative fibroblast migratory behavior is shown in Fig. 2. Soon after seeding, the cell had a round shape and was positioned on the top surface of the square islands.
between the grooves \((t = 0 – 50\) min). Then, the cell body penetrated into the horizontal groove and started to migrate \((t = 100\) min). The outline of the cell followed the side wall of the groove \((t = 100 – 150\) min). Next, the front region of the cell moved forward and the rear of the cell gradually thinned and elongated \((t = 200 – 350\) min) until finally the trailing edge retracted into the cell body.

For all other cells tested, cytoplasmic protrusion was not limited to the direction of forward movement but rather frequently occurred into the groove normal to the direction of movement, as shown at \(t = 200\) min in Fig. 2. In some cases, the orthogonal-oriented cytoplasmic protrusion became dominant, and the direction of the migration changed to the orthogonal direction (data not shown). Additionally, thinning and elongation of the rear of the cell \((t = 200 – 400\) min in Fig. 2) was commonly observed in other cells tested. Cytoplasmic protrusion followed by trailing edge retraction indicates front protrusion-driven migration of the fibroblast in the lattice-type groove.

3.2 Actin organization in a fibroblast in a lattice-type groove

Previous studies have shown that the actin cytoskeleton contributes to cytoplasmic protrusion and trailing edge retraction in cells migrating on a flat surface (Ridley et al., 2003). Swiss3T3 fibroblast cells spread well on a flat PDMS substrate. In these fibroblasts, actin stress fibers were formed in the cytoplasm, and thin threadlike actin filaments, called filopodia, protrude normal to the cell outline (data not shown). The effect of intersecting branched grooves on fibroblast migration was evaluated by observing alterations in actin cytoskeleton distribution in the fibroblasts in a lattice-type groove.

As shown in Fig. 3, actin structures similar to those observed on a flat PDMS substrate were observed in the lattice-like grooved structures. Bundled actin filaments, similar to the stress fibers observed in fibroblasts on the flat PDMS substrate, were formed along the side wall to bridge a portion of the branch at the junction of the intersecting groove (e.g., red arrowheads in Fig. 3a). The XZ-projected image (Fig. 3d) showed that this actin bundle was formed away from the bottom of the groove and the YZ-projected image (Fig. 3b and c) showed that the actin filaments accumulated at the cell periphery. In addition, thin threadlike actin filaments, similar to the filopodia observed in fibroblasts on the flat PDMS substrate, were observed in cells in the lattice-like grooved structure. These protrusions extended into both grooves containing the cell body (e.g., blue arrow in Fig. 3a) and other branched grooves (e.g., blue arrow heads in Fig. 3a).

Taken together, the results show that a grooved structure can affect the orientation of actin stress fibers and the direction of filopodia protrusions.
Three dimensional cellular distribution of the actin cytoskeleton in fibroblasts in the lattice-type intersecting grooved structure. The groove width, spacing, and depth were 5 μm, 5 μm, and 13 μm, respectively. (a) XY projection image. The images acquired using a confocal microscope are projected in the depth direction. Scale bar, 10 μm. (b, c) YZ projection image along the vertical yellow dot lines in (a). (d) XZ projection image along the horizontal yellow dot line in (a). (b, c, d) The top face of the grooved structure was defined as z = 0 and the in-depth direction is indicated by the negative sign.

3.3 The effect of groove dimensions on cell shape and the formation of actin stress fibers and filopodia

Next, a comb-like grooved pattern with a major groove and branched sub-grooves with three different combinations of width, length, and spacing (Fig. 1b and c) was designed to investigate the effect of the groove dimensions, specifically groove width and groove spacing, on the cellular distribution of the actin cytoskeleton and resulting migratory behavior. The major groove was designed to guide cell body penetration, and the branched grooves were designed to guide cellular protrusion into these grooves. The cells exhibited more varied shapes than expected in these comb-like grooved structures. Therefore, the cell shapes were first classified and then the cellular organization of the actin stress fibers and filopodia protrusions for each type of cell was determined.

3.3.1 Cell shape classification

The cell shapes were classified into six categories based on visual observation: 1) “Major-Groove-type”, showing an elongated cell body in the major groove and little cytoplasmic protrusion into the branched sub-grooves; 2) “Balance-type”, showing penetration into both the major groove and the branched sub-grooves; 3) “Sub-Groove-Type”, showing an elongated cell body in the branched sub-groove; 4) “Rounded-type”, showing a spherical shape in the major groove; and 5) “Upper-type”, spreading on the top of the square islands surrounded by the groove; and 6) those unidentified.

The fibroblasts spread well on a flat PDMS surface and thick actin bundles were observed throughout the cell. Dot-like fluorescent areas of the phospho-myosin light chain were co-localized with the actin bundles (data not shown). Thick actin bundles co-localized with phosphorylated myosin are contractile stress fibers that drive retraction of the rear of migrating cells (Vallenius, 2013). In addition, thin actin protrusions with no phosphorylated myosin typical of filopodia (Jacquemet et al., 2015) were observed. We investigated how these actin cytoskeletal structures changed in fibroblasts in the comb-like grooved structures.

3.3.2 Major-Groove-type

A typical cellular distribution of the actin cytoskeleton and phosphorylated myosin in the Major-Groove-type cells is shown in Fig. 4.

Actin bundles, including phosphorylated myosin traversing the cell body, were observed in all the Major-Groove-type cells irrespective of the groove dimensions (4 cells for GRM=5, m=5, s=4, 2 cells for GRM=6, m=5, s=4, 1 cell for GRM=6, m=5, s=5). These actin bundles must be stress fibers, and the stress fibers were formed to bridge the
opening portion between the sub-grooves (e.g., red arrow head in Fig. 4d).

Another feature of Major-Groove-type fibroblasts was thin actin protrusions into the branched sub-grooves. As shown in the enlarged view in Fig. 4f-h, phosphorylated myosin was present at the base of the thin protrusions and was not present in the interior of the protrusions. This morphological feature indicates that the thin protrusions must be filopodia. Additionally, as shown in Fig. 4f-h, multiple filopodia extended from almost the same position into one sub-groove frequently. The formation of multiple filopodia from the same position observed in the Major Groove-type cells was not frequently observed in cells on a flat PDMS, indicating that the branched sub-groove helps promote filopodia formation compared with the flat PDMS surfaces.

![Fig. 4](image)

Fig. 4 Cellular distribution of the actin cytoskeleton and the phosphorylated myosin in a Major-groove type cell in the $GR_{w=5, s=4}$ Comb-type intersecting grooves. (a) Binary image of the cell. The position of the major groove is indicated by magenta, and that of the ridge between the sub-grooves is indicated by blue. (b) Actin fluorescence image. (c) Phosphorylated myosin light chain fluorescence image. (d) Merged image of the fluorescence image of the actin filament (green) and that of the phosphorylated myosin light chain (red). (e) Differential interference contrast microscope image. Scale bar in (e): 10 µm. (f, g, h) Enlarged images of the boxed areas in (b, c, d). Scale bar in (h): 1 µm.

### 3.3.3 Balance-type

A typical cellular distribution of the actin cytoskeleton and phosphorylated myosin in the Balance-type cells is shown in Fig. 5.

Actin bundles, including phosphorylated myosin traversing the cell body, were observed in 79% of Balance-type cells (9 of the 11 cells for $GR_{M=5, w=5, s=4}$, 4 of the 5 cells for $GR_{M=6, w=5, s=4}$, 2 of the 3 cells for $GR_{M=6, w=5, s=5}$). These actin cytoskeletal structures must be stress fibers. The stress fibers crossed the major groove, as shown in the red arrow in Fig. 5d. In other cases, the stress fibers in the balance-type cells sometimes formed along the long axis of the major groove to bridge the opening at the junction of the sub-grooves, similar to that observed for the Major-Groove-type

![Fig. 5](image)

Fig. 5 Cellular distribution of the actin cytoskeleton and the phosphorylated myosin in a Balance-type cell in the $GR_{w=6}$ $w=5, s=4$ comb-type intersecting grooves. (a) Binary image of the cell. The position of the major groove is indicated by magenta, and that of the ridge between the sub-grooves is indicated by blue. (b) Actin fluorescence image. (c) Phosphorylated myosin light chain fluorescence image. (d) Merged image of the fluorescence image of the actin filament (green) and that of the phosphorylated myosin light chain (red). (e) Differential interference contrast microscope image. Scale bar, 10 µm.
cells (data not shown).

The thin protrusions lacking phosphorylated myosin (blue arrowhead in Fig. 5d) were filopodia and were observed in 84% of cells (11 of the 11 cells for $GR_{M=5, w=5, s=4}$, 3 of the 5 cells for $GR_{M=6, w=5, s=4}$, 2 of the 3 cells for $GR_{M=6, w=5, s=5}$). The filopodia protruded into both the major groove and the sub-grooves. In some cases, multiple filopodia protruded into one major or one sub-groove (data not shown).

The stress fibers and filopodia did not necessarily form concomitantly in Balance-type cells, suggesting that the formations of the stress fibers and filopodia are independent events.

### 3.3.4 Sub-Groove-Type

Two typical cellular distributions of the actin cytoskeleton and phosphorylated myosin in the Sub-Groove-type cells are shown in Fig. 6. As shown in Fig. 6a - e, actin bundles, including phosphorylated myosin traversing the cell body (red arrow in Fig. 6d), were observed in 44% of the Sub-Groove-type cells (4 of the 7 cells for $GR_{M=5, w=5, s=4}$, none of the 2 cells for $GR_{M=6, w=5, s=5}$). These actin cytoskeletal structures must be stress fibers. Bundled actin filaments did not form in the other cells (Fig. 6f – i). The filopodia formation were not usual in the Sub-Groove-type cells (3 of the 7 cells for $GR_{M=5, w=5, s=4}$, none of the 2 cells for $GR_{M=6, w=5, s=5}$).

![Fig. 6](image_url)

**Fig. 6** Cellular distributions of the actin cytoskeleton and the phosphorylated myosin in two representative Sub-Groove-type cells in the $GR_{W=6, m=5, s=4}$ comb-type intersecting grooves. (a, f) Binary images of the cells. The position of the major groove is indicated by magenta, and that of the ridge between the sub-grooves is indicated by blue. (b, g) Actin fluorescence images. (c, h) Phosphorylated myosin light chain fluorescence images. (d, i) Merged image of the fluorescence images of the actin filament (green) and that of the phosphorylated myosin light chain (red). (e, j) Differential interference contrast microscope images. Scale bars, 10 μm.

### 3.3.5 Rounded-type

A typical cellular distribution of the actin cytoskeleton and phosphorylated myosin in the Rounded-type cells is shown in Fig. 7. Actin bundles, including phosphorylated myosin, were not observed in 78% of the Rounded-type cells, and filopodia formation was rare (19%). Instead, the actin filaments accumulated in the cell cortex and co-localized with phosphorylated myosin.

![Fig. 7](image_url)

**Fig. 7** Cellular distribution of the actin cytoskeleton and the phosphorylated myosin in a Round-type cell in the $GR_{W=6, m=5, s=5}$ comb-type intersecting grooves. (a) Binary image of the cell. The position of the major groove is indicated by magenta. (b) Actin fluorescence image. (c) Phosphorylated myosin light chain fluorescence image. (d) Merged image of the fluorescence image of the actin filament (green) and that of the phosphorylated myosin light chain (red). (e) Differential interference contrast microscope image. Scale bar, 10 μm.

### 3.3.6 Upper-type
A typical cellular distribution of the actin cytoskeleton and phosphorylated myosin in the Upper-type cells is shown in Fig. 8. The actin filaments accumulated along the lateral side of the major and sub-grooves. Based on the z-sliced images, actin accumulated in the in-depth direction up to the cell periphery (data not shown), and this accumulation is likely due to a lamellipodia-like structure formed at the leading edge of the cell extending in the depth direction. This observation suggests that the penetration of the fibroblasts into a concave rail consisting of the intersecting lattice groove was not result from just falling down of the cell body, but spontaneous cell migration.

![Fig. 8](image)

**Fig. 8** Cellular distribution of the actin cytoskeleton and the phosphorylated myosin in an Upper-type cell in the $GR_{w=5, \, w=5, \, s=4}$ comb-type intersecting grooves. (a) Binary image of the cell. The position of the major groove is indicated by magenta, and that of the ridge between the sub-grooves is indicated by blue. (b) Actin fluorescence image. (c) Phosphorylated myosin light chain fluorescence image. (d) Merged image of the fluorescence image of the actin filament (green) and that of the phosphorylated myosin light chain (red). (e) Differential interference contrast microscope image. Scale bar, 10 μm.

### 3.3.7 Dependence of cytoplasmic protrusion into the groove on the groove dimensions

In Fig. 9, cell shape in comb-type intersecting grooves, depending on the groove dimension is summarized. The comb-like grooved structure guided cell body penetration into the groove for 70% of the cells ($N = 86$), as intended. On the other hand, the body of the remaining 30% of the cells (all 15 of the Upper-type cells and 11 of the unidentified cells) were present on the top surface of the square islands surrounded by the grooves, contrary to expectation.

Of the 70% of cells that penetrated the grooves, the shape adopted by these penetrating cells differed depending on the dimensions of the comb-like grooves. The cell shape in the $GR_{W=6, \, w=5, \, s=4}$ grooves was dominated by Balance-type cells co-existing with Major-Groove-type and Sub-Groove-type cells. This result indicates that the major groove and the sub-grooves, both of which were 5 μm wide, were equally effective in guiding filopodia protrusion and the resulting cytoplasmic penetration into the groove. Taken together with the result that cytoplasmic penetration into 5 μm wide grooves was observed with the lattice-type grooved structures (Fig. 2, Fig. 3), the protrusive activity of the fibroblasts into the 5 μm-groove remained unchanged even though the groove spacing was changed from 10 μm, 5 μm to 4 μm. Actin stress fibers formed in the penetrated cytoplasm and traversed the cell body. These stress fibers bridged the opening at the junction of the intersecting grooves (Fig. 4, Fig. 5).

The $GR_{W=6, \, w=5, \, s=4}$ grooves had a 1 μm wider major groove than the $GR_{W=5, \, w=5, \, s=4}$ groove. Balance-type cells were still observed but the Sub-Groove-type cells were replaced with Rounded-type cells. Comparison of the results obtained for $GR_{W=6, \, w=5, \, s=4}$ with those for $GR_{W=5, \, w=5, \, s=4}$ indicated decreased cytoplasmic protrusion activity into the sub-groove when the major groove was 6 μm wide.

The $GR_{W=6, \, w=5, \, s=4}$ grooves had the same major and sub-groove dimensions as those of $GR_{W=6, \, w=5, \, s=4}$ grooves, but a 1 μm larger spacing between the sub-grooves. The number of the fibroblast movement into the former grooves increased compared to the latter. However, the number of Balance-type and Major-Groove-type cells was decreased and Rounded-type cells were dominant, indicating decreased cytoplasmic protrusion activity into the major and sub-grooves.
4. Discussion
In this study, we designed a model microenvironment comprising a feature of a complex porous structure with branched openings. We fabricated a cell culture substrate with intersecting grooves whose size allowed individual Swiss3T3 fibroblasts to penetrate into the groove. The fibroblasts exhibited guided migration inside a concave rail consisting of the intersecting lattice grooves that were 5 μm wide, 13 μm deep and spaced 10 μm apart. The actin cytoskeleton and phosphorylated myosin light chain are keys for generating the force required to drive cell migration (Stoitzner, Pfäffler et al., 2002; Friedl, Wolf et al., 2007; Schwartz and Chen, 2013). Thus, to understand the mechanism underlying guided migration, the effects of the branched grooved structure on the cellular organization of the actin filaments and phosphorylated myosin light chain were analyzed. Furthermore, we investigated how strengthen of the effect is altered depending on the groove width and spacing.

4.1 The effects of the intersecting grooved structure on fibroblast migration
Based on the observation of the actin and phosphorylated myosin organization in the Swiss3T3 fibroblasts in the lattice type groove with 5 μm in width and 5 – 10 μm in spacing (Fig. 3), and in the $GR_{w=5, s=4}$ groove of the comb types (Fig. 4), the effects of the intersecting branched grooves to guide fibroblast migration are: 1) enhancement of filopodia protrusion into the groove; and 2) guidance in orienting the stress fibers to bridge the opening of the junction of the intersecting grooves.

An earlier study into the mechanism underlying filopodia protrusion indicated that collisions of the barbed ends of actin filaments lead to gradual clustering of the barbed ends, resulting in multimerization of the associated tip complexes and convergence of the filaments to form a filopodium (Svitkina et al., 2003). The same mechanism would hold for filopodia formation in the grooved structure. In the intersecting groove, the membrane is unconstrained at the groove junctions, allowing membrane protrusion only at these sites. This would increase the frequency of collisions of the barbed ends in the cytoplasm just under the unconstrained area of cell membrane, thus leading to multiple filopodia protrusions.

Stress fiber formation is likely initiated by the contact between the cytoplasm and the side walls of the groove. Then, the increase in actin bundling at the unconstrained edge at the opening at the groove junction increases the contractility of the stress fibers, and determines the orientation of the stress fibers inside the cytoplasm occupying the intersecting grooved structure. This increase in the contractility of the stress fibers in the adhesion-free edge was previously demonstrated using a two-dimensional micro-patterned cell culture substrate (Thery et al., 2006).

The strength of the effects of the intersecting grooved structure on filopodia protrusion and orientation of the stress fibers changed depending on the groove dimensions (Fig. 9). Efficient cytoplasmic protrusion was observed in intersecting grooves 5 μm wide and 4 μm apart arranged in a comb-like structure (Fig. 4, $GR_{w=5, s=4}$), and 5 μm wide and 5 μm or 10 μm apart in a lattice arrangement.

The efficiency of cytoplasmic protrusion into a sub-groove decreased when the width of the major groove increased by 1 μm ($GR_{w=6, s=4}$). The main cause of this decrease will not be a decrease in filopodia protrusion but rather a decrease in cytoplasmic protrusion, since filopodia protrusion into the sub-groove was observed in both Major-Groove-type and Sub-Groove-type cells in the $GR_{w=6, s=4}$ comb-like structure.
cells in intersecting grooves with a wider, 6 \( \mu m \) major groove, was greater in proportion to the whole cell compared with that of cells in the 5 \( \mu m \)-major groove. Consequently, higher contractile force in the cortex of the cell body in the major groove might be required for cytoplasmic protrusion into the branched sub-groove. A decrease in the efficiency of cytoplasmic protrusion into the sub-groove was also observed in another intersecting grooved structure with a 6 \( \mu m \)-major groove (\( GR_{w=6, w=5, s=1} \)). The decrease in efficiency in the \( GR_{w=6, w=5, s=1} \) structure was more significant than in \( GR_{w=6, w=5, s=4} \), and the majority of cells were Rounded-type.

The corner at the groove junction of the comb-type grooves was more rounded than that of the lattice-type. In our experimental condition, both lattice-type and comb type had the same effect to enhance the filopodia protrusion regardless of the difference in the shape of the corner. However, in other cases, the difference in the shape of the corner can change the effect to guide filopodia protrusion, probably due to affecting the accumulation of the focal adhesions at the corner. Indeed, a study by using shallow V-shaped grating patterns with a sharp corner on the PDMS substrate has demonstrated that the number and the size of the focal adhesions in MC3T3-E1 osteoblast increase at the bending tip, which leads to change in the direction of migration at the tip (Zhou et al., 2016).

Taken together, our analysis demonstrated that the intersecting branched grooves with 5 \( \mu m \) in width had the effects to enhance cytoplasmic protrusion into the groove, and to guide the orientation of the stress fibers to bridge the opening of the junction of the intersecting grooves. The strength of the effect was unchanged, regardless of the groove spacing within the range from 4 \( \mu m \) to 10 \( \mu m \). On the other hand, guiding effectiveness of the cytoplasmic protrusion and stress fiber formation were suppressed in the intersecting branched grooves with a wider major groove with 6 \( \mu m \). In these grooves, the mode of cell migration seemed to change from mesenchymal type to pseudopodial amoeboid type (Friedl and Wolf, 2010). The reason for this remains to be clarified and is an important topic for future investigations aimed at understanding the extracellular environmental determinants of cell migration, and how the mode of cell migration is tuned by the three-dimensional extracellular microenvironment in vivo.

4.2 Proposed mechanism of fibroblast migration in an intersecting grooved structure

In Fig. 10, we propose a hypothesis on the mechanism of the fibroblast migration guided by the intersecting branched groove with a dimension that effectively enhances cytoplasmic protrusion into the groove, and guides the orientation of the stress fibers. Observation of Upper-type cells (Fig. 8) suggests that, after seeding, the cells migrate toward the bottom of the groove by protruding lamellipodia-like leading edge into the groove (Fig. 10b). Inside the groove, the fibroblast laterally protruded filopodia into the groove, as seen in Major-Groove-type (Fig. 4), Balance-type, and Sub-Groove-type cells. In some cases, multiple filopodia protruded from almost the same points into a groove (Fig. 4f - h). Multiple filopodia protrusion was not commonly observed in fibroblasts on a flat PDMS surface and indicates that filopodia protrusion was enhanced by the groove. In the penetrated cell body, stress fibers form along the side wall of the groove (Fig. 10c). The stress fibers tended to thicken to bridge the opening of the junction of the filamentous.

![Proposed mechanism for the migration of a fibroblast in the intersecting grooved structure.](image)

**Fig. 10** Proposed mechanism for the migration of a fibroblast in the intersecting grooved structure. (a) Just after seeding, the cells are present on the top of the grooved structure. Then, (b) the leading edge of the cell protrudes along the side wall of the groove. (c) Filopodia and stress fibers are formed in a guided manner inside the groove. Filopodia formation is enhanced by a micro-groove. Stress fibers form along the side wall of the groove. (d, e) The filopodia pioneer the path followed by the cytoplasmic protrusion. Some of the filopodia retract. Some stress fibers increase the thickness to bridge the opening of the junction of the intersecting groove. Cytoplasmic protrusion and stress fiber contraction lead to displacement of the cell body. (f) New filopodia and stress fiber are formed in the protruded cytoplasm in a guided manner, and cell migration enter a new cycle.
Filopodia are known to probe the microenvironment and may grow into cellular protrusions (Mattila and Lappalainen, 2008; Mattila and Lappalainen, 2008). Consistent with this, the filopodia of fibroblasts in the intersecting grooves grew into cellular protrusion, whereas others retracted into the cell body (Fig. 10c - e). Concomitantly, contraction of the stress fibers drives the retraction of the rear of the cell and promotes the cytoplasmic protrusion (Fig. 10d - e). Filopodia and stress fibers newly form in the translocated cell (Fig. 10f). In this cycle, Swiss3T3 fibroblasts could effectively migrate, guided by the grooves.

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