Left-Right Symmetry Breaking in Tissue Morphogenesis via Cytoskeletal Mechanics

Ting-Hsuan Chen, Jeffrey J. Hsu, Xin Zhao, Chunyan Guo, Margaret N. Wong, Yi Huang, Zongwei Li, Alan Garfinkel, Chih-Ming Ho, Yin Tintut, Linda L. Demer

Rationale: Left-right (LR) asymmetry is ubiquitous in animal development. Cytoskeletal chirality was recently reported to specify LR asymmetry in embryogenesis, suggesting that LR asymmetry in tissue morphogenesis is coordinated by single- or multi-cell organizers. Thus, to organize LR asymmetry at multiscale levels of morphogenesis, cells with chirality must also be present in adequate numbers. However, observation of LR asymmetry is rarely reported in cultured cells.

Objectives: Using cultured vascular mesenchymal cells, we tested whether LR asymmetry occurs at the single cell level and in self-organized multicellular structures.

Methods and Results: Using micropatterning, immunofluorescence revealed that adult vascular cells polarized rightward and accumulated stress fibers at an unbiased mechanical interface between adhesive and nonadhesive substrates. Green fluorescent protein transfection revealed that the cells each turned rightward at the interface, aligning into a coherent orientation at 20° relative to the interface axis at confluence. During the subsequent aggregation stage, time-lapse videomicroscopy showed that cells migrated along the same 20° angle into neighboring aggregates, resulting in a macroscale structure with LR asymmetry as parallel, diagonal stripes evenly spaced across the culture. Removal of substrate interface by shadow mask-plating, or inhibition of Rho kinase or nonmuscle myosin attenuated stress fiber accumulation and abrogated LR asymmetry of both single-cell polarity and multicellular coherence, suggesting that the interface triggers asymmetry via cytoskeletal mechanics. Examination of other cell types suggests that LR asymmetry is cell-type specific.

Conclusions: Our results show that adult stem cells retain inherent LR asymmetry that elicits de novo macroscale tissue morphogenesis, indicating that mechanical induction is required for cellular LR specification. (Circ Res. 2012;110:551-559.)

Key Words: adult stem cells ■ cell culture ■ development ■ migration ■ morphogenesis

Left-right (LR) asymmetry often occurs in embryonic and tissue morphogenesis, such as in helices of snail shells and internal organs in most extant metazoan animals. In some vertebrates, LR asymmetry is believed to be generated by motile cilia, whose beating generates a leftward fluid flow that step up downstream morphogen gradients. Another model is that LR asymmetry is motivated by intracellular events originating by cytoplasmic organization at an even earlier stage. Recently, cytoskeletal chirality and planar cell polarity were shown to specify the LR axis at early embryogenesis, suggesting that LR asymmetry is coordinated by single- or multi-cell organizers and propagates through the rest of the tissue architecture, as evidenced by epithelial cell chirality underlying the hindgut rotation and chiral movement of blastomeres. Thus, to organize LR asymmetry at multiscale levels of morphogenesis, cells with chirality must also be present in adequate numbers. However, although LR asymmetry is ubiquitous, from whole body and visceral handedness to myocyte fiber orientation, chiral behavior from nonembryonic cells after enzymatic isolation in culture has been seen rarely in past decades.

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To investigate such LR asymmetry and cellular chirality, we cultured vascular mesenchymal cells (VMCs) on a micropatterned substrate. VMCs differentiate and self-
organize into periodic multicellular aggregates resembling patterns in normal tissue architecture. We report that, on migration across an interface between adhesive and non-adhesive substrates in culture, VMCs preferentially turn right and self-organize into periodic multicellular structures with remarkably consistent alignment on the principal diagonal axis. This de novo LR asymmetry in nonembryonic cells required stress fiber accumulation on encountering the substrate interface, suggesting that mechanical induction, possibly resembling contextual instruction from native tissue architectures, is required to regain chirality in culture for morphogenesis after cells are enzymatically isolated from their native tissue.

Methods

Micropatterning

A glass substrate with hexamethyldisilazane and polyethylene glycol (PEG) substrate was prepared. Prior to plating cells, the hexamethyldisilazane/PEG substrates were first incubated with solutions of extracellular matrix, consisting of fibronectin (FN), collagen types I and IV, or laminin-1, to allow adsorption on hexamethyldisilazane. The protein-coated chip was then plated with VMCs, bovine vascular endothelial cells (BVECs), NIH 3T3 fibroblast (3T3), or mouse bone marrow stromal cell line (ST2) with brief washings such that only cells adhering to the extracellular matrix regions remained.

Shadow-Mask Plating

The mask was made of stainless steel (2 cm × 2 cm × 100 μm, NW Etch, WA) containing 25 parallel windows (300 μm × 1.5 cm) spaced 300 μm apart. Prior to plating, the tissue culture dish was first uniformly coated with FN solution. Thereafter, cells were plated through the mask followed by removal of the shadow mask.

Cell Culture

VMCs, BVECs, and 3T3 cells were isolated and cultured as described and ST2 cells were commercially purchased (Cell Bank, Riken Bioresource Center, Japan). All cells were grown in Dulbecco’s Modified Eagle’s Medium supplemented with 15% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin (10,000 IU/10,000 μg/mL; all from Mediatech, VA). Cells were incubated at 37°C in a humidified incubator (5% CO₂ and 95% air) and passaged every 3 days.

Multicellular Pattern Formation

Each culture was prepared on 1 of 4 substrates: 1) 35-mm plastic dishes, 2) binary substrates consisting of PEG alternating with purified extracellular matrix protein (FN, collagen I, collagen IV, or laminin-1), 3) uniformly coated FN substrate on chips with titanium markers on the reverse side of chip, or 4) shadow-mask plating as described elsewhere in methods, with media changes every 3 days. For inhibition of stress fibers or intervention of morphogen activities, Y27632, blebbistatin, bone morphogenetic protein-2 (BMP-2), noggin, and warfarin were added at day 0 and replenished with each media change. After 10 to 14 days, cultures were stained with hematoxylin to reveal multicellular aggregates.

Green Fluorescent Protein Transfection

VMCs were transfected with a plasmid encoding green fluorescent protein (pmaxGFP; Amaxa Biosystems, Germany) using the Effectene Transfection Reagent (Qiagen, CA).

Time-Lapse Videomicroscopy

Cultures were incubated in a microscopic thermal stage (HCS60, Instec, CO) at 37°C and continuously supplied with premixed 5% CO₂. Images were acquired at 5 minutes intervals for a total of 22.5 hours using charge-coupled devices and an inverted microscope in bright field.

Immunofluorescent Staining

Cells were cultured on FN/PEG substrates or shadow-mask plated in normal growth medium, or on FN/PEG substrates in growth medium supplemented with Y27632 or blebbistatin. Cells were fixed in cold methanol, blocked with Image-iT™ FX signal enhancer (Invitrogen, CA) at room temperature, then labeled with monoclonal anti-α-tubulin-FTTC antibody (Sigma-Aldrich, St. Louis, MO; 1:50; for polarity assay on VMCs and BVECs), pericentrin polyclonal antibody (Covance, CA; 1:500; for polarity assay on 3T3 cells and ST2 cells), or nonmuscle myosin-IIa (NMM-IIa) antibody (Covance, CA; 1:1000; for stress fiber distribution on all cell types) at room temperature for 1 hour. The pericentrin and NMM-IIa antibodies were subsequently labeled with appropriate secondary antibodies. Samples were mounted with DAPI. For stress-fiber distribution of NMM-IIa, images were stacked using etched microgrooves on the reverse surface for registration. For shadow-mask plating experiments, images were stacked using automated edge detection for registration.

Orientation Analysis

Images were processed by segmentation and boundary tracing to allow the identification of cells based on the size of the closed-loop regions. The orientation angle was calculated by the method of “minimum circumscribed rectangle” in which the shape of a given cell can be approximated by circumscribing rectangles of different sizes. The orientation angle, θ, was defined as the longitudinal axis of the rectangle of minimal area relative to the horizontal axis of the image (and of the interface).

Stacking Images of Immunofluorescence NMM-IIa After Shadow-Mask Plating

Images were processed by segmentation and boundary tracing to binary the image and identify the sheet of cells by excluding small closed-loop regions. We selected a level of 300 bright pixels per row as the threshold to identify the edge of the cell sheet. The midline of the cell sheet, defined as equidistant between 2 edges, was used to register and stack the images.

Mathematical Model

See online-only Data Supplement.

Results

LR Asymmetry in Pattern Formation of VMCs

VMCs, when homogeneously plated on conventional tissue-culture plastic, aligned locally with their neighbors at confluence (Figure 1A). After 2 weeks, they differentiated and self-organized into periodic aggregates in labyrinthine configurations...
as seen in reaction-diffusion phenomena (Figure 1C). To test effects of initial plating distribution, we microengineered glass substrates with functional surfaces composed of alternating stripes (300 μm-wide) of cell-adhesive FN and nonadhesive PEG (Figure 1B; black stripes=titanium lines on reverse side indicating FN/PEG interfaces) as described previously. After 1 week, cells spread beyond the FN onto the PEG regions toward confluence. After 10 to 14 days, instead of the expected labyrinthine pattern, cells aggregated into long, straight, and parallel ridges, consistently aligned on a principal diagonal relative to the interfaces—a striking, unidirectional LR asymmetry (Figure 1D). Of note, the formation of ridges with LR asymmetrical alignment was independent of passage number for P16 to P26. For example, Online Figure IE and IF, and Figure 1D are from 3 different passages. It was also unaffected by treatments with BMP-2, noggin (BMP-2 inhibitor), or warfarin (inhibitor of matrix γ-carboxyglutamic acid protein) (Online Figure IA to ID). Furthermore, it was unaffected by substituting other cell-adhesive substrata in lieu of FN (Online Figure II).

Coherent Single-Cell Orientation Perpendicular to the Axis of Diagonal Ridges Directing the Subsequent Migration Toward Aggregates

To study earlier stages of this novel asymmetry, individual VMCs were tracked by green fluorescent protein transfected at low efficiency. At confluence, cells oriented coherently and consistently in an antidiagonal direction. This angle of cell orientation, , was defined relative to the titanium lines (Figure 2A). From days 2 to 5, mean increased and its standard deviation decreased (6°±30°→19°±14°, Figure 2C and Online Figure III), indicating a coherent orientation toward an angle perpendicular to the alignment of multicellular ridges (90°±90°≈110°, Figure 1D). This cell orientation appeared earlier on FN then later on PEG, ultimately occurring throughout the plate (Online Figure IV). On plates with wider (300–600 μm) FN/PEG stripes, cells also oriented coherently with the same angle (Online Figure V). In contrast, on uniform FN substrates, cells oriented randomly (Figure 2B and 2D). Thus, the coherent orientation of individual cells perpendicular to multicellular ridges seems to be dependent on the presence of FN/PEG interface but independent of the interface spacing.

In addition, to assess tightness of the control of cell orientation by interface angle, we created 2 orthogonal sets of interfaces on the same chip (Online Figure VIA and VIB) and found that multicellular aggregates aligned in a stripe pattern at 110° relative to the local interfaces up to a transition zone (~300 μm wide) at the junction, where cell alignment smoothly curved and nodules arose (Online Figure VIC and VID). These results indicate the importance of substrate interface orientation.

To test whether the coherent orientation contributes to ridge formation, time-lapse videomicroscopy was performed at confluence to capture the time course of LR alignment. Cells migrated toward discrete aggregates preferentially following the coherent orientation , and gradually formed parallel ridges aligned at 90° relative to the local interfaces up to a transition zone (~300 μm wide) at the junction, where cell alignment smoothly curved and nodules arose (Online Figure IVC and VID). These results indicate the importance of substrate interface orientation.

Figure 1. Left-right (LR) asymmetry in pattern formation by vascular mesenchymal cells (VMCs). Phase contrast microscopy of VMCs on (A) conventional plastic substrate at confluence and (B) fibronectin (FN)/polyethylene glycol (PEG) substrates (interfaces identified by black titanium lines) showing preferential attachment to FN domain immediately after plating. Scale bar, 300 μm and 200 μm (inset). After 10 to 14 days, development of regularly spaced aggregates (C) in a labyrinthine configuration and (D) in a stripe pattern along principal diagonal axis in bright field (multicellular ridges stained with hematoxylin). Insets: higher magnification images of multicellular aggregates. Scale bar, 2 mm and 300 μm (inset).
for cells on collagen IV (orientation angle: collagen IV, 22±7°; collagen I, 25±15°; laminin-1, 28±17°; mean ± SD, n >500 each), as demonstrated by its smaller standard deviation.

To study how reaction-diffusion together with preferential migration could give rise to parallel ridges with asymmetrical alignment, we developed a mathematical model. The activities of a slowly-diffusing activator, BMP-2, u, its rapidly-diffusing inhibitor, matrix γ-carboxyglutamic acid protein, v, and cell density, n, reflecting proliferation, cytokinetic diffusion and chemotactic migration toward activators, were modeled as functions of a 2-dimensional domain (x, y) following reaction-diffusion kinetics14,18,19:

\[
\begin{align*}
\frac{\partial u}{\partial t} &= D \nabla^2 u + \gamma \left( \frac{nu^2}{v(1+ku)} - cu \right) \\
\frac{\partial v}{\partial t} &= \nabla^2 v + \gamma (nu^2 - ev) \\
\frac{\partial n}{\partial t} &= \sum A_i \left[ \nabla^2 \left( D_i \nabla^2 n - \frac{X_i n}{(kn + u)} \nabla u \right) \right] + r(n)(1-n) \\
A &= \begin{bmatrix}
\frac{b_1}{b_1-b_2} & \frac{b_2}{b_1-b_2} \\
\frac{b_2}{b_1-b_2} & \frac{b_1}{b_1-b_2}
\end{bmatrix}
\end{align*}
\]

where D is the ratio of activator to inhibitor diffusion coefficients; \(D_u\) is the ratio of cytokinetic to inhibitor diffu-

**Figure 2. Early stage of coherent single-cell orientation perpendicular to the axis of diagonal ridges.** Fluorescence microscopy of green fluorescent protein (GFP)-transfected vascular mesenchymal cells (VMCs) on (A) fibronectin (FN)/polyethylene glycol (PEG) substrates showing coherent individual cell orientation, relative to the interface axis (black lines) and (B) control substrate as homogeneous FN. Scale bar, 200 μm. Histogram of θ for (C) FN/PEG, showing convergence to 19±14° (n=81 cells; day 5; mean ± SD) and (D) control substrate, showing nonconvergence (n=83 cells; day 5).

**Figure 3. Cell migration toward aggregates directed by the coherent orientation.** Time-lapse videomicroscopic images at (A) t=0, (B) t=150, (C) t=300, and (D) t=625 minutes. Scale bar, 200 μm. PEG indicates polyethylene glycol; FN, fibronectin.
LR Polarity is Associated With Stress Fiber Accumulation in VMCs at FN/PEG Interface

Because intracellular polarization of the cell-motility apparatus directs migration, it may also explain the coherent orientation and migration of VMCs. Just as cells at the leading edge of a “wound” are polarized with their microtubule organizing centers (MTOC) facing the wound, VMCs may also polarize with their MTOC facing the bare PEG substrate (outward from the FN stripes). We reasoned that if the MTOC also had a LR polarization accompanying outward polarity, it might account for the orientation angle θ. We visualized the MTOC by α-tubulin immunofluorescence and classified its polarity relative to the nucleus centroid (Online Figure VII) into quadrants of Right/Out, Right/In, Left/Out, and Left/In for cells past, near or remote from the FN edge (Figure 5A). Six hours after plating, significantly more cells that were past the interface polarized toward Right/Out compared with cells remote from the FN edge (Figure 5B) (P<0.0001, Fisher’s Exact test). This was seen as early as 2 hours after plating (Figure 5C). Although outward polarization is consistent with known cell behavior in wound healing, the rightward preference is novel. In addition, there was a gradient of increasing polarization as the cells approached and passed the interface (Figure 5B), possibly because of the propagation of biased polarity by cell-cell interaction. As expected, this right-turning relative to outward migration occurred at both edges of a FN stripe. Thus, intracellular compartmental polarization, specifically the MTOC relative to the nuclear centroid, appears to contribute to the development of coherent orientation. Preferential outward migration competes with haptotaxis, which tends to retain cells on FN adhesion sites. Given that actomyosin contractility is required for cell migration, we hypothesized that cells encountering the nonadhesive PEG assembled additional actomyosin stress fibers, thereafter triggering the expression of LR polarity. We visualized stress fiber distribution by immunofluorescence of NMM-IIa 6 hours after plating (Figure 6A). Image stacking, with registration to create a heat map of local stress (n=45), showed accumulation of actomyosin stress fibers at the FN/PEG interface (Figure 6B). Inhomogeneous cell distribution was excluded by nuclear staining (Figure 6C and 6D; single and stacked images).
Because accumulation of stress fibers is localized at FN/PEG interfaces, resembling the spatial distribution of LR polarity, it may contribute to the LR polarity gradient.

**Abrogation of LR Polarity and Multicellular Alignment With Removal of Interface or Inhibition of Stress Fiber Accumulation**

To test whether the stress fiber accumulation and LR polarity are associated with FN/PEG interfaces, but not with inhomogeneous cell-cell contacts, we used an additional method to restrict cell plating to specific regions. A thin shadow mask, containing 25 long, rectangular windows, was overlaid on a homogeneous FN substrate before plating cells. The mask was removed after 30 minutes, leaving cells only within the 25 rectangular domains (Figure 7A and Online Figure VIII). After 6 hours, cells at the edge of cell sheets polarized outward, but showed no LR polarity (Figure 7B) or stress fiber gradient (Figure 7C). After 14 days, consistent with the lack of LR polarity or stress fiber gradient, the multicellular ridges failed to produce parallel ridges along the principal diagonal, instead,
forming a labyrinthine pattern as in conventional cultures (Figure 7D). Thus, the FN/PEG interface is essential for expression of stress fiber accumulation, LR polarity, and multicellular aggregates with asymmetrical alignment.

To further test whether stress fiber accumulation is required for LR polarity, we used the nonmuscle myosin-II inhibitor, blebbistatin, and the Rho kinase (ROCK) inhibitor, Y27632, to inhibit stress fiber accumulation specifically on nonmuscle myosin-II phosphorylation in response to Rho kinase.27–30 In the presence of either inhibitor, results showed loss of stress fiber accumulation (Figure 8A and 8B), loss of LR polarity of cells near the interface (Figure 7B), and loss of the coherent orientation (Online Figure IVB and IVC). In addition, the multicellular aggregates failed to exhibit asymmetrical alignment, instead, forming a labyrinthine pattern in the presence of Y27632 and a more disorganized pattern in the presence of blebbistatin (Figure 8C and 8D).
To test for cell-type specificity, BVECs, 3T3 cells, and ST2 cells were cultured on the FN/PEG substrates. Stress fiber accumulation at the interface was seen in all cell types, but ST2 cells showed leftward-biased polarity whereas BVECs and 3T3 cells showed neutral polarity near the interface (Online Figure IX). Because ST2 cells are multipotent but do not self-assemble into aggregates under our culture conditions, it suggests that LR asymmetry is not tied to self-assembly but possibly to multipotency. Together with results that inhibition of stress fiber formation abrogated LR polarity and the alignment of VMC aggregate patterns, it indicates that cytoskeletal reorganization is necessary and cell-type specific for the LR asymmetry.

**Discussion**

Our findings suggest an inherent, cryptic chirality in VMCs that is revealed by an unbiased extracellular mechanical transition and mediated by cytoskeletal reorganization, analogous to chemically induced chirality seen in neutrophil-like cells. To our knowledge, this is the first demonstration of an association between LR asymmetry and cytoskeletal reorganization, triggered by an unbiased mechanical interface, and the first demonstration that a microscale dynamic asymmetry unfolds into a de novo, consistently oriented and periodic macroscale pattern resembling tissue architecture. In VMCs, the rightward-biased turning required stress-fiber accumulation at the FN/PEG interface, suggesting that chirality may be in the architecture of the actin filament assembly at the macroscale level, say as clockwise or counterclockwise orientation. Alternatively, it may arise from chirality at the micro- or nanoscale, such as helicity of microfilaments, or chiral rotagen molecules, such as dynemin or myosin, in which a molecular chirality directly drives the LR asymmetry. Given that the chirality at the molecular level may be similar among cell types, our observation that chirality is cell-type specific, together with findings of Wan et al. suggests that the cell-type dependence of right/leftward bias may depend on macroscale cytoskeletal chirality, which may vary with cell-type. Thus, instead of “provoking” the LR asymmetry, the substrate interface may merely amplify the LR asymmetry already present in the cytoskeletal assembly.

On the other hand, the 20° steady-state orientation is an independent phenomenon. The evolution to this orientation may result from equilibrium between competing forces, specifically the lateral movements produced by cellular chirality and the outward movement created by random cell motility. It likely involves a combination of matrix traction, cell migration, intercellular signaling, and intercellular mechanical stress. It is not surprising that cells settle into a specific orientation relative to mechanical signals, given that tissue aligns to mechanical stress in vivo, as in skeletal, muscle, and vascular tissues. During the subsequent aggregate stage, the observed coherent migration into patterns of multicellular aggregates may involve both reaction-diffusion and matrix traction. Given that mechanical inductions introduced from native tissue architecture are lost when cells are enzymatically harvested, our findings may explain why chirality is rarely seen in cultured adult cells.

Cellular LR asymmetry is believed to amplify into tissue LR asymmetry through various means, such as planar cell polarity, an epithelial patterning phenomenon that involves asymmetrical distribution of proteins and cross-talk via the cytoskeleton, which determine the apical-basal axis in tissues. In vivo examples include drosophila hindgut rotation and the coherent orientation of cardiomyocytes, which form individual concentric layers of fibers, with incrementally increasing angle in each layer from the epicardium to the endocardium. At a broader level, this robust capacity of differentiated cells to self-organize into multicellular structures with LR asymmetrical alignment may have a fundamental role in embryogenesis and postnatal development, allowing cell-based engineering of regenerative tissues that are architecturally and functionally more authentic than previously possible.

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**Disclosures**

None.

**References**


Novelty and Significance

What is Known?

- The emergence of left-right (LR) asymmetry is a critical step in tissue morphogenesis.
- This asymmetry has been attributed to a hypothetical innate cellular chirality, and pattern formation has been attributed to reaction-diffusion phenomena.
- LR asymmetry is rarely observed in conventional cell culture.

What New Information Does This Article Contribute?

- Vascular mesenchymal cells preferentially turn right on migration across an unbiased substrate interface and then orient at 20° to the interface.
- In the presence of multiple parallel interfaces, they further self-organize into parallel diagonal stripes evenly spaced throughout the culture.
- This rightward bias requires accumulation of stress fibers at the interface, suggesting that mechanical signals play a critical role in this asymmetrical morphogenesis.

It is not clear how cells distinguish left versus right to produce tissue and organ asymmetry. We have found that vascular cells encountering a substrate interface preferentially turn right and that, with multiple interfaces, they self-organize into a regular diagonal pattern of macroscopic stripes. In this manner, asymmetry in cells may be translated and amplified into a de novo LR asymmetry at the tissue level. This LR bias required stress fiber accumulation in cells at the interface, potentially explaining the lack of LR asymmetry in conventional cultures where substrate interfaces, such as those in our experiments and in nature, are absent. This phenomenon may be a simple model for the LR asymmetry seen in the spatial plan of many organs. In addition to serving as a model of how tissue is laid out in development, these findings offer novel mechanisms of cellular self-organization and may guide cell-based therapy for tissue repair.
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SUPPLEMENTAL MATERIAL

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Short Title:
Left-right asymmetry in vascular cells

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Detailed Methods

Micropatterning. A glass substrate (Precise Glass and Optics, CA) was cleaned and coated with HMDS, followed by spin-coating with photoresist (AZ4620). The photoresist was exposed by ultraviolet, developed (AZ-400K), and treated with oxygen plasma (500 mTorr, 200 W) for 2 min. The remaining photoresist was rinsed with acetone, IPA, and deionized water. For PEG coating, the HMDS/glass substrates were immersed in 3 mM of C₃H₉O₃Si(C₂H₄O)₆-9CH₃ (Gelest, Inc., PA) dissolved in anhydrous toluene with 1% triethylamine (v/v) (Sigma-Aldrich, St. Louis, MO) for 4 hours, followed by ultrasonication in anhydrous toluene, ethanol and deionized water for 5 min, respectively.¹ After drying, the HMDS/PEG substrates were diced into 2 cm × 2 cm chips and stored in desiccators. Prior to plating cells, the HMDS/PEG substrates were first incubated with FN solution (50 μg/ml, Sigma-Aldrich, St. Louis, MO) in calcium-/magnesium-free phosphate-buffered saline (Mediatech, Inc., VA) at 4°C for 15 min. The same protocol was used for other ECM proteins including collagen types I and IV (Santa Cruz Biotechnology, Inc., CA) and laminin-1 (Sigma-Aldrich, St. Louis, MO). The protein-coated chip was plated with VMCs (200,000 cells per chip), BVECs (400,000 cells per chip), 3T3 cells (300,000 cells per chip), or ST2 cells (200,000 cells per chip) for 30 min in 500 μl media. After brief washings, only cells adhering to the ECM regions remained. The FN/PEG interface was identified by either titanium lines (for multicellular pattern formation, orientation analysis, and time-lapse videomicroscopy) or etched microgrooves (for assays of polarity and stress fiber) on the reverse side of the chip fabricated before the preparation of ECM/PEG substrates.

Shadow-mask plating. The mask was made of stainless steel (2 cm × 2 cm × 100 μm, NW Etch, WA) containing 25 parallel windows (300 μm × 1.5 cm) spaced 300 μm apart. Prior to plating, the tissue culture dish was first uniformly coated with FN solution (50 μg/ml) at 4°C for 15 min. After brief washings, a permanent magnet was attached underneath the culture dish to immobilize the mask. Thereafter, cells were plated through the mask for 30 min (200,000 cells in 500 μl) followed by removal of the shadow-mask. Schematic diagram for detailed procedure and results are provided in Online Figure VIII.

Cell culture. VMCs, BVECs, and 3T3 cells were isolated and cultured as described²⁻⁴ and ST2 cells were commercially purchased (Cell Bank, Riken Bioresource Center, Japan). All cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 15% heat inactivated fetal bovine serum and 1% penicillin/streptomycin (10,000 IU/10,000 μg/ml; all from Mediatech, Inc., VA). Cells were incubated at 37°C in a humidified incubator (5% CO₂ and 95% air) and passaged every three days.

Multicellular pattern formation. Each culture was prepared on one of four substrates: 1) 35-mm plastic dishes, 2) binary substrates consisting of PEG alternating with purified ECM protein (FN, collagen
I, collagen IV, or laminin-1), 3) uniformly coated FN substrate on chips with titanium on the reverse side of chip, or 4) shadow-mask plating as described elsewhere in methods, with media changes every three days. For inhibition of stress fiber or intervention of morphogen activities, Y27632 (10 μM, Ascent Scientific, NJ), blebbistatin (10 μM, Sigma-Aldrich, St. Louis, MO), BMP-2 (200 ng/ml, R&D systems, MN), noggin (100 ng/ml, R&D systems, MN), and warfarin (50 μM, Sigma-Aldrich, St. Louis, MO) were added at day zero and replenished with each media change. After 10-14 days, cultures were stained with hematoxylin (Sigma-Aldrich, St. Louis, MO) for 15 min to reveal multicellular aggregates. The panorama images (1.5 cm × 1.5 cm in visual field) were taken using a series of images (4.4 mm × 3.3 mm in visual field) with 1-2 mm offset, and recombined by panoramic stitching software (PTGui, New House Internet Services BV, Rotterdam, Netherlands). Images were acquired using an inverted microscope (Olympus IX71) with a color digital bio-imaging camera (Exi Aqua) in bright field.

**Green fluorescent protein transfection.** VMCs were transfected with a plasmid encoding GFP (pmaxGFP; Amaxa Biosystems, Germany) using the Effectene Transfection Reagent (Qiagen, CA). One day prior to transfection, 500,000 cells were plated on a 60-mm plastic culture dish. For transfection, the GFP plasmid was mixed with the transfection reagent following the manufacturer’s instructions and applied to the cells for 3 days. Each image was captured using the CCD (Coolsnap ES, Photometrics, AZ) equipped with an inverted microscope (Eclipse TE 2000, Nikon Instruments Inc., CA) with an excitation wavelength of 420-495 nm.

**Time-lapse videomicroscopy.** Cultures were incubated in a microscopic thermal stage (HCS60, Instec, Inc., CO) at 37° C and continuously supplied with premixed 5% CO₂. Images were acquired at 5 min intervals for a total of 22.5 hours using the CCD and inverted microscope (as above) in bright field. To verify adequacy of the on-stage incubator, proliferation of 3T3 cells in the thermal stage was compared with that in a conventional incubator by hemocytometry. Over 100 hours of culture, proliferation in the thermal stage remained comparable to that in the conventional incubator (Online Figure X).

**Immunofluorescent staining.** Cells were cultured on FN/PEG substrates or shadow-mask plated in normal growth medium, or on FN/PEG substrates in growth medium supplemented with Y27632 (10 μM) or blebbistatin (10 μM). FN/PEG stripes were 600 μm wide for the polarity assay and 300 μm for stress fiber distribution. Cells were fixed in cold methanol (-20° C) for 10 min, blocked with Image-iT™ FX signal enhancer (Invitrogen, CA) at room temperature for 30 min, then labeled with monoclonal anti-α-tubulin-FITC antibody (Sigma-Aldrich, St. Louis, MO: 1:50; for polarity assay on VMCs and BVECs), pericentrin polyclonal antibody (Covance, CA: 1:500; for polarity assay on 3T3 cells and ST2 cells), or NMM-IIa antibody (Covance, CA: 1:1000; for stress fiber distribution on all cells) at room temperature for 1 hour. The pericentrin and NMM-IIa antibodies were subsequently labeled with
secondary antibodies for 30 min (Alexa Fluor 555 anti-rabbit IgG antibodies, Invitrogen, CA; 1:500). Samples were mounted by ProLong GOLD antifade with DAPI (Invitrogen, CA). Images were acquired using the CCD and inverted microscope (as above) with appropriate excitation wavelengths (420-495 nm for anti-α-tubulin-FITC; 510-560 nm for NMM-IIa and pericentrin; and 330-380 nm for DAPI). For stress fiber distribution of NMM-IIa, images were stacked using etched microgrooves on the reverse surface for registration. For shadow-mask plating experiments, images were stacked using automated edge detection for registration (described below). After stacking images, NMM-IIa distributions were represented as the intensity value normalized by the number of images, and the scale bar in different conditions was scaled to the same maximum/minimum value in the control.

**Orientation analysis.** Images were processed using image segmentation, boundary tracing, and orientation calculation. Specifically, given the intensity histogram from a gray scale image of a GFP-transfected cell (Online Figure XI A), we identified the peak frequency (gray level 75 in Online Figure XI B) as the representative value for background, and the second peak (gray level 255 in Online Figure XI B) as the representative value for cells. To segment the image into binary images, the threshold for image segmentation was set at the midpoint between these two peaks (Online Figure XI C). Next, a boundary tracing algorithm, using an 8-connected neighborhood, was applied to locate the contours of bright regions, allowing identification of cells based on the size of the closed-loop regions. The orientation angle was calculated by the method of “minimum circumscribed rectangle” in which the shape of a given cell (blue region in Online Figure XI D) can be approximated by circumscribing rectangles of different sizes (position 1, 2, or 3 in Online Figure XI D). Thus, the orientation angle, \( \theta \), was defined as the longitudinal axis of the rectangle of minimal area relative to the horizontal axis of the image (i.e. the axis of the interface demarcated by the black titanium line; position 3 in Online Figure XI D). Finally, the histogram of \( \theta \) distribution was determined over all GFP-transfected cells (Online Figure XI E).

**Stacking images of immunofluorescence NMM-IIa after shadow-mask plating.** Due to the absence of a conventional registration marker, immunofluorescence images for NMM-IIa after shadow-plating were aligned to achieve registration using automated edge detection (Online Figure XII A). Specifically, as for orientation analysis above, a segmentation threshold was defined by the midpoint between the frequency peaks for cells and background (Online Figure XII B). Automated boundary tracing was then applied to locate the contours of bright regions, and the sheet of cells was identified by excluding small closed-loop regions. Subsequently, the edges of each cell sheet were identified by plotting the number of bright pixels as a function of row index of the image (blue curve in Online Figure XII C). We selected a level of 300 bright pixels as the threshold to identify the edge of the cell sheet (red dotted line in Online Figure XII C). In each image, the upper and lower edges of the cell sheet were defined by the
two intersections of the red dashed line with the blue curve (Online Figure XII C). The midline of the cell sheet, defined as equidistant between two edges, was used to register and stack the images (Online Figure XII D).

Mathematical model. The reaction-diffusion phenomenon was qualitatively modeled by a system of partial differential equations. VMCs secrete two morphogens, the slowly-diffusing activator, BMP-2, and its rapidly-diffusing inhibitor, MGP. Over this 2-dimensional domain, the reaction and diffusion of these morphogens create a spatiotemporal pattern of morphogenetic activities that mediate pattern formation. Specifically, the production of BMP-2 obeys an autocatalytic reaction and saturates at a high level of BMP-2. BMP-2 induces expression of MGP and is inhibited by MGP. The morphogen activities are proportional to cell density and also subject to degradation. Under the influence of these chemical activities, cells proliferate, diffuse in proportion to local cell density, and chemotactically migrate along a gradient of BMP-2 activity.

Based on the above, cell density and activities of the activator and inhibitor were formulated as functions of space, \( N(x, y) \), \( a(x, y) \), and \( h(x, y) \), respectively:

\[
\frac{\partial a}{\partial t} = D_a \nabla^2 a + \frac{N \rho_a a^2}{h(1 + q^2 a^2)} - \mu_a a \\
\frac{\partial h}{\partial t} = D_h \nabla^2 h + N \rho_h a^2 - \mu_h h \\
\frac{\partial N}{\partial t} = D_N \nabla^2 N - \nabla \left( \frac{\chi N}{(k_N + a)^2} \nabla a \right) + r_N N \left( 1 - \frac{N}{N_c} \right)
\]

\( D_a \) and \( D_h \) are the diffusion coefficients of activator and inhibitor, and \( D_N \) is the coefficient of cytokinetic diffusion. In Equation 1, the production of activator follows an autocatalytic reaction kinetic and is also regulated by the activity of inhibitor. Therefore, we use a sigmoidal form \( \frac{N \rho_a a^2}{h(1 + q^2 a^2)} \), where \( \rho_a \) is coefficient of autocatalysis and \( q \) denotes the constant for autocatalytic saturation. The inhibition of \( a \) by \( h \) is modeled by placing the \( h \) term in the denominator. In Equation 2, the production of inhibitor is formulated as a function of \( a^2 \) where \( \rho_h \) is the coefficient of this induced-reaction. In the first two equations, the production of activator and inhibitor are proportional to the cell density \( N \) and degrade in a first-order manner expressed by \( \mu_a \) and \( \mu_h \). In Equation 3, the chemotactic migration in response to the gradient of activator is regulated by the factor \( \chi \) and saturates at high levels of \( a \). \( k_N \) denotes the constant for the saturation of chemotaxis. \( r_N \) is the maximum rate of cell proliferation, and \( N_c \) is the cell density at confluence.
To derive corresponding equations that allow for migration of cells with preferred directions, the cell motility equation (Eqn. 3) may be modified by the rotation of axes $x$ and $y$ and weighting the migration coefficients toward one axis. First, the cell motility equation may be restated as a dot product of a derivative operator and a gradient vector, which may represent the gradient of cell density or the gradient of BMP-2 in the 2-dimensional domain. Thus:

$$\frac{\partial N}{\partial t} = \nabla \cdot [D_N \nabla N - \frac{\chi N}{(k_N + a)^2} \nabla a] + r_N N\left(1 - \frac{N}{N_c}\right)$$

4

where $G$ represents the combinatory gradient vector consisting of the gradient of cell density and BMP-2. While the axes $x$ and $y$ are rotated to $(\cos \theta, \sin \theta)$ and $(-\sin \theta, \cos \theta)$ (red axes in Figure 4A), $G$ is decomposed with respect to these rotated axes to allow scaling of directional coefficients. To scale anisotropic migration in the rotated axes, the directional coefficients, $b_1$ and $b_2$, were introduced for the decomposed vectors in rotated axes $(\cos \theta, \sin \theta)$ and $(-\sin \theta, \cos \theta)$, respectively. The scaled gradient vector, $G$, is then rearranged with respect to the original axes $x$ and $y$. The above may be summarized as follows:

$$G_m^T = RFR^TG^T$$

5

where $G_m$ means the modified gradient vector $G$, $F$ is the matrix of directional coefficients, and $R$ is the rotational matrix. With this modified gradient vector, the temporal variation of $N$ is recalculated by the dot product of derivative operator and the modified gradient vector.
\[
\frac{\partial N}{\partial t} = \nabla \cdot G_m + + r_N N \left(1 - \frac{N}{N_c}\right)
\]

\[
= \sum_{ij} A_j \left[\nabla^T \otimes \left( D_N \nabla N - \frac{X_N}{(k_N + a)^2} \nabla\right)\right]_{ij} + r_N N \left(1 - \frac{N}{N_c}\right)
\]

\[
A = \begin{bmatrix}
    b_1 \cos^2 \theta + b_2 \sin^2 \theta & (b_1 - b_2) \cos \theta \sin \theta \\
    (b_1 - b_2) \cos \theta \sin \theta & b_1 \sin^2 \theta + b_2 \cos^2 \theta
\end{bmatrix}
\]

Of note, this modified equation is a more generalized form. The original equations of isotropic migration may be stated as a special case where \(b_1 = b_2 = 1\). Together with the equations of morphogenetic dynamics, the reaction-diffusion mechanism is then described in a series of partial differential equations with anisotropic cell migration:

\[
\frac{\partial a}{\partial t} = D_a \nabla^2 a + \frac{N \rho_a a^2}{h(1 + q^2 a^2)} - \mu_a a
\]

\[
\frac{\partial h}{\partial t} = D_h \nabla^2 h + N \rho_h a^2 - \mu_h h
\]

\[
\frac{\partial N}{\partial t} = \sum_{ij} A_j \left[\nabla^T \otimes \left( D_N \nabla N - \frac{X_N}{(k_N + a)^2} \nabla\right)\right]_{ij} + r_N N \left(1 - \frac{N}{N_c}\right)
\]

\[
A = \begin{bmatrix}
    b_1 \cos^2 \theta + b_2 \sin^2 \theta & (b_1 - b_2) \cos \theta \sin \theta \\
    (b_1 - b_2) \cos \theta \sin \theta & b_1 \sin^2 \theta + b_2 \cos^2 \theta
\end{bmatrix}
\]

In non-dimensional form, this becomes:

\[
\frac{\partial u}{\partial t^*} = D \nabla^2 u + \gamma \left(\frac{nu^2}{v(1 + ku^2)}\right) - cu
\]

\[
\frac{\partial v}{\partial t^*} = \nabla^2 v + \gamma (nu^2 - ev)
\]

\[
\frac{\partial n}{\partial t^*} = \sum_{ij} A_j \left[\nabla^T \otimes \left( D_n \nabla^* n - \frac{X_n}{(k_n + u)^2} \nabla^* u\right)\right]_{ij} + r_n n (1 - n)
\]

\[
A = \begin{bmatrix}
    b_1 \cos^2 \theta + b_2 \sin^2 \theta & (b_1 - b_2) \cos \theta \sin \theta \\
    (b_1 - b_2) \cos \theta \sin \theta & b_1 \sin^2 \theta + b_2 \cos^2 \theta
\end{bmatrix}
\]

where the dimensionless variables are:
\[ \nabla^* = L^2 \nabla, \quad t^* = \frac{D_h}{L^2} t, \quad u = \frac{\rho_h}{\rho_a} a, \quad v = \frac{\rho_h}{\rho_a} N_c t_c, \quad n = \frac{N}{N_c}, \quad \gamma = \frac{L^2}{D_h t_c}, \quad k = \frac{q^2 \rho_r^2}{\rho_h^2}, \quad c = \mu_a t_c, \]

\[ e = \mu_a t_c, \quad D = \frac{D_u}{D_h}, \quad D_n = \frac{D_u}{D_h}, \quad \chi_0 = \frac{\rho_h}{D_h \rho_a} \chi, \quad k_n = \frac{\rho_h}{\rho_a} k_N, \quad r_n = \frac{L^2}{D_h} r_N, \]

and where \( L \) is the linear dimension of the domain, and \( t_c \) is the characteristic timescale of biosynthetic kinetics. We used this series of partial differential equations to model anisotropic cell migration as described in the text.

The partial differential equations were solved via the finite difference method. The 2-D spatial domain was discretized with a uniform mesh (200 \( \times \) 200). For all simulations, non-flux boundary conditions were used, and initial values of \( u, v \) and \( n \) were uniformly distributed with a 2\% random fluctuation.

Parameter values were estimated from the biological literature as described previously, with some modifications to correct for our revised culture platform and conditions.\(^5\) The ratio of diffusivities and the degradation terms were described previously. To account for the smaller dimension of the culture plates, we reduced the domain length, \( L \), to 1.5 cm. To allow for reduced diffusion through purified substrates, the diffusion coefficient for the inhibitory morphogen, \( D_h \) was estimated as \( 3 \times 10^{-8} \) cm\(^2\)/sec.

Using the same approximate timescale of biosynthesis (3600 seconds), the non-dimensional scaling factor, \( \gamma \), becomes 20000. We estimated \( D_N \) as \( 2 \times 10^{-9} \) cm\(^2\)/sec, as described previously.\(^9\) Assuming cytokinetic diffusion and chemotaxis to be on the same order of magnitude, we then obtained \( D_n = 0.06 \) and \( \chi_0 = 0.06 \).\(^6\) The chemotaxis constant, \( k_n \), was set at 1, as suggested by the literature.\(^6\) Using \( r_N = 0.015 \) hr\(^{-1} \) leads to \( r_n = 322 \) in dimensionless form. The total time \( t^* = 1 \) for each simulation.

The autocatalytic constant, \( k \), which sets the saturating value at specific values of \( v \), was estimated using the parametric space (Turing space) in which the pattern formation is driven by Turing instability, given that the activity of MGP in the experiments may not be measurable. The suitable range of \( k \) is calculated using the mathematic conditions that satisfy Turing space:

\[ f_u + g_v < 0 \]
\[ f_e g_v - f_v g_e > 0 \]
\[ f_u + D g_v > 0 \]
\[ (f_u + D g_v)^2 - 4D(f_u g_v - f_v g_u) > 0 \]

where the subscript denotes the first-order derivative with respect to \( u \) or \( v \). Note that the inequalities are evaluated at steady state \((u_0, v_0)\), in which the temporal change of morphogen activities and rates of
diffusion are zero. Thus, the mathematical components in the inequalities can be written as:

\[
\begin{align*}
    f_u &= \frac{2u_0}{v_0(1 + ku_0^2)^2} - c \\
    f_v &= -\frac{u_0^2}{v_0^2(1 + ku_0^2)} \\
    g_u &= 2u_0 \\
    g_v &= -e.
\end{align*}
\]

The steady state values \( u_0 \) and \( v_0 \) are functions of \( k, e \) and \( c \). Given \( D = 0.005 \), the Turing space can be plotted as function of \( k \) and \( e/c \) (Online Figure XIII). Based on our pre-selected values, \( e/c = 2 \) (red dotted line in Online Figure XIII) gives the acceptable range of \( k \) as \( 0 < k < 0.34 \) (red line within the blue region in Online Figure XIII). In our simulation, we chose \( k = 0.28 \).
Online Figure I. The consistent LR asymmetry of ridge formation from different passages and chemical treatments. Phase contrast microscopy of multicellular ridges (A) in normal medium and in the presence of (B) exogenous BMP-2, (C) exogenous noggin, and (D) warfarin. Scale bar, 600 μm. Light microscopy of multicellular ridges stained with hematoxylin in VMCs cultured from (E) passage 16 and (F) passage 21. Scale bar, 2 mm.
Online Figure II. Tests of matrix substrates showing persistence of LR asymmetry on extracellular matrices other than FN. Hematoxylin stain of ridges on (A) collagen I, (B) collagen IV, and (C) laminin-1. Scale bar, 2 mm.
Online Figure III. Histograms of coherent orientation $\theta$ from day 2 and day 4.  

**A**, Day 2 ($6 \pm 30^\circ$; $n = 79$ cells; mean ± s.d.).  

**B**, Day 4 ($15 \pm 20^\circ$; $n = 113$ cells).
Online Figure IV. Cell orientation at confluence with stress fiber inhibition. A, Coherent orientation at confluence when cultured on FN/PEG substrates. The loss of coherent orientation on FN/PEG substrates with inhibitor (B) Y27632 or (C) blebbistatin. Scale bar, 200 μm.
Online Figure V. Histogram of coherent orientation $\theta$ on FN/PEG stripes with various width. The stripe width is (A) 300 $\mu$m (20 $\pm$ 13.3°; n = 66 cells; mean ± s.d.), (B) 400 $\mu$m (24 $\pm$ 18°; n = 82 cells), (C) 500 $\mu$m (21 $\pm$ 20°; n = 46 cells), or (D) 600 $\mu$m (25° $\pm$ 17°; n = 44 cells). The images were taken on day 7.
Online Figure VI. Transition of local alignment at the junction of two orthogonal sets of interfaces. Phase contrast microscopy of VMCs on FN/PEG substrates (interfaces identified by black titanium lines) at day 0 at (A) low magnification (scale bar, 600 µm) and (B) high magnification (scale bar, 300 µm). C, Light microscopy of hematoxylin-stained cells at day 12, showing alignment of aggregates in a stripe pattern at 110° relative to interfaces up to the edge of the transition zone, where nodules arose and cell alignment smoothly curved over a distance of about 300 µm. Scale bar, 2 mm. D, High magnification at the junction zone of two orthogonal sets of interfaces. Scale bar, 1.2 mm.
**Online Figure VII. The polarity of VMCs.** Immunofluorescence microscopy of α-tubulin in VMCs showing (A) MTOCs and (B) the direction of polarity (red arrow) based on MTOC orientation relative to the nuclear centroid. Scale bar, 20 μm.
Online Figure VIII. Shadow-mask plating.  A, Uniform coating of FN on the tissue culture dish.  B, A magnet underneath the tissue culture dish immobilizes the shadow-mask above.  C, Cell plating through the mask allows only cells within the windows to adhere to the FN.  D, Cells remain in the specific domains of the windows after mask removal.
Online Figure IX. Stress fiber accumulation of BVECs, 3T3 cells and ST2 cells at FN/PEG interface. (A) Polarity of BVEC (n = 3; > 175 cells each), 3T3 (n = 5; > 245 cells each) and ST2 (n = 3; >265 cells each) near the FN/PEG interface. Stacked images of immunofluorescence microscopy of NMM-IIa in (B) BVECs (n = 45), (C) 3T3 fibroblasts (n = 50), and (D) ST2 cells (n = 35) on FN/PEG substrate. Scale bar, 100 μm.
Online Figure X. Proliferation of 3T3 cells in a conventional incubator compared with that on a thermal stage (mean ± s.d.).
Online Figure XI. Image processing procedure for orientation analysis. 

A, The original gray scale image containing GFP-transfected cells. B, The histogram of the gray level in (A). C, Image segmentation with the threshold determined from the histogram of gray level. D, The orientation defined as the angle of the longitudinal axis of the minimal circumscribed rectangle relative to the horizon axis of the image. E, The original image superimposed on the calculated orientation angle $\theta$. 
Online Figure XII. Image processing procedure for registration of NMM-IIa images from shadow-mask plating. A, The original gray scale image of immunofluorescence NMM-IIa. B, Image segmentation with exclusion of isolated cells. C, Number of bright pixels vs. row index of the image. The edges of cell sheet were identified by two intersections between the red dotted line (300 pixels) and the blue curve. D, Midline of cell sheet, defined as equidistant between the two edges, was used to register and stack the images.
Online Figure XIII. The Turing space plotted as a function of $k$ and $e/c$. The red dotted line represents $e/c = 2$, and the blue area represents the Turing space. The range of $k$, $0 < k < 0.34$, was determined by the span of the red dotted line which overlaps the blue area.
Supplemental References

1. Li N, Ho C-M. Photolithographic patterning of organosilane monolayer for generating large area two-dimensional B lymphocyte arrays. *Lab Chip.* 2008;8:2105-2112.


Legends for Video files

**Online Video I.** Time-lapse observation of cell migration over 22.5 hours. Cells coherently migrated following the orientation angle 22° relative to FN/PEG interface, gradually aggregating into parallel ridges perpendicular to the orientation.

**Online Video II.** Numerical simulation of $n(x, y)$ for isotropic cell migration ($b_1 = 1, b_2 = 1$).

**Online Video III.** This movie shows the numerical simulation of $n(x, y)$ for anisotropic cell migration ($b_1 = 1, b_2 = 10^{-6}$).