APPLICATION NOTE

A1/A1 R, AX/AX R Confocal Microscope

Morphogenesis of stable 3D epithelial sheet domes induced by osmotic gradients, as captured by phase contrast and confocal microscopy

In the process of organ morphogenesis in living organisms, flat epithelial sheets are bent, folded, and elongated to form the complex 3D shape of organs. Dome formation is an example of morphogenesis due to flexion, but many of its mechanisms remain unclear. In recent years, the osmotic gradient has been suggested as a factor in dome formation. Specifically, it is thought that the dome structure is formed through the following steps: (1) increased ion concentration at the basal side due to transcellular transport, and the accompanying generation of an osmotic gradient, (2) fluid influx to the basal side due to the osmotic gradient, and (3) generation of hydrostatic pressure by fluid influx. But the specific behavior of the epithelial sheets in the presence of an osmotic gradient has not been investigated. Furthermore, the stable 3D dome structure seen *in vivo* has not been reproduced, and the role of the osmotic gradient in dome formation has not been fully verified.

This application note introduces a report by Dr. Hisashi Haga, Dr. Seiichiro Ishihara, and Dr. Sumire Ishida-Ishihara of Hokkaido University. In this report, they used phase contrast and confocal microscopy to examine whether the application of an osmotic gradient to epithelial sheets in an environment close to *in vivo* induces the formation of stable domes, and investigated the formation mechanism thereof.

Keywords: Phase contrast and confocal microscopy, Epithelial cells, Osmotic gradient, Extracellular matrix (ECM), Swelling, Aquaporin

Overview

The extracellular matrix (ECM) plays a role in supporting the 3D morphogenesis of epithelial sheets *in vivo*. ECM (Genipin-cross-linked Matrigel) was placed inside a culture vessel (Transwell) with a 0.4 µm pore membrane, and sheets of MDCK epithelial cells were produced thereon. Then, an osmotic gradient was applied to the epithelial sheets, and live imaging was performed with phase contrast and confocal microscopy to see if formation of a stable dome structure occurred. In addition, in order to investigate the contribution of intracellular contractile force to dome formation, a double phosphorylated myosin-regulated light chain (2P-MRLC), which is known to enhance actomyosin contractility, was fixed and stained.

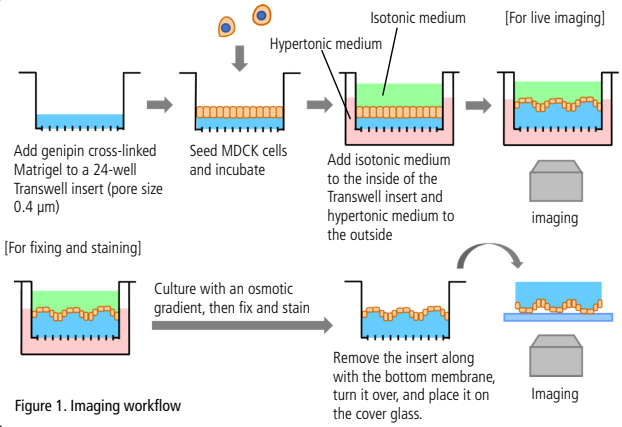

Figure 1. Imaging workflow

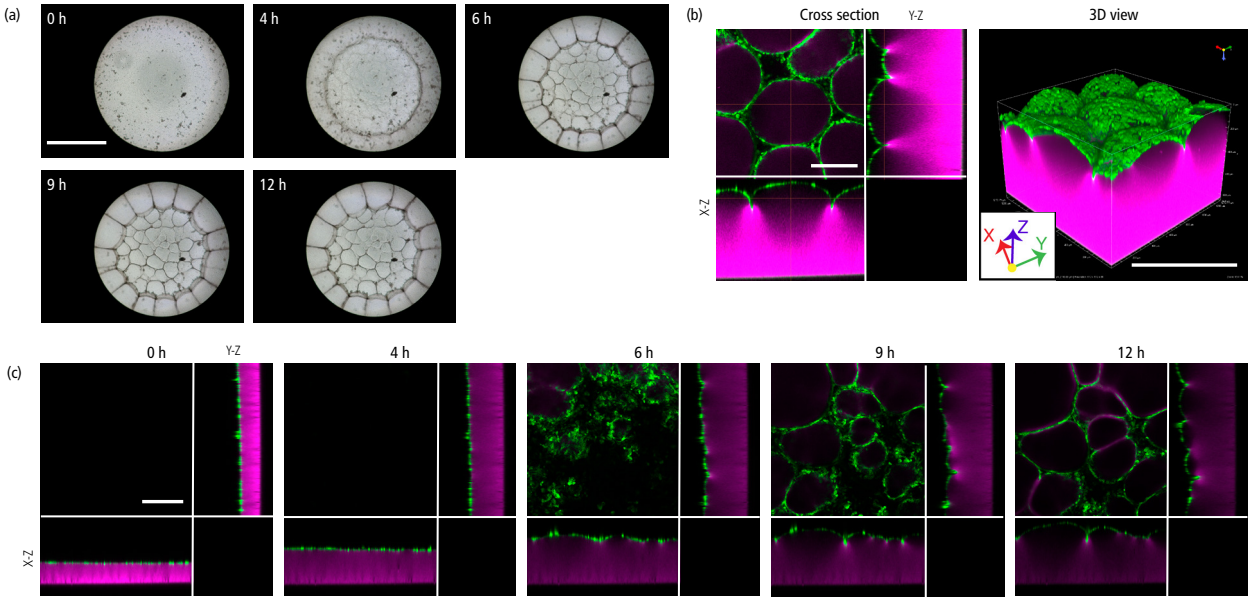

Figure 2. Live imaging of cell sheets after osmotic gradient loading
(a) Time-lapse images by phase contrast microscopy, Scale bar = 2 mm
(b) Fluorescence 3D images by confocal microscopy, Scale bar = 200 µm
(c) 3D live images by confocal microscopy, Green: MDCK cells (cell membrane was labeled with green fluorescent protein), Red/Magenta: Genipin cross-linked Matrigel, Scale bar = 200 µm
Objective: (a) CFI Plan Fluor 4X (NA 0.13) PhL DL, (b) and (c) CFI Plan Fluor 10X (NA 0.30) DIC L

Results

Time-lapse imaging with a phase contrast microscope revealed that multiple round structures appeared simultaneously on the epithelial sheets to which osmotic stress was applied, and that these structures were maintained without breaking (Fig. 2a). Each of these round structures exhibited a hemispherical dome-shaped structure when observed three-dimensionally with a confocal microscope using MDCK cells whose cell membranes were fluorescently labeled (Fig. 2b). Furthermore, the results of 3D live imaging revealed that the ECM under the epithelial sheets swelled unevenly during the formation process of the stable dome structure (Fig. 2c).

Epithelial sheets transport water via aquaporins (AQP) in response to osmotic stress. Therefore, when water transport by AQPs was inhibited by mercury ions, the swelling of ECM and dome formation were inhibited (Fig. 3). Furthermore, mathematical model analysis showed that the non-uniform swelling was because the lower the cell height, the more water was transported to the ECM side.

Next, the contribution of actomyosin, which is known to play a key role in morphogenesis, was investigated. Since 2P-MRLC is known to increase the contractile force of actomyosin, when the localization of 2P-MRLC was investigated by fixing and staining, partial localization was observed in the cell sheet (Fig. 4a). However, administration of the myosin inhibitors Blebbistatin and Y27632 did not prevent dome formation (Fig. 4b-c), suggesting that intracellular contractile force did not contribute to dome formation.

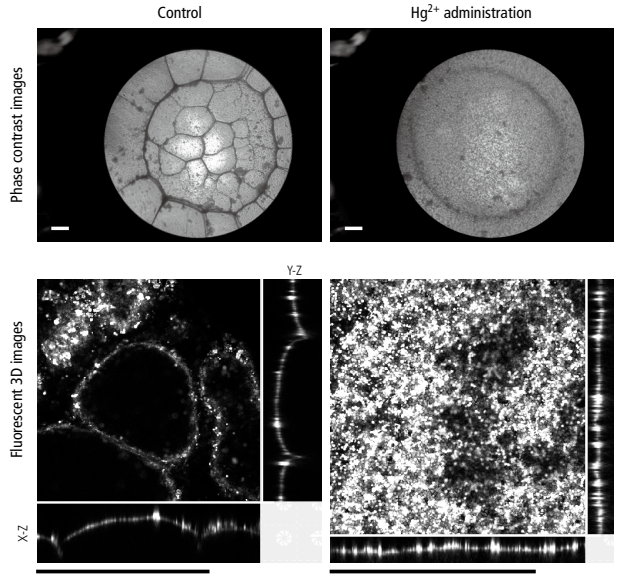

Figure 3. Dome formation during aquaporin inhibition

Phase-contrast and fluorescent 3D images of epithelial sheets after administration of aquaporin inhibitor mercury ion (Hg^{2+}). (Fluorescent image) White: calcein-AM, Scale bar = 0.5 mm
Objective: CFI Plan Fluor 4X (NA 0.13) PhL DL (phase contrast image),
CFI Plan Fluor 10X (NA 0.30) DIC L (fluorescent 3D image)

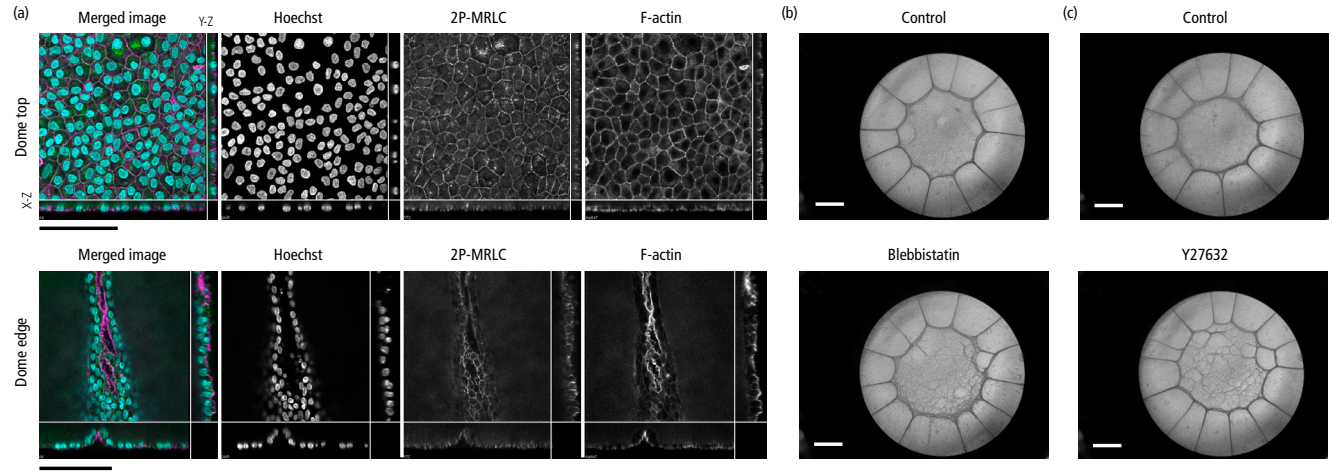

Figure 4.

(a) Fixed and fluorescent stained images of double phosphorylated myosin-regulated light chain (2P-MRLC). Cyan: Hoechst, Green: 2P-MRLC, Magenta: F-actin, Scale bar = 100 μm
(b) Phase-contrast images of epithelial sheets with 20 μM blebbistatin added. Scale bar = 1 mm
(c) Phase-contrast images of epithelial sheets with 20 μM Y27632 added. Scale bar = 1 mm
Objective: (a) CFI Plan Apochromat VC 60XC WI (NA 1.20), (b) and (c) CFI Plan Fluor 4X (NA 0.13) PhL DL

Summary

The overall image of the stable dome structure formed on the epithelial sheets on the ECM by osmotic gradient induction was captured using a confocal microscope and a low magnification objective, even in the thick state where cells were further seeded on the gel placed inside the insert.

The results of confocal observations show that non-uniform water transport by AQP causes local swelling of the ECM, resulting in the emergence of these dome structures. The osmotic gradient is a phenomenon that exists universally in the living body, but its relationship with morphogenesis is still largely unknown. This study raises the importance of osmotic gradients in *in vivo* morphogenesis.

References

"Osmotic gradients induce stable dome morphogenesis on extracellular matrix"
Sumire Ishida-Ishihara, Masakazu Akiyama, Kazuya Furusawa, Isao Naguro, Hiroki Ryuno, Takamichi Sushida, Seiichiro Ishihara and Hisashi Haga
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