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Short Communication

Time-lapse observation of branching morphogenesis of the lung bud epithelium in mesenchyme-free culture and its relationship with the localization of actin filaments

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ABSTRACT It has been shown that branching morphogenesis of the lung bud is mediated by epithelial-mesenchymal interaction via such molecules as FGF10, BMP4 and Shh. However, a recent study showed that the isolated lung epithelium still undergoes branching morphogenesis *in vitro* even in the absence of mesenchyme (Nogawa and Ito, 1995). In the present study, we observed *in vitro* the dynamic movement of the isolated lung epithelium of the fetal mouse using time-lapse recording, and investigated the roles of actin filaments in branching of the lung bud. First, time-lapse observation of the initial phase of lung branching morphogenesis revealed that at the sites of cleft formation, the epithelial surface was retracted inward from its original position. From this observation we assumed that there should be some structures which exert a physical force on the epithelium, and the localization and arrangement of actin fibers in the cultured lung epithelium were examined at various stages of branching morphogenesis. At the prebudding (6 h) and onset-budding (24 h) stages, no specific localization of actin filaments was observed in the lung bud epithelium, but at the postbudding stage (48 h) they were localized densely in the cells at the tip of the branched lung epithelium. The cell density was not different between the tip and cleft regions of the lung bud epithelium. When cultured with FGF-soaked beads, an actin-rich region was induced at the tip of the lung bud which was growing toward an FGF-soaked bead. These results indicate that actin fibers do not play a significant part in cleft formation but can be secondarily induced by FGF in the surrounding matrix and play some roles at later shaping of the branch in lung morphogenesis.

KEY WORDS: *lung, morphogenesis, actin fiber, organ culture.*

Branching morphogenesis in the embryonic lung and other glandular organs has been extensively studied as an example of epithelial-mesenchymal interaction. Several classical studies indicated that extracellular matrices play essential roles in determining the branching patterns in various organs (Gilbert, 2000). Recent studies have identified some secretory signaling molecules for epithelial-mesenchymal interaction, such as BMP4, Shh and FGF10, and they have been shown to contribute to determining the branching pattern (for review, see Hogan, 1999). For example, BMP4 is expressed at the tip of the branching epithelium (Bellusci *et al.*, 1996) while FGF10 is expressed in the mesenchyme near the tip of the epithelium and has a chemotactic activity for tip formation in the epithelium (Bellusci *et al.*, 1997b). BMP4 and FGF10 are shown to play opposing roles during branching morphogenesis (Weaver *et al.*, 2000). Shh is expressed at the tip of the epithelium and is assumed to promote cell proliferation (Bellusci *et al.*, 1997a). However, it remains to be elucidated what kind of mechanism initially determines the tips and clefts in the growing lung bud epithelium and regulates the distribution of these signaling molecules.

Recently it was revealed that isolated epithelium of the embryonic lung has the capacity to undergo branching morphogenesis even in the absence of mesenchyme (Nogawa and Ito, 1995), and we utilized this culture system as a model of branching morphogenesis because the observed results could be directly compared with various computer simulations of branching morphogenesis, which are well studied in theoretical biology (for review, see Ball, 1999).

Abbreviations used in this paper: BMP, bone morphogenetic protein; FGF, fibroblast growth factor; SHH, Sonic Hedgehog.

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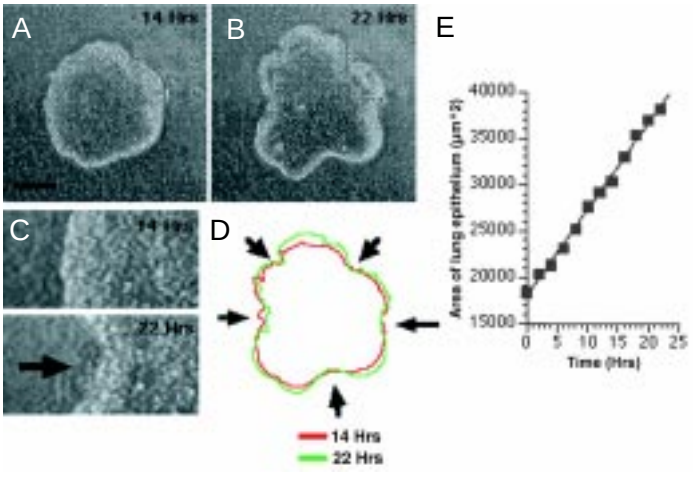

Fig. 1. The lung epithelium retracts inward at the sites of cleft formation. (A,B) *Sequential changes of lung epithelium morphogenesis. A, 14 h, B, 22 h. Numerous clefts were formed in the epithelium. Scale bar, 100 μm.* **(C)** *High magnification views of the site of cleft formation. The epithelium retracted at the site of cleft formation (arrow).* **(D)** *Sequential recording of contour lines of the epithelium revealed that the epithelium retracted inward from its original position at the sites of cleft formation (arrows).* **(E)** *The size of cultured lung epithelia during cleft formation. The growth rate appeared constant and no specific change was observed during cleft formation.*

Nogawa and Ito (1995) cultured the lung bud epithelium in Matrigel supplemented with FGF1 and observed branching morphogenesis *in vitro*. FGF1 has been found to be expressed in the fetal lung (Bellusci *et al*., 1997b) and to have a similar activity as FGF10 (Nogawa *et al.,* 1998) which is expressed in the fetal lung and plays a key role in establishing the lung branching pattern. Several other studies utilized this *in vitro* experimental system to explain the mechanism of *in vivo* branching morphogenesis. For example, Cardoso *et al.* (1997) revealed that FGF1 and FGF7 have different effects on the isolated embryonic lung epithelium and attributed the difference to the differential distribution of FGF receptors. Therefore it seems widely accepted that pattern formation *in vitro* has some correlation with that *in vivo*.

At first, we carefully observed pattern changes of explanted lung epithelia to identify the mechanisms which exert a physical force during morphogenesis. There are several candidate factors which can influence branching morphogenesis such as cell proliferation, actin filament contraction and extracellular matrix turnover (Alberts *et al*., 1994). Nogawa *et al.* (1998) examined cell proliferation by BrdU labeling and failed to show any specific localization of cell proliferation in the lung epithelium at the onset of budding, suggesting that cell proliferation does not play a critical role in determining the initial budding in cultured lung epithelium.

Time-lapse observation revealed that the cultured lung bud "shrunk" at the sites of cleft formation (Fig. 1). To make observation and quantitative analysis easier, we employed a culture condition in which epithelium grows two-dimensionally in the flat gel. An initial event in branching morphogenesis was the formation of clefts which occurred 6 h after culture, as described by Nogawa and Ito (1995). The movement of the epithelium was recorded from 0 until 24 h of culture and its contour lines were analyzed sequentially. Fig. 1 C,D shows that at the sites of cleft formation, the lung epithelium was retracted inward from its original position (arrows). This phenomenon could not be induced by simple expansion of the epithelium caused by cell division. Moreover, the formed clefts were usually sharp and it was unlikely that extracellular matrices were produced specifically at the point of cleft to form sharp invagination. We quantified the growth rate of the epithelium by measuring the area of the lung epithelium, but the growth appeared constant and no specific reduction or acceleration of growth occurred during cleft formation (Fig. 1E). Similar retraction of the epithelium occurred also in the presence of the mesenchyme (data not shown).

From the observation described above we assumed that some cytoskeletal structure should exert a physical force at the clefts of the cultured epithelium. We postulated that actin filaments may be a candidate factor involved in cleft formation since actin is the most common molecule to generate physical forces in the cell, and examined the distribution of actin filaments in the cultured epithelium.

Phalloidin-rhodamine staining revealed that actin filaments were neither localized specifically nor formed a particular structure at the clefts, but were localized at the tip of the branching epithelium (Fig.

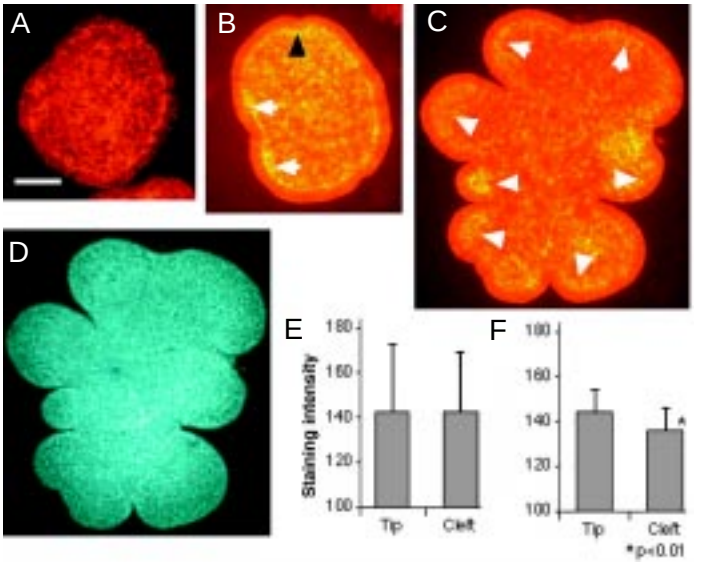

Fig. 2. The distribution of actin filaments in the lung epithelium at various stages of culture. (A) *At the prebudding stage (6 h) no cleft formation was observed and there was no specific localization of actin fibers. Scale bar, 100 μm.* **(B)** *At the onset-budding stage (24 h) some areas with densely stained actin fibers were observed on the apical side of the epithelial cells. However, in some cases they were localized at the tip (white arrowheads) and in other cases at the cleft of the epithelium (black arrowheads); therefore, it was not clear whether the localization of actin filaments was correlated with cleft formation.* **(C)** *At the postbudding stage (48 h), branch morphology was established and the apical side of the epithelial cells was densely stained at the tip of the branched epithelium.* **(D)** *Counter staining of a 48 h -cultured sample with Hoechst 33342 showed no specific distribution of nuclei, indicating that the above finding (C) was not an artifact caused by inhomogeneous staining.* **(E)** *Difference in the fluorescence intensity between cleft and tip regions after 24 h of culture. The standard deviation was large and no significant difference could be detected between the cleft and tip regions.* **(F)** *Difference in the fluorescence intensity between cleft and tip regions after 48 h of culture. Tip regions were more densely stained and the difference from the cleft regions was statistically significant.*

2). We employed the staging of the cultured lung epithelium proposed by Nogawa *et al.*(1995), i.e., the prebudding (6 hr), onset-budding (24 hr) and postbudding (48 hr) stages. At the prebudding stage, clefts were not recognizable yet and no specific localization of actin filaments was seen (Fig. 2A). At the onset-budding stage, clefts had been formed and actin fibers were found to condense near the apical surface of each epithelial cell (Fig. 2B). However, in some cases the actin condensation was localized at the tip (white arrowheads in Fig. 2B) while in other cases it was localized at the cleft of the branch (black arrowheads in Fig. 2B). At the postbudding stage, each bud had elongated and clearly there were regions with strongly stained actin filaments at the tip of the branch (white arrowheads in Fig. 2C). Counter staining did not reveal any specific localization of cell nuclei, indicating that the observed condensation of actin fibers was not an artifact due to inhomogeneous staining (Fig. 2D). We could not find any specific actin arrangement at the clefts even with the aid of a confocal microscope (data not shown).

Image processing revealed a statistically significant difference in the staining intensity between the tip and cleft regions at the postbudding stage, but not at the onset-budding stage (Fig. 2 E,F). Eight onset-budding and 4 postbudding stage epithelia were stained with Phalloidin-rhodamine. Their images were captured using a CCD camera in the same exposure time, and their average staining intensity at the tip and cleft regions was quantified using the NIH-Image software. At the onset-budding stage, the intensity was variable as predicted by visual inspection and no statistically significant difference was detected between the tip and cleft regions. On the other hand, in the postbudding stage epithelia, the staining intensity was stronger at the tip region than in the cleft region and the difference was statistically significant ($p<0.05$).

Since the localization of actin fibers became apparent at the tip of the epithelium only at later stages, we assumed that this could be induced by exogenously applied FGFs. Recent studies have shown that FGF-soaked beads have a chemotactic activity upon lung epithelium (Park *et al.*, 1998), so we undertook phalloidin-rhodamine staining of the lung bud moving toward the FGF source. Densely stained actin filaments were seen to localize at the growing tip of the lung epithelium (Fig. 3). When FGF1-soaked beads were applied, the chemotactic activity was not clear in 24 h and no specific distribution of actin fibers were seen (Fig. 3A). After 48 h of culture, the growing tip of the epithelium moved toward the FGF1-soaked beads and densely stained actin fibers were seen to localize around the beads (Fig. 3B, arrowheads). Counterstaining appeared homogeneous, indicating that the observed actin localization was not an artifact due to the uneven thickness of the epithelium. FGF-10 soaked beads exerted more strong chemotactic effects on the lung bud, and within 24 h the lung bud moved toward the FGF10 bead. Actin fibers were observed to concentrate at the tip of the epithelium which was moving toward an FGF10 bead. After 48 h of culture, the epithelium reached the FGF10 bead and bifurcated to surround the bead, and actin filaments were seen at the tips of the bifurcated lung bud (Fig. 3F, arrowheads).

Our data indicated that actin fibers can take part in shaping buds in the lung epithelium, which occurs later than cleft formation. In this regard, it is interesting to note that the shape changes of each epithelial cell are essential for morphological changes of organs, as is seen in neural tube closure (Karfunkel, 1972) and gastrulation (Nakajima and Burke, 1996). For example, an aggregation of square

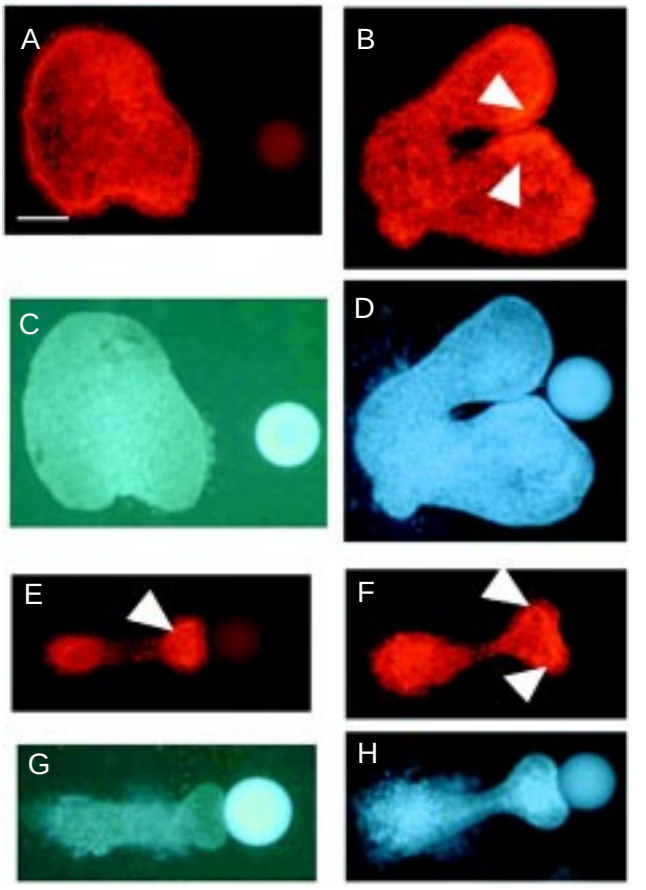

Fig. 3. The distribution of actin filaments in the lung epithelium migrating toward FGF beads. (A) *A lung epithelium cultured for 24 h with an FGF-1 soaked bead. No specific distribution of actin fibers is seen. Scale bar, 100 μm.* **(B)** *A lung epithelium cultured for 48 h with an FGF-1 soaked bead. Dilated lung buds moved toward the FGF-1 soaked bead and actin fibers were densely stained near the FGF-soaked bead (arrowheads).* **(C,D)** *Counterstaining of lung epithelia cultured for 24 (C) and 48 (D) h. No inhomogeneous staining could be seen.* **(E)** *A lung epithelium cultured for 24 h with an FGF-1 soaked bead. The lung bud moved toward the FGF-soaked bead and attached to it.* **(F)** *A lung epithelium cultured for 48 h with an FGF-10 soaked bead. The lung bud bifurcated around the FGF-1 soaked bead and dense actin fibers were observed at the tips near the FGF-soaked bead (arrowheads).* **(G,H)** *Counterstaining of lung epithelia cultured for 24 (G) and 48 (H) h. No inhomogeneous staining could be seen.*

epithelial cells is energetically most stable when the epithelial sheet is flat. However, if the adhesion belt (Alberts *et al.,* 1994) of the epithelial cells shrinks (which results in actin condensation) and epithelial cells become wedge-shaped, the energetically most stable state of the collection of such cells is a curved structure, in which the condensation of actin filaments should be present at the apical side of the tip, as we experimentally observed (Fig. 2). We have shown that FGF1 and FGF10 can induce condensation of actin filaments at the tip of the branch. It should be interesting to investigate whether these signaling molecules regulate such genes as Rho, which controls the assembly of actin fibers in the epithelium of the lung bud.

Some theoretical biologists suggest that pattern formation of the lung can be explained as a purely physical phenomenon (Lubkin *et al.,* 1995), but our present results do not support this

hypothesis, at least at a very early phase of morphogenesis. Branching morphogenesis of non-living materials is well studied in theoretical biology (Ball, 1998) and applied physics (Kessler *et al.,* 1988). Lubkin *et al.* (1995) propose that lung branching morphogenesis can be explained as a physical phenomenon called viscous fingering. When a less viscous fluid is pushed into a more viscous fluid, their interface becomes unstable to form branch-like morphology, and Lubkin *et al.* (1995) claimed that lung branching morphogenesis was homologous to this purely physical phenomenon. In this model, the sufficient condition for pattern formation is that a less viscous fluid (=epithelium) expands toward a more viscous fluid (=Matrigel) and it is not necessary for cells to differentiate before cleft formation occurs. It is attractive to explain seemingly complicated biological phenomena in terms of simple physical mechanisms, but it is unlikely that a condition exists where the interface *retreats* at the sites of cleft in this viscous fingering phenomenon. Some additional factors should be involved in the viscous fingering model, or another model needs to be developed that can explain the retraction of the lung epithelium at the onset-budding stage.

There should be factors other than actin fibers which possibly exert a physical force in the epithelium during cleft formation. Three cytoskeletal components are known to act as a source of mechanical tension, i.e., actin filaments, microfilaments and intermediate filaments (Alberts *et al.,* 1994). It is unlikely that intermediate filaments are involved in the initial pattern formation since they are known to mechanically stabilize the cell. While microtubules contribute mainly to intracellular transport of materials, they have been shown to influence tissue morphogenesis as in the case of neural tube closure (Gilbert, 2000). Since whole-mount immunohistochemical methods for two-dimensional epithelial cell culture are not available, further technical advance is needed to elucidate the mechanisms of branching morphogenesis.

Experimental Procedures

Lung epithelium culture

The culture of the lung epithelium was carried out basically as described by Nogawa *et al.* (1995) with some modification. The lung epithelia were prepared from ICR mouse fetuses (SLC Japan Inc., Shizuoka, Japan). Pregnant day-11.5 mice (plug day = day 0) were killed by cervical dislocation and their uteri were removed and placed in Tyrode buffer solution. The lung buds of the embryos were dissected with microscissors, washed several times with Tyrode buffer and then incubated with 1 unit/ml dispase (Gibco) for 15 min at 37ºC. After that the epithelium and mesenchyme of the lung bud were separated using sharp needles, and the epithelium was collected and placed on a 10 mm tissue culture insert (NUNC) in a 4 μl drop of Tyrode buffer. Then the epithelium was covered by 20 μl of 50% Matrigel / 50% DMEM/F-12, allowed to gel for 30 min at 37ºC, and then flooded with 400 μl of DMEM/F-12 supplemented with 1% BSA and 500 ng/ml FGF-1 (UBI) in a 4-well dish (NUNC). In some cases the lung epithelium was cultured with Heparin beads (Sigma) loaded with 100 μg/ml FGF1 or 200 μg/ml FGF10 as described by Park *et al.* (1998)

Time-lapse observation

The lung epithelia were conditioned as described above, and the culture dishes placed on an inverted microscope (Nikon TMD), which was covered with an incubation chamber. The atmosphere in the chamber was kept at 37ºC and CO2 was supplied periodically to maintain the culture medium around pH 7.0. The images of the cultures were captured with a CCD camera every 10 min, and stored in a magneto-optical disk. All procedures were controlled using a Macintosh Quadra 700 computer and the NIH Image program (developed at U. S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image).

Staining of actin fibers and cell nuclei

The staining of actin fibers and cell nuclei was carried out as described by Itasaki *et al.* (1989). The cultured epithelia were fixed in 4% paraformaldehyde for 1 h, washed with PBS, and then incubated in 5 unit/ml phalloidin-rhodamine (Molecular Probes) with 10 μg/ml Hoechst 33342 for 1 h at 37ºC. The culture insert was then washed once in PBS, and the membrane was separated and mounted on a slide glass. We used a fluorescence microscope (Zeiss Axioplan 2) and a confocal microscope (BIO-RAD Radiance 2000) for observation of cultures. The pictures were digitized using a cooled CCD camera with the same exposure time and the relative intensity of fluorescence was compared using the NIH Image software.

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