

# In vitro morphological bud formation in organ-like three-dimensional structure from mouse ES cells induced by FGF10 signaling

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**Abbreviations:** EB, embryoid body; EB3 cells, undifferentiated embryonic stem cells; ES, embryonic stem; FGF10, fibroblast growth factor 10; FGFR2b, fibroblast growth factor receptor 2b; iPS, induced pluripotent stem; LIF, leukemia inhibitory factor; PBS, phosphate-buffered saline

Embryonic stem (ES) cells have a pluripotent ability to differentiate into a variety of cell lineages in vitro. Using an embryoid body (EB) culture system, we developed a gut-like three-dimensional structure from mouse ES cells (the ES 3-D structure). Genetic studies implicate fibroblast growth factor 10 (FGF10)-FGF receptor 2b (FGFR2b) signaling as a critical regulator of lung bud morphogenesis in the embryonic foregut. The aim of the present study was to form a putative respiratory tract in the ES 3-D structure. By local application of FGF10 protein, we successfully demonstrated in vitro morphological formation of putative primitive respiratory tract-like processes, or buds, in the ES 3-D structure. Such organs that are differentiated from ES cells may provide new insights into tissue engineering and regenerative medicine.

## Introduction

Signaling by fibroblast growth factor 10 (FGF10) and its receptor FGF receptor 2b (FGFR2b) is critical for bud morphogenesis in many developing structures.<sup>1</sup> During organogenesis, FGF10 is expressed by the mesoderm and subsequently diffuses to activate FGFR2b in adjacent endodermal- or ectodermal-derived cells and induce budding.<sup>1-4</sup> In the earliest stages of lung development, FGF10 regulates branching morphogenesis via its receptors on the foregut, and disruption of FGF10-FGFR2b signaling is lethal at birth and results in defects in multiple organs, including the lungs.<sup>5-7</sup>

Recently, embryonic stem (ES) cells were shown to spontaneously give rise to a functional gut-like unit called the ES gut, which consists of a broad array of enteric derivatives from all three embryonic germ layers, including epithelial cells (endoderm), smooth muscle cells and interstitial cells of Cajal (mesoderm), and a small number of diffusely distributed enteric neurons (ectoderm).<sup>8,9</sup> We successfully reproduced in vitro morphological development of mouse ES cell-derived three-dimensional structure similar to ES gut, which we designated the ES 3-D structure. Although nothing is known about the targets of FGF10-FGFR2b signaling in the ES 3-D structure, we hypothesized that primitive lung bud formation may be induced on the ES 3-D structure as a

result of FGF10-FGFR2b signaling activation. In the present study, we demonstrate for the first time that local application of FGF10 induces differentiation of bud-like processes from the inner layer of ES 3-D structure. Our results open up a new vista of possibilities in the fields of tissue engineering and regenerative medicine.

## Materials and Methods

Undifferentiated ES cells (EB3 cells) were maintained on gelatin-coated dishes without feeder cells in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Gibco/BRL), 0.1 mM 2-mercaptoethanol (Wako), 0.1 mM nonessential amino acids (Gibco/BRL), 1 mM sodium pyruvate (BioWhittaker Molecular Applications), and 1,000 U/ml leukemia inhibitory factor (LIF; Chemicon). The EB3 cells originated with Dr. Hitoshi Niwa, Center for Developmental Biology, Riken, Kobe. To induce embryoid body (EB) formation, dissociated ES cells were cultured in hanging drops as previously described, with minor modifications.<sup>10</sup> The cell density of one drop was 500 cells per 15 µl ES cell medium, without LIF. EBs were formed after 7 d in hanging drop culture. These EBs were transferred to outgrowth cultures on 100-mm gelatin-coated plastic dishes and allowed to attach. In each outgrowth culture,

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cell clusters underwent a dramatic transformation into cyst-like structures with a cavity containing fluid and solids. After observation of small cyst-like structures (on day 14), we planted heparin acrylic beads (Sigma), which were pre-soaked for 4 h in either control buffer (phosphate-buffered saline; PBS) or FGF10 (100 ng/ $\mu$ l, Sigma), near the cyst-like structures. Cells and organs were maintained in a humidified incubator at 37°C with 5% CO<sub>2</sub> and images were acquired using a phase-contrast microscope (Nikon).

## Results and Discussion

ES cells were cultured for 7 d in a hanging drop culture system and allowed to form aggregates called EBs, which were subsequently transferred to gelatin-coated dishes. After 5 to 7 d in culture, multiple clusters within each developing EB began to contract spontaneously with an irregular rhythm as the cells differentiated into beating cardiac muscle cells. Consistent with previous reports,<sup>8-11</sup> each contracting cluster then underwent a dramatic transformation into a hemispherical dome-like 3-D structure (Fig. 1A).

After observation of small cyst-like structures on day 14, we planted heparin-coated beads that were pre-soaked in either PBS or FGF10 near the cyst-like structures. As expected, the control heparin beads were not able to induce differentiation of bud-like organs in the ES 3-D structures (Fig. 1B). In contrast, local application of FGF10 induced differentiation of bud-like processes from the intestinal inner layer in ES 3-D structures on day 16 (Fig. 1C–E). These results suggest that the formation of bud-like processes may be induced by activation of FGF10 signaling in the ES 3-D structure.

Genetic data indicate that FGF10-FGFR2b signaling is required for bud formation in multiple developing structures during organogenesis.<sup>1-4</sup> In the developing respiratory tract of the mouse, FGF10 is first detected at embryonic day 9.5 during primary bud formation.<sup>7,12,13</sup> The importance of FGF10 in lung

development is demonstrated by the fact that FGF10 null mice die at birth due to numerous defects, one of which is the absence of lung buds.<sup>14,15</sup> Using lung explant culture, it has been demonstrated that FGF10 acts as a chemo-attractant for the epithelium in lung buds *in vitro*. Furthermore, FGF10 expression studies suggest that FGF10 signaling plays an iterative role during lung branching morphogenesis *in vivo*, as FGF10 is expressed in a dynamic pattern at the tip of each forming bud.<sup>5-7,12-16</sup>

Our results provide the first evidence that FGF10 signaling may play a role in morphological formation of buds in the ES 3-D structure. We presume that these buds can be regarded as primitive respiratory tract-like processes, although we lack the biochemical evidence needed to definitively identify their molecular composition.

Recent studies have shown that induced pluripotent stem (iPS) cells can be generated from adult human dermal fibroblasts and other human somatic cells, and can be maintained on isogenic parental feeder cells.<sup>17,18</sup> In combination with iPS cells, primitive lung bud formation within the 3-D structure represents a potential revolution in the fields of tissue engineering and regenerative medicine; for example, by opening the way for functional reconstruction after total laryngectomy.

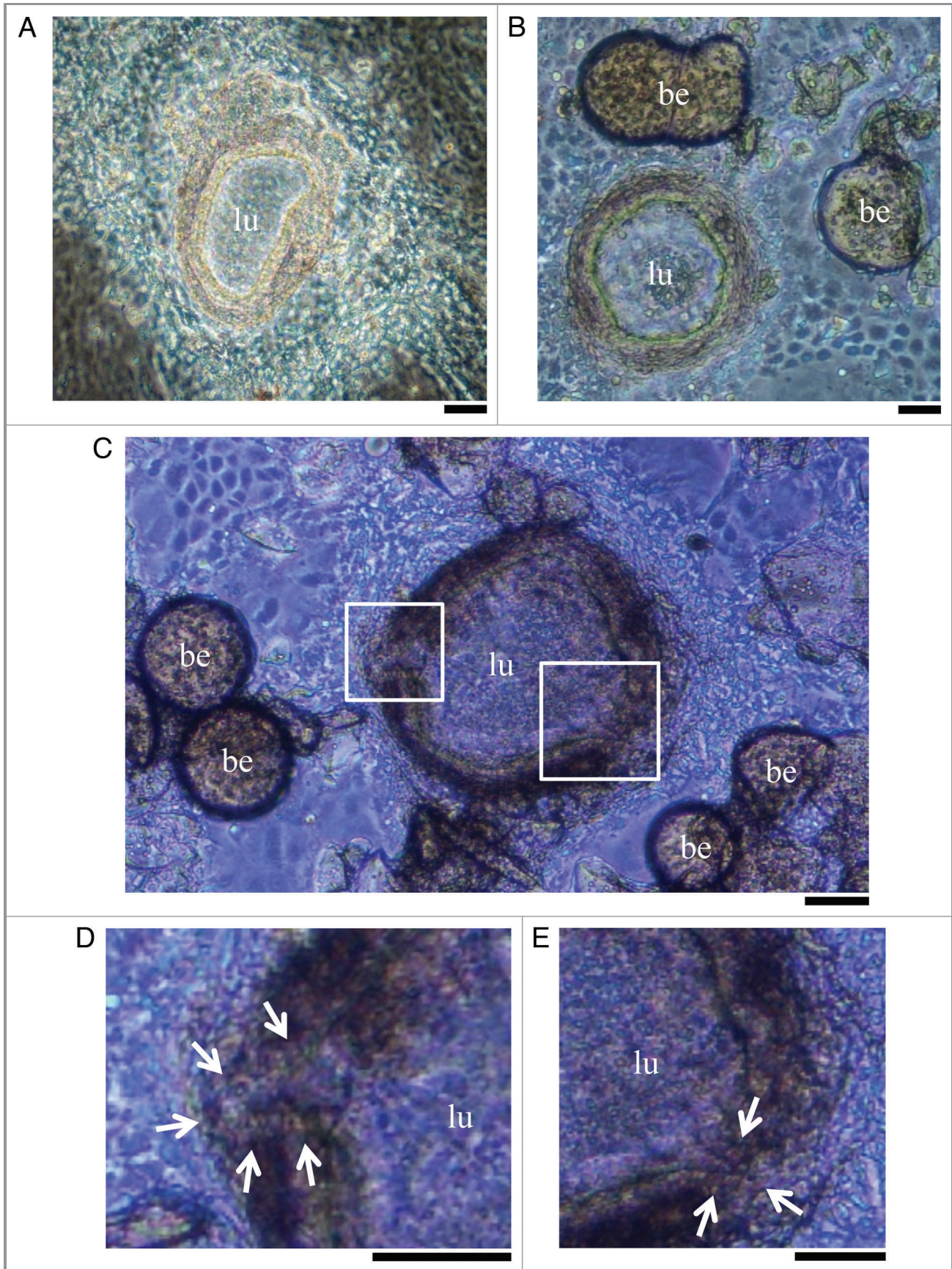
### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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**Figure 1 (See opposite page).** (A) A typical mouse ES cell-derived three-dimensional structure (ES 3-D structure) on day 16 of embryoid body (EB) outgrowth culture. (B) Representative example of ES 3-D structure planted control heparin beads (day 16). No morphological differences were observed among these conditions during our culturing protocol. (C) Representative example of ES 3-D structure treated with heparin beads soaked in FGF10 (day 16). Bud-like processes extending from the structural inner layer were observed. (D and E) Higher magnification of the bud-like processes within the white squares in (C). (lu) structural lumen; (be) heparin-coated bead. Scale bar indicates 100  $\mu$ m. Arrows indicate the bud-like processes.



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