In vitro self-organization of submandibular gland cells for reproducing branching morphogenesis

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Abstract

The development of specific tissue architecture is orchestrated by multiple mechanical and chemical interactions, which progressively pattern the shape and geometry of the developing tissue. Recent advances in 3-dimensional (3D) organogenesis models have elucidated the central role of collective multicellular behaviour in many organs development, including glandular tissues such as the submandibular salivary gland (SMG). Here, we modified a simple and highly reproducible in vitro 3D culture model of primary SMG cells self-organization to dissect the cellular dynamics during tissue patterning. On a time-scale of hours, we observed marked size and shape transformations in the developed 3D glandular structures. Further, we found that this morphological transformation resulted in a spatially-controlled growth differential from the centre to the periphery of the formed aggregates. As extracellular matrix (ECM) proteins are known to regulate diverse cellular functions, we subsequently investigated the effect of fibronectin (FN) on SMG cells self-organization using our culture model. Interestingly, we found novel roles of FN in inducing duct-like elongation during initial stages of SMG bud formation. To confirm these findings, epithelial buds were isolated and supplemented with FN, and similar ductal elongations were observed. Thus, this in vitro model provides a simple and valuable tool for organogenesis studies.
Introduction

During embryogenesis, tissues and organs develop through a complex and coordinated set of events that govern growth, proliferation, migration, and apoptosis in a plethora of tissue-forming cells. Such a complex set of events ultimately results in the formation of specific cell patterns and well-organized structures. In this context, branching morphogenesis - a well-known developmental process - has been extensively investigated due to its unique pattern of repetitive self-similar bifurcations of the epithelium into the surrounding mesenchyme, that result in distinct tree- or bush-like organ geometries. Mouse submandibular salivary gland (SMG), in particular, has been used as a preferred model for investigating glandular branching morphogenesis. SMG development initiates at embryonic day 11 (E11) as a solid epithelium bud protruding into the thickened underlying mesenchyme. Recent studies have revealed the dynamic aspects of SMG branching, including cell-cell and cell-matrix interactions, as well as cell migration and proliferation. These processes are controlled by the action of numerous growth factors, including epidermal growth factor (EGF), fibroblast growth factors (FGFs) and transforming growth factor β (TGF-β), that can induce differentiation of cells as well as changes in ECM properties and composition (e.g., collagens, laminin, and fibronectin). In particular, fibronectin (FN) - a glycoprotein known to mediate cellular adhesion, migration, growth, and differentiation - is known to play a crucial role in cleft formation and progression in SMG tissue branching by converting cell-cell adhesions to cell-matrix adhesions in those cleft-specific regions.

Understanding of the underlying mechanisms in these processes involved in branching morphogenesis proved to be the cornerstone for the emergence of functional organ regeneration therapies. Therefore, several in vitro model systems have been developed. Among these, the long established ex vivo embryonic organ cultures, which utilize either isolated intact whole SMG or SMG epithelium, have been used successfully to study the molecular basis of SMG organogenesis. Recently, a newly established self-organization model of SMG cells forming an organized SMG-like structure, proved to be a considerably valuable model, especially because it has been utilized in recent attempts to fabricate functional salivary gland organoids. However, many fundamental aspects involved in SMG cells self-organization, such as the dynamic interaction between epithelial
and mesenchymal cells at the earliest stages of bud formation, and the spatio-temporal steps involve in the specific tissue patterning, still need further elucidation. In this context, we herein initially developed a simple and highly reproducible 3D SMG culture system to investigate the self-organization process of embryonic SMG mesenchymal and epithelial cells into SMG tissue-like structures, in order to obtain a deeper understanding of epithelial bud formation at the very early stages of SMG development. Additionally, we used this system to evaluate the effect of FN during the early stages of cell aggregation and duct formation of SMG development. Interestingly, we identified a novel role of FN in regulating not only bud branching, but also duct elongation.

**Methods**

**Tissue isolation**

ICR mice were purchased from Charles River Laboratories (Japan). Mouse care and animal handling were performed in accordance with “Guidelines for Animal Experiments at Okayama University” and all experimental protocols were approved by the Animal Care and Use Committee Okayama University (OKU-2013039 and OKU-2015540). Embryos from pregnant ICR strain female mice were harvested at E12.5 or E13, and SMGs were isolated from embryos under dissecting microscope (STZ-40Tba Shimadzu, Japan). For experiments in which epithelial rudiments were used, isolated SMGs were treated with 4 U/mL dispase 1 (Roche, Switzerland) for 5 min at room temperature and then completely separated into epithelial and mesenchymal tissues using a pair of fine forceps. Isolated epithelial tissues were then washed with phosphate-buffered saline (PBS) solution and maintained in Dulbecco’s modified Eagle’s medium/Nutrient Mixture F-12 (DMEM/F12) (Wako Pure Chemical, Japan), supplemented with 1% penicillin/streptomycin (Nacalai Tesque, Japan) (DMEM/F-12/PS medium), until further use.

**Whole SMG dissociation**

E13 SMGs were further dissociated into individual cells through the following procedure: whole SMGs were first treated twice with 100U/ml collagenase I (Worthington, NJ) in PBS solution,
without calcium or magnesium, at 37°C for 10 min on a rotary shaker and then treated with 0.25% trypsin-EDTA (Sigma-Aldrich, MO) for 5 min at 37°C on a rotary shaker. After all enzymatic treatments, tissues were re-suspended with (DMEM/F-12/PS) medium containing 10 µg/mL DNase I (Roche, Switzerland). Finally, tissues were dissociated into single cells by gentle pipetting, followed by filtering using 70 µm nylon filter (BD Falcon, NC). Cell concentration of the dissociated whole glands was measured using haemocytometer and aliquoted into grease coated 1.5 ml micro tubes to yield 2, 3, and $4 \times 10^4$ cells per tube. The aliquoted cells were pelleted by centrifugation at 900 g for 3 min and the obtained cell pellets were seeded using a 20 µl pipette inside a 40 µl drop of Matrigel (BD Biosciences, MA) diluted 1:1 with DMEM/F-12/PS medium and incubated at 37°C for 72 h.

**Quantification of self-assembly process of cell aggregates**

Branching morphogenesis of each cell aggregate was observed under the microscope (TE-2000, Nikon, Japan). Number of buds per aggregate and duct elongation per unit area were quantified at each time-point using the Image J software (NIH, MD). Each experiment was repeated at least 5 times and the average of multiple experiments was used for comparison. Dissociated E13 whole SMG cell aggregates were incubated at 37°C supplemented with 5% CO₂ and 95% humidified air in a self-made environmental chamber fitted onto an inverted microscope (Eclipse Ti, Nikon). Images were acquired using bright field optics through 4× or 10× objectives once every 15 min for 72 h and were assembled into movies in AVI format using the Image J software. Changes in aggregate size and shape were observed by performing serial measurements of the aggregate area from 0 h. Change in aggregate circularity ($C$) was measured as $4\pi A/ L^2$, where $A$ is the aggregate projected area and $L$ is the contour line length.

Cell migration stage was identified as the period of vigorous cell motility to establish initial cell-cell contact resulting in a sudden and rapid decline on cell aggregate area (Figure 2b, blue dotted line). The compaction stage was defined as the following period which showed condensation of the loosely formed aggregate, with a slow and gradual decrease in the aggregate area (Figure 2b, area between the two dotted lines).
Shorter movies (average 23 frames) were prepared for cell migration tracking. Aggregates were divided based on observation of each aggregate area into three layers (Suppl. Fig. S1a). Fifteen single cells from each layer were manually tracked through the entire short movie or until they could no longer be tracked. Total distance travelled by cells (T) and cell velocity (T/t) were calculated from the stacks of calibrated images using the Image J software. Average migratory persistence identified as (D/T) was calculated, where D is the distance between the first and final points of each cell track (Suppl. Fig. S1b).

**Immunofluorescent staining**

Samples were fixed with 4% paraformaldehyde for 20 min at room temperature and washed with PBS containing 1% bovine serum albumin (BSA) (Nacalai Tesque) and 0.1% Triton X-100 (PBS X). Samples were then blocked with blocking solution One Histo (Nacalai Tesque), followed by incubation with primary antibodies diluted into PBS-X: rabbit anti-Ki67 (1:1000, Abcam) and Anti-Cytokeratin 7 antibody (1:200, Abcam). Antibody binding was detected using Alexa Fluor-conjugated secondary antibodies (Life Technologies, NY) and images were obtained using a confocal microscope (C1, Nikon). For nucleus staining, DAPI (Life Technologies) was used. For staining of F-actin, Rhodamine-conjugated phalloidin (1:50, Molecular Probes) was used.

**Whole cell aggregate and epithelial rudiment culture**

Cell aggregates and epithelial rudiments were cultured individually in 96-well plate inside a 40 µl drop of matrigel diluted 1:1 with DMEM/F-12/PS medium and incubated at 37ºC for 72 h. Medium was replaced every 24 h. Recombinant mouse FN (3, 6, 12.5, and 25 µg/ml, Sigma-Aldrich) were added to the medium. Branching morphogenesis of each cell aggregate was quantified as described earlier. In experiments where epithelial rudiments or the whole SMG tissue were used, branching was quantified as a fold change in the number of buds at each time-point (24, 48, and 72 h) divided by number of buds at 0 h.

**Statistical analysis**
Quantitative analyses were conducted in proportion and mean values with standard deviations were then calculated. Statistical significance was determined by using one-way analysis of variance (ANOVA) and post hoc tests, Scheffé’s F or Fisher’s PLSD tests, when required. The p-values are indicated in the figure legends.

Results

Analysis of cell movements and aggregate patterning during SMG self-driven morphogenesis

In order to develop a simplified culture model that closely mimics the initial stages of SMG branching morphogenesis in vivo, we modified a previously reported model. Briefly, we first isolated the whole SMG tissue from E13 embryos and enzymatically dissociated the SMG tissue to obtain single cells. Cells were then centrifuged to obtain aggregates in a 40 µl drop of Matrigel, and cultured in a 96-well plate for up to 72 h. In order to optimize the culture conditions of this 3D culture system, we first tried different cell concentrations. We found that a larger number of cells in the aggregate was required to develop branched buds that closely resemble native SMG development (Fig. 1a). Analysis of the rate of branching (quantified as the number of formed buds per cell aggregate) showed a directly proportional ratio between the initial cell concentration per aggregate and the final number of formed buds (Fig. 1b). Consequently, an initial concentration of $4 \times 10^4$ cells per aggregate was established as the optimal concentration that allowed the reproducibility and resemblance of the native E15 gland in terms of size and structure of the buds (Fig. 1a).

Next, we used time-lapse imaging to track cell movements and analyse the self-assembly of SMG cells into their tissue-like 3D patterns. As shown in Fig. 2a, the self-assembly process has shown the previously reported 3 stages; cell migration, aggregate compaction and bud branching stages. Time-course analysis of the changes in aggregate size (area, Fig. 2b) and shape (circularity, Fig. 2c) during tissue self-assembly showed that the total cell aggregate area markedly decreased in the initial migratory stage (0–6 h) (Fig. 2b; diagonal striped area). Tracking of individual cells at this initial migratory stage showed that cells performed rapid inward migration with marked differences between those cells in the outer and inner layers of the aggregate. In comparison to cells in the inner
core of the aggregate, cells in the outermost layer exhibited a 4-fold faster and 2.5-fold more directionally persistent movements in their speed and migration persistence, respectively (Fig. 2d).

In the subsequent aggregate compaction stage, further aggregate shrinkage resulted in highly dense aggregate, reaching the lowest projected area (of approximately 80% shrinkage from the initial aggregate area) at 16 h (Fig. 2b; horizontal striped area). This condensation typically occurs as a result of increasing cell-cell adhesion. During the compaction stage, a small number of epithelial buds started to be formed (Fig. 2a; white dotted circle). In the later stage of bud branching, the formed epithelial buds expanded through bud division. Concomitantly, we observed a repulsion of the scattered bud-free cells, mostly from mesenchymal cell population, to the outer side of the aggregate (Fig. 2a; black arrows), which reflected in a gradual increase in the total aggregate area in later stages (Fig. 2b; black dotted area).

The analysis of the aggregate circularity (a structural indicator of how close the aggregate shape is to that of a complete circle) showed a marked decrease during the early migration stage, and reached its minimum value of 0.2 at $t \leq 2$ h (Fig. 2c). Subsequently, the circularity gradually increased until it reached a constant value of 0.95 at $t \leq 26$ h (Fig. 2c; black dotted line). Observation of dynamic shape transformation up to this point $t \leq 26$ h suggests that during development, the cell aggregate reaches a state of equilibrium between the increasing condensation force derived from the cell-cell junction and the adhesion strength of cell-extracellular matrix environment. Of note, it has been generally proposed that tissues develop via a succession of equilibrium states in which the sum of the mechanical forces is in balance.

**Analysis of bud formation in self-assembled SMG tissues**

Based on our observation of the aggregate development, it appeared that the formed buds would attain different morphological characteristics according to their location within the developing aggregate. Individual buds were tracked and their respective sizes were measured throughout the culture time (0–72 h) (Fig. 3a). Buds within the inner circle of the aggregate (termed inner buds) were significantly smaller than those at the outer most layer (termed outer buds) (Fig. 3b). Additionally,
analysis of the frequency of the buds, in terms of their size, showed a gradual increase in the number
of larger buds in the outer bud group, which was evident at 72 h (Fig. 3c).

To address whether there was a correlation between the increase in bud size and cell number
inside each bud, we performed quantitative analysis DAPI-stained cell nuclei. The results showed that
outer buds presented a significantly higher number of cells compared to inner buds, from 24 h onward
(Fig. 3d and 3e). We hypothesized that this evident variation in cell number could be an indication of
differential cell proliferation rates between the inner and outer buds. Indeed, cell proliferation analysis
showed enhanced expression of the cell proliferation marker, Ki67, in cells in the outer buds
compared to cells in the inner buds (Fig. 3f).

Taken together, these results suggest that the inner and outer buds could be subjected to
different levels of compaction depending on their location within the aggregate, and that could also
affect the proliferation of epithelial cells inside the buds, which eventually would reflect the size
difference between the inner and outer buds.

**FN induces branching of epithelium and marked ductal elongation**

FN promotes branching morphogenesis in the entire SMG\(^{10}\). However, reports about the
effect of FN on the development of other tissues suggest that FN still could have unknown roles in
SMG branching. To address this possibility, we investigated the effect of FN on the very initial steps
of SMG branching morphogenesis using our developed 3D SMG cell aggregate system. Cell
aggregates were supplemented with exogenous FN (3, 6, 12.5, and 25 µg/ml) and cultured for up to
72 h. Interestingly and unexpectedly, we observed that exogenous FN induced significant ductal
elongation in branched SMG aggregates with as little as 3 µg/ml dose (Fig. 4a and 4b). However, a
dose of 25 µg/ml FN induced an inhibitory effect, compared to 3, 6, and 12.5 µg/ml FN. To confirm
the ductal tubulogenesis, we performed an immunostaining analysis of the developing aggregate for a
specific duct marker cytokeratin 7 (ck7) and filamentous actin (F-actin), which delineates the
cytoarchitecture, to show organized ductal structures with group of aligned ck7 positive cells with
prominent F-actin filaments (Fig. 4c).
To eliminate the hypothesis that these effects of FN on cell aggregates could be due to the culture condition in this 3D SMG cell aggregate model, we tested the effect of FN supplementation in cultures of epithelial rudiments with similar doses (3, 6, 12.5, and 25 µg/ml FN). The FN concentration needed to induce the maximum ductal expansion effect in case of epithelial rudiments was found to be higher than that needed for SMG cell aggregates (12.5 and 3 µg/ml, respectively) (Figs. 5a-c). Based on these results, we established that our developed 3D self-assembly model would be a valuable tool in understanding the mechanisms of SMG tissue development.

Discussion

The term “self-organization” generally refers to the spontaneous formation of well-ordered structures from individual elements. This phenomenon has been widely observed throughout nature in a variety of non-living matter as well as in multiple biological systems. For instance, 3D culture of Madin-Darby canine kidney (MDCK) cells and aggregate culture of glandular tissues such as mammary epithelium have been reported to acquire the ability to autonomously form ordered structural patterns in the absence of external guidance. Recently, this concept of self-organization has garnered considerable attention as it has proved to be advantageous in organ regeneration therapy, especially in the attempts to reconstitute 3D organ buds (organoids). In our study, we optimized a 3D culture model system to study the self-organizing nature of embryonic SMG tissue development, including the effect of mechanical and chemical cues on self-organization. In particular, we investigated the effect of FN on SMG development, and identified the novel role of FN in ductal formation.

The self-organizing nature of SMG cells has been described by Wei C. et al. Using a culture model of embryonic SMG epithelial cells in Matrigel, they demonstrated the capacity of these cells to self-organize into branched SMG-like structures in an ordered, multistep process that typically starts with an initial β1 integrin-dependent cell migration stage in which cells persistently migrate to form a loose aggregate. This is followed by an E-cadherin-dependent compaction stage in which aggregate contraction occurs to form a condensed solid mass of epithelial cells. Finally, it progresses...
to the branching stage, which is characterized by the branching of multiple buds that resemble those formed during natural SMG development\textsuperscript{16}.

On the other hand, in order to obtain a deeper understanding of the complex and multi-scaled process of tissue self-organization, it is necessary to analyse the dynamics of multicellular interactions in real time and space\textsuperscript{33}. For instance, in a multicellular tissue-like SMG, different epithelial-mesenchymal cellular interactions typically occur during self-driven morphogenesis\textsuperscript{34}. Such intercellular interactions would be responsible for the generation of local internal mechanical forces, which in turn would lead to dynamic and cooperative tissue morphological responses\textsuperscript{33}. This fact indicates the need to build a more sophisticated branching model for SMG development that account for the dynamic spatio-temporal interactions between the epithelial and mesenchymal cells, which a model, based on only epithelial cell aggregate, will not be able to provide adequately.

In this context, we conducted this study using a model of 3D whole SMG cell aggregate in a Matrigel substrate to provide a platform to re-create and understand the dynamic nature of SMG development. Thorough observations of this process showed interesting results: the highly dynamic SMG cell aggregate self-organization affects the morphology of the formed tissue through location-dependent bud size variation. This variation marked the difference between the group of buds which occupies the inner core of the aggregate (inner buds) and those at the outermost layer of the aggregate (outer buds). Furthermore, it results in a spatially controlled growth differential between cells in both outer and inner buds, in the form of a significant location-dependent variation in the number of cells forming each bud. In regard to the mesenchymal cell population, it appears that the spatio-temporal cellular re-arrangements allowed epithelial buds to occupy the core of the developed aggregate leading to repulsion most of these mesenchymal cells to outer layers after conveying their inductive signalling to the developing epithelium.

Several mechanisms have been reported, through which the mechanical compression can influence cell proliferation/differentiation and thus generate such spatial growth differential within tissues\textsuperscript{22}. For instance, tissue compression and internal stresses were found to play a pivotal role in regulating size and morphology in tissues such as the \textit{Drosophila} imaginal disc, where a compression gradient resulted in a homogenous proliferation rate across the tissue, and consequently, can control
its size\textsuperscript{22}. In addition, the higher levels of compaction applied on the inner buds can disrupt isometric condition of their basement membrane. This state of dynamic equilibrium is maintained by the balance between collective cell traction forces generated by the constituent cells of each bud and the resisting forces applied by the ECM\textsuperscript{35}. Therefore, any external force that disrupts this dynamic equilibrium consequently feeds back on the isometric tension of the cells themselves. Disruption of the cellular cytoskeleton tension balance can negatively impact cellular growth and inhibit epithelial morphogenesis in embryonic salivary gland\textsuperscript{36}. An alternative mechanism involves “cell competition”, which is a prevalent feature in epithelial tissues\textsuperscript{37}. However, in this model, cells of the inner buds are not completely eliminated, but are surpassed in growth by the outer buds. As aggregate development proceeds, inner buds are trapped in the inner core and get gradually distanced from sources of nutrition, having no direct contact with the surrounding Matrigel environment and inductive mesenchymal cells. Consequently, these buds get outcompeted by the outer buds, which benefit in these conditions and grow larger, occupying more space at the expense of the inner buds. Moreover, it is likely that inner cells adopt slower proliferation rates as a result of their local density, which substantially increases during the compaction stage. This phenomenon is known as ‘contact inhibition of proliferation’\textsuperscript{38}. Nevertheless, it is more likely that a combination of physical and chemical factors may account for this growth differential. Therefore, our findings would be helpful in developing a biophysical model to analyse the dynamics of multicellular interactions in 3D cell aggregates.

Previous reports regarding salivary branching identified FN as a key regulator of cleft formation\textsuperscript{10}. However, reports about other glandular tissues branching suggested that FN played a wider role in branching morphogenesis. For instance, FN expression has been shown to affect the acinar size in mammary gland branching by modulating proliferation of acinar cells\textsuperscript{39}. Furthermore, FN was found to induce ureteric bud cells tubulogenesis in 3D gel cultures\textsuperscript{40}. We previously showed that the integrin-binding domain of FN, the tri-peptide sequence arginine-glycine-aspartic acid (RGD), can successfully modulate in vitro SMG tissue growth and morphology\textsuperscript{23}. This result was noteworthy because RGD enhanced SMG growth along with both, bud formation and duct elongation. Taken together, these findings strongly suggest possible wider roles of FN in SMG branching. In accordance, here, we report that low concentrations of FN can in fact induce tubulogenesis in 3D
SMG cell aggregates. In addition, FN can directly work on isolated SMG epithelial rudiments and induce marked duct elongation.

This effect of FN may have been masked in earlier reports by the effect of other additional growth factors supplementations, such as FGF7 and EGF\textsuperscript{10}. FGF10 has been shown to induce ductal elongation in developing SMG through the FGF10/FGFR2b-mediated signalling\textsuperscript{41}. However, FGF10 was also found to bind to FGFR1b\textsuperscript{42}. Interestingly, FN has shown an ability to activate FGFR1b, independent of FGF ligand, in liver endothelial cells\textsuperscript{43}. It is possible that FN induces ductal elongation in epithelial rudiment cultures through a similar mechanism. Moreover, heparan sulphate (an ECM glycoprotein) was reported to function as a co-receptor or cofactor that can regulate the activity of FGFs\textsuperscript{44} and specifically allow FGF10 to stimulate cells of SMG branches\textsuperscript{41}. It is likely that FN, which is another ECM glycoprotein\textsuperscript{10}, can similarly work as a cofactor and regulate FGF10 function. However, a detailed mechanism of the role of FN remains to be determined. This would be very important, especially since multicellular self-organization has become an emerging concept in the field of tissue regenerative therapy.

In summary, we present a simple and valuable \textit{in vitro} 3D culture system which can be used effectively in organ generation studies. The efficiency of this system was demonstrated by analysing the intercellular dynamics during early SMG tissue development as well as revealing a novel role of FN in SMG ductal expansion.
References


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**Competing financial interests**

The Authors declare no competing financial interests.
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Figure 1
Generation of in vitro 3D SMG cell aggregate a) Evaluation of self-driven morphogenesis of E13 SMG cell aggregates with different initial cell concentrations. b) Quantification analysis of bud number per aggregate at each time point. Branching increased significantly with increased number of cells per aggregate (* p<0.01, ANOVA, Scheffé’s F test). (Scale bar; 200 µm)

Figure 2
Time-lapse analysis of self-driven cell migration and aggregate development. a) Cells aggregate undergo marked size and morphology changes during the culture period. As aggregate develops, a cloud of scattered mesenchymal cells (black arrows), can be seen surrounding the core of the formed epithelial buds. (Scale bar, 100 µm). b) Structural analysis of 3D aggregate area changes. A is the aggregate projected area. Blue dotted line marks the extent of the migration stage, red dotted line marks the smallest area at 16 h. following the compaction stage. c) Aggregate circularity changes. Circularity was defined as $4\pi A$ divided by the square of the contour line length; $L^2$. Red dotted line marks the closest point of the aggregate as a circle. The source movie is Movie S1. d) Average cell velocity for each layer was measured (µm/min) (* p<0.01, ANOVA, Scheffe’s F test). e) Persistence measured as displacement divided by total track length (D/T) (* p<0.01, ANOVA, Scheffe’s F test). The source movie is Movie S2.

Figure 3
Evaluation of temporal and spatial morphological changes and subsequent cell proliferation in the cell aggregates a) Outer buds exhibit morphologically larger areas than inner buds. White dotted line represents line of demarcation between the two bud groups (Scale bar; 200 µm). b) Size quantification of both groups shows significantly larger outer bud area after 48 h culture period (* p<0.01, ANOVA, Scheffe’s F test). c) Time-course size frequency analysis of inner and outer buds showing a gradual increase in frequency of outer buds with larger areas (> 0.006 mm$^2$) over the culture period. d) Time-course immunostained images of whole aggregate with nuclear staining DAPI (blue) showing outer
buds (upper panel) and inner buds (Lower panel). White inserts showing magnified images of developing buds (Scale bar; 100 µm). e) Quantification analysis of cell number per bud in inner and outer buds over the entire culture period. Starting from 48 h, outer buds show significant increase in cell number (* p<0.01, ANOVA, Fisher’s PLSD test). e) Cell proliferation evaluation using cell proliferation marker Ki 67 and counterstained with counterstained for F-actin with rhodamine phalloidin. At 72 h, outer buds show more cells positively expressing Ki67 cells in inner buds, suggesting a reduction in cell proliferation at the inner area of the aggregate. White inserts marking location of inner and outer buds (Scale bar; 100 µm).

Figure 4
Fibronectin induces branching of epithelium and marked ductal elongation in SMG cell aggregate. a) Effect of FN on SMG cell aggregates growth was evaluated by culturing aggregates inside matrigel with different concentrations of recombinant FN for 72 h. White dots demarcates the expanded ducts (Scale bar; 200 µm). b) Quantitative analysis of ductal elongation showing that 3 µg/ml concentration induced highest ductal expansion. (* p<0.01, ANOVA, Scheffé’s F test). c) Duct formation in the developing aggregates. Left; bright field image shows the elongating structure in cell aggregate supplemented with 3µg/ml FN. White dots demarcates the expanded ducts. Middle; aggregates were stained with an antibody to the duct-specific marker, cytokeratin 7 (ck7) (green), and counterstained for F-actin with rhodamine phalloidin (red) (Scale bar = 100 µm). Right; high magnification of regions of F-actin localization overlapped with K7 staining (white arrows) confirming identification of these extended areas as ductal structures (Scale bar = 50 µm).

Figure 5
Fibronectin induces branching of epithelium rudiments with marked ductal elongation, epithelial rudiments were dissociated from early E13 mice. a) FN action was tested by culturing rudiments inside drop of Matrigel with different concentrations of recombinant FN for 72 h. (Scale bar; 200 µm). b, c) Quantitative analysis of ductal elongation and bud number showing that 12.5 µg/ml
concentration induced significant increase in ductal expansion and bud growth at 72 h. (* p<0.01, ANOVA, Scheffe’s $F$ test).