Published in final edited form as:

Nat Rev Mol Cell Biol. 2014 October ; 15(10): 647–664. doi:10.1038/nrm3873.

Three-dimensional organotypic culture: experimental models of mammalian biology and disease

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Abstract

Mammalian organs are challenging to study as they are fairly inaccessible to experimental manipulation and optical observation. Recent advances in three-dimensional (3D) culture techniques, coupled with the ability to independently manipulate genetic and microenvironmental factors, have enabled the real-time study of mammalian tissues. These systems have been used to visualize the cellular basis of epithelial morphogenesis, to test the roles of specific genes in regulating cell behaviours within epithelial tissues and to elucidate the contribution of microenvironmental factors to normal and disease processes. Collectively, these novel models can be used to answer fundamental biological questions and generate replacement human tissues, and they enable testing of novel therapeutic approaches, often using patient-derived cells.

The anatomical basis of life was first studied by natural historians who identified and named organs across species. A crucial simplification came when Bichat recognized that organs represented combinations of a few fundamental tissues¹. Compound microscopes enabled Virchow to define epithelium, connective tissue, nerve, muscle and blood as the universal tissues², and by 1900, the microscopic anatomy of humans was well known³. However, it remains difficult at a cellular and molecular level to understand how mammalian organs form during development and how they change during disease.

Compared with the transparent embryos of externally developing species, mammalian tissues and organs are fairly inaccessible to experimental manipulation and optical observation. Furthermore, mammalian development occurs over the time range of days to years. These limitations led Harrison *et al.* to develop twodimensional (2D) culture techniques in 1907 (REF. 4). 2D culture enabled biologists to observe and manipulate mammalian cells and laid the foundation for cell and molecular biology. However, 2D cultures do not completely recapitulate the three-dimensional (3D) organization of cells and extracellular matrix (ECM) within tissues and organs. Consequently, there is a large gap between our detailed knowledge of sub cellular processes and our incomplete understanding of mammalian biology at the tissue level. Dynamic analyses of organogenesis have instead

relied on model systems, such as *Caenorhabditis elegans*, *Drosophila melanogaster*, *Xenopus laevis* and zebrafish.

The goal of reconstituting organ function *ex vivo* is broadly shared, and there are successful examples for most tissues and organs (TABLE 1). In pursuit of this goal, a wide range of techniques has been developed that are referred to as 3D culture, organotypic culture or organoid culture. Various subfields use these terms either interchangeably or distinctly; for example, in the field of mammary gland biology, the term organoids refers to primary explants of epithelial ducts into 3D ECM gels⁵. Conversely, in studies of intestinal biology, organoids can refer to clonal derivatives of primary epithelial stem cells that are grown without mesenchyme⁶ or can refer to epithelial–mesenchymal co-cultures that are derived from embryonic stem (ES) cells or induced pluripotent stem cells (iPS cells)⁷.

In this Review, we first provide an overview of the commonly used cellular inputs and culture formats. We then discuss how these experimental systems have been used to visualize the cellular mechanisms that drive epithelial tissue development, to study the genetic regulation of cell behaviours in epithelial tissues and to evaluate the role of microenvironmental factors in normal development and disease. Finally, we provide examples of how 3D culture techniques can be used to build complex organs, to generate replacement human tissues and to advance therapeutic approaches.

Cellular inputs into 3D culture

To understand how mammalian organs can be cultured *ex vivo*, it is useful to consider their constituent parts. The external surfaces of the body and the linings of organs are built from epithelial tissues⁸. Epithelial cells are connected to each other by intercellular junctions and are located within a specialized ECM, which is known as the basement membrane. These cell–cell and cell–ECM interactions are not completely modelled in 2D culture. Epithelial tissues are avascular and exist in close proximity to vascularized connective tissue⁸. In contrast to the epithelium, connective tissue contains an abundance of ECM and a diverse population of stromal cells, including fibroblasts, immune cells and adipocytes⁸. Epithelium and connective tissue are functionally interdependent units within organs and are integrated with nerves and muscle to varying degrees depending on their organ-specific function.

The first crucial design choice in 3D culture is the extent to which the full *in vivo* complexity of the organ is recapitulated. Organ function results from cooperation among different tissues, but it can be difficult to isolate the roles of specific genes or cell behaviours *in vivo*. One approach is to deconstruct organs into their parts (for example, epithelium, stromal cells, vasculature or ECM) and then selectively recombine these parts in 3D culture. Embedding epithelial cells within an ECM gel enables the cells to self-assemble into tissues and to both interpret and remodel the ECM⁹. Similarly, endothelial cells and perivascular cells can be patterned into functional, perfused networks within ECM gels to model vascular development within connective tissue^{10–12}. Alternatively, multiple tissue components can be combined into the same culture.

Whole-organ and organ-slice cultures

Conceptually, the simplest unit that can be explanted into 3D culture is the whole organ (FIG. 1a). This approach was successfully used to study skeletal development as early as 1929 (REF. 13) and was extended to the kidney, lung, salivary gland, liver, pancreas and mammary gland by the mid-twentieth century^{14–16}. However, the limited diffusion of extracellular molecules into thick tissues restricts these studies to embryonic or thin organs. Alternatively, organs such as the lung¹⁷, small intestine^{18,19}, colon^{19,20}, brain^{21,22} and aorta²³ can be sectioned into tissue ‘slices’ and mounted onto siliconized filter paper²⁴ or porous culture membranes²⁵ for mechanical support. Slices from the same organ can be subjected to different experimental conditions, which enables the evaluation of both matrix-bound and soluble paracrine signals; for example, the lactogenic hormones were defined by their ability to induce milk production in whole mammary gland cultures derived from virgin mice²⁶. Although these culture formats enable optical access to the tissue and experimental interventions within native stromal tissues, it can be challenging to maintain tissue viability, and the interpretation of experimental manipulations is more difficult within complex organs.

Tissue organoids

A fundamental developmental question is whether epithelial cells determine organ pattern or whether the pattern emerges from a dialogue between the epithelium and the connective tissue or mesenchyme. To answer this question, epithelial tissues are isolated and cultured without their corresponding stromal cells; this approach is known as mesenchyme-free or organoid culture (FIG. 1b). Tissue organoids are freshly isolated from primary mammalian organs for every experiment. Each tissue organoid contains several hundred cells, which are accessible to signalling molecules and can be genetically modified using robust lentivirus- and adenovirus-based techniques^{27–31}.

Organoid protocols have been developed for the mammary gland^{5,32}, salivary gland³³, kidney³⁴, lung³⁵, small intestine^{6,36}, colon^{36,37}, liver³⁸, stomach³⁹, pancreas⁴⁰ and prostate⁴¹. Tissue organoids are typically explanted into commercial matrices, such as Matrigel⁴² or collagen I⁴³, which enable optical imaging. As pieces of tissue are explanted intact into culture, the resulting organoids contain diverse epithelial cell types that are organized in their normal spatial configurations as observed *in vivo*. This culture format has been used to study the cell movements that drive organogenesis and to model the cell and tissue consequences of genetic changes.

Stem cell organoids

Organoids can also be expanded from ES cells, from iPS cells or from primary stem cells that have been purified from organs (FIG. 1c); for example, primary tissue-derived, intestinal stem cells that express Leu-rich repeat-containing G protein-coupled receptor 5 (LGR5) clonally generate crypt–villus architecture in 3D culture⁶. This approach was extended to the stomach³⁹, colon³⁷, pancreas⁴⁰ and liver³⁸. Investigators have also developed robust techniques to generate stem cell organoids from ES cells and iPS cells (TABLE 1). An advantage of iPS cell-derived organoids is that they can be generated from cells from the patient. ES cell- or iPS cell-derived organoids have also been used to

demonstrate the self-organization of the retina^{44,45}, cerebral cortex^{46,47} and pituitary⁴⁸. However, *in vivo* organs do not expand from single isolated stem cells, and therefore the mechanisms that drive the formation of stem cell organoids may be distinct from organogenesis *in vivo*. For example, cortical organoids that are formed from ES cells generate stratified structures with layer-specific neuronal differentiation⁴⁶; however, the inside-out pattern of layer formation that is observed *in vivo* is reversed in 3D culture⁴⁶. Nonetheless, the extent to which brain anatomy can be recapitulated from defined cellular and molecular starting materials is remarkable^{46,47}. An additional issue is the timing of molecular interventions in tissues compared with that in single cells, as differences in timing could easily change phenotypes.

Reaggregated single-cell suspensions

Clonal expansion from a single ES cell or iPS cell requires many rounds of cell division to generate organoids. Accordingly, many 3D culture assays start from suspensions of single cells, such as cell lines, stem cells or freshly isolated primary cells (FIG. 1d). Classic amphibian embryology experiments showed that disaggregated single cells would spontaneously reaggregate and recapitulate their normal tissue architecture⁴⁹. Similarly, mammalian kidney⁵⁰ or mammary⁵¹ cell lines readily form acini from single cells when cultured on top of Matrigel. These epithelial models were used to dissect the molecular basis of epithelial adhesion and polarity^{50,51}. A single-cell suspension can also generate more complex tissues; for example, isolated epidermal or oesophageal keratinocytes organize into a stratified epithelium with highly realistic tissue architecture^{52–54}. Similarly, dissociated cells from mouse embryonic kidneys reaggregate to form renal structures that contain epithelial-derived ureteric buds and mesenchyme-derived nephrons^{55,56}. These approaches facilitate the formation of chimeric tissues that consist of different cell types or cells with distinct genetic modifications.

Culture formats for 3D culture

In addition to the cellular inputs, culture formats can be varied independently in order to answer specific biological questions (TABLE 1).

2.5D cultures

The addition of basement membrane proteins to the medium in 2D cultures is sufficient to induce tissue-specific differentiation of diverse epithelial cells, including mammary⁵⁷, kidney⁵⁸ and lung⁵⁹ (FIG. 2a). Most experiments rely on a commercial basement membrane protein source, such as Matrigel⁴². Conversely, epithelial tissues often lose their differentiated state and migrate individually when cultured in a stromal matrix such as collagen I (REF. 60). These observations led to the development of diverse assays in which single cells are plated on top of Matrigel, with additional Matrigel in the medium^{50,51,61}. This technique is frequently referred to as two and one-half-dimensional (2.5D) culture or ‘drip’ culture, in reference to the ECM in the medium (FIG. 2b). This format does not perfectly model the *in vivo* environment, as the cells contact a large fluid reservoir that they would not encounter *in vivo*. As a result, fluid-facing surfaces of the cell lack contact with the ECM, and cell-generated paracrine factors are diluted. Despite these limitations, 2.5D

assays are experimentally convenient, they induce cells to form a more physiological tissue architecture than 2D assays and the cells remain accessible for molecular analysis⁵¹.

3D-embedded cultures

To better model *in vivo* tissue organization, cells can be fully embedded within 3D ECM gels (FIG. 2c). Tissue organoids that are embedded in 3D ECM have been used to study the branching morphogenesis of the mammary^{5,32,62}, salivary^{33,63}, kidney⁶⁴, lung³⁵ and pancreatic ductal⁶⁵ epithelium. In this format, both the composition and mechanics of the ECM environment can be varied^{66–71}. Collagen I models the ECM of connective tissue, basement membrane extracts (such as Matrigel) recapitulate the tissue context of epithelial ducts, and fibroblast-conditioned matrix, which is rich in fibronectin, models the microenvironment during wound healing. Embedded cultures also support a broader and more complex range of tissue architectures than is typically observed in 2.5D formats. However, owing to the location of the cells within a thick ECM gel, both optical imaging⁷² and the recovery of cells are more complex than in 2.5D cultures.

Mechanically supported cultures

Both whole-organ explant cultures and stratified epithelial cultures that have been reconstituted from single cells are typically cultured on top of mechanical supports. These cultures were historically grown on top of siliconized paper^{14,16,24} and, more recently, have been grown on cell culture inserts with semipermeable membranes (FIG. 2d; TABLE 1). For organ explants, these membranes provide flexible support and enable the delivery of nutrients and signalling molecules to the bottom surface of the tissue, and optionally to the top surface. Epidermal keratinocytes that are seeded on ECM-coated culture inserts form a monolayer when submerged, and they stratify and differentiate into epidermis when exposed to an air–liquid interface^{52,53}. The addition of fibroblasts to a floating collagen lattice enables the formation of an underlying dermis and the self-organization of full-thickness human skin⁵³. Similar approaches generate histologically realistic normal and neoplastic models of oral, oesophageal and cervical epithelium^{54,73} (TABLE 1). These reconstituted tissues are used to study normal development and disease processes, and they can also be used for toxicity assays.

Bioengineering-inspired culture systems

Although we cannot review them in detail here, a diverse range of engineered culture assays has also been developed, both to answer fundamental biological questions and to function as platforms for constructing replacement tissues. Important examples include synthetic polymer systems for 2.5D and 3D cultures^{74–76}, integration of microfluidic systems in complex cultures^{77–79}, cell surface-modification techniques to pattern the assembly of epithelial tissues⁸⁰, microfabricated 3D environments to control tissue geometry and mechanics⁸¹ and 3D patterning of perfused vascular networks^{10,11}.

Imaging in 3D cultures

Imaging in 3D cultures enables a continuous cellular and molecular description of tissue-level development over a time period of days to weeks⁷². Imaging is typically carried out

using an inverted microscope and requires the robust control of temperature, humidity, CO₂ and evaporation. A major obstacle is the scattering of light in thick 3D cultures. One strategy to minimize scattering is to simplify the culture to its core components, such as by using tissue organoids rather than whole organs^{62,82}. A second approach is to match the index of refraction of the immersion medium to that of the culture medium. Finally, the working distance of the lens must be sufficient to image structures throughout the culture.

Different microscope systems can be used in a complementary way. Differential interference contrast enables optical sectioning in thick ECM gels and label-free visualization of collagen I fibres. Confocal imaging enables multicolour 3D time-lapse imaging up to 70–100 µm deep within the tissue^{82–85}, and two-photon microscopy facilitates deeper imaging and visualization of fibrillar ECM using second harmonic signals^{43,86}. However, limitations of two-photon microscopy include increased cost and high levels of energy deposition at the plane of focus. Finally, 3D culture samples can be prepared for transmission electron microscopy and scanning electron microscopy^{27,59,87,88}.

Tailoring the culture format

3D culture assays would ideally be perfectly representative of the *in vivo* situation, easy to manipulate and inexpensive. However, in practice, trade-offs must typically be made, especially with primary human tissues. It is useful to identify the main experimental goal and then tailor the 3D culture to achieve that goal; for example, replacement human tissues need to be large in size and incorporate into the host vasculature, and the host response to the graft needs to be minimized. By contrast, 3D cultures that are designed for drug screening should ideally fit within 384-well plates, have a low cost per well and predict the results of testing in preclinical animal models and human clinical trials. Important factors to consider include the throughput that is required in the assay, the ease of molecular manipulations and molecular readouts, the necessary recapitulation of the *in vivo* histology and the availability of primary cells and tissues for that specific organ. For a close match to *in vivo* histology, the more complex embedded-culture or culture-insert models are most suitable. For genome-scale molecular-interference approaches, the least complex culture system enables the most rapid analysis. Efficient use of limited starting materials is required if the cultures involve primary human tissues. If optical imaging is crucial, the 3D culture needs to be relatively thin and transparent. Finally, the goals of the experiment will determine which cell types need to be incorporated. Many published assays are essentially monocultures of epithelial cells, endothelial cells or neurons. However, each assay format can incorporate additional cell types; for example, cancer cells can be incorporated to study the interactions between normal and neoplastic cells, and stromal cells can be incorporated to study the interactions between epithelial and stromal cells^{54,89}.

The great variety in 3D culture techniques reflects both the various requirements of the different organs and the distinct goals of the investigators. A large body of work has been published on 3D, organotypic or organoid culture, the results of which cannot be comprehensively reviewed here. However, a few common themes emerge that both capture the challenges that motivated the development of 3D culture techniques and illustrate the various biological questions that can be uniquely answered using these approaches.

Organ morphogenesis

Understanding how cells build tissues and organs is of fundamental interest. However, the long timescale of mammalian development and its location deep within an opaque animal limit most organogenesis studies to comparisons of fixed sections from different animals. Owing to the large number of cells and the morphological heterogeneity of developing organs, these studies often cannot distinguish among several mechanistic possibilities of organ formation. Furthermore, an imprecise understanding of the normal developmental trajectory necessarily limits our ability to identify the molecular and cellular differences that define the mutant phenotype. Accordingly, many 3D culture techniques were initially developed to enable the direct observation of developmental processes. In the examples below, we mainly focus on epithelial tissues, as their formation is extensively described in the literature, and highlight common mechanistic themes as well as species- and organ-specific differences.

Mechanisms that drive epithelial tube elongation

A good example of the application of 3D cultures in developmental biology is the study of epithelial tubulogenesis. This process involves an increase in epithelial surface area and changes in tissue shape (FIG. 3a). Tube elongation can, in principle, be accomplished through various combinations of changes in cell number, cell shape and cell size. Terminal branching of the *D. melanogaster* trachea occurs by subcellular branching of the leading cell⁹⁰, without proliferation, whereas mammalian tubulogenesis in the mammary gland, salivary gland, kidney and lung involves a large increase in epithelial tissue size (FIG. 3b–e). The iterative branched structure of these tubular networks and the simple epithelial organization of the resulting ducts initially led to the concept of a conserved process of branching morphogenesis⁹¹.

However, it remained an open question whether conceptually related processes of ductal elongation and bifurcation occurred via a common sequence of cellular events across these organs. Answering this question was challenging as it was difficult to be certain that a duct was elongating at the moment it was fixed. 3D confocal imaging of 3D cultures of the mammary gland, salivary gland, kidney and lung has in fact shown that these organs have distinct cellular mechanisms of branching morphogenesis. Mammary ducts first undergo a simple to stratified transition, elongate as a stratified epithelium and then polarize to re-establish simple ductal architecture^{62,82,88} (FIG. 3b). Salivary glands start as a stratified epithelium that first clefs and then progressively polarizes to form a network of simple ducts^{29,92} (FIG. 3c). The kidney ureteric bud transitions from simple to pseudo-stratified, elongates as a pseudostratified epithelium and then polarizes to a simple epithelium^{93,94} (FIG. 3d). By contrast, epithelial buds initiate and elongate as a polarized simple epithelium during monopodial branching in the chick lung⁸⁵ (FIG. 3e). Therefore, these tubes have distinct tissue architectures and use different cellular mechanisms during elongation.

Cell proliferation in tubulogenesis

The imaging analyses that are discussed above generated a general framework for branching morphogenesis that enabled researchers to identify the underlying cellular mechanisms. In

turn, cell division was shown to have distinct roles in different epithelial organs; for example, asymmetric divisions within mammary luminal epithelial cells initiate stratification and lead to a loss of polarity during both development and ERBB2-induced oncogenic stratification⁸². Salivary gland epithelial cell proliferation is increased at ductal tips and involves heparan sulphate-mediated regulation of FGF10–FGFR2 signalling; disruption of heparin signalling results in an expanded zone of proliferation and abnormal branching⁹⁵ (FIG. 3f). In the ureteric bud, proliferating cells delaminate into the luminal space, and daughter cells reintegrate at non-adjacent locations, which leads to mixing of different cellular populations⁹⁶ (FIG. 3g). The requirement for proliferation in organ development also varies across species. In the chick lung, monopodial branching occurs even in the absence of proliferation⁸⁵ (FIG. 3h), whereas in the mouse lung, both domain branching and bud bifurcation involve polarized cell divisions⁹⁷ (FIG. 3i). These species-specific and tissue-specific differences in cell proliferation during tubulo-genesis highlight the importance of real-time analysis as a framework for interpreting molecular interventions. Understanding the role of cell proliferation in development is also relevant in cancer, as oncogenic activation of proliferation might have different consequences in different organs.

Gene regulation of cell behaviour

3D culture techniques have also been developed to elucidate the molecular mechanisms that regulate tissue organization and function in developmental and disease states. Although *in vivo* studies can test whether a gene is required for organ development, they cannot always determine how the gene regulates cell behaviours to change tissue architecture and function. Conversely, the roles of individual proteins in biochemical pathways have mostly been elucidated in 2D culture studies. However, these molecular interactions can differ in a 3D tissue context; for example, the molecular composition and phosphorylation status of focal adhesions within fibroblasts are different on 2D surfaces compared with 3D ECM gels⁹⁸. RNAi, Cre–*lox*-based recombination and lentiviral short hairpin RNA (shRNA) approaches have all recently been adapted to 3D culture, which has enabled the evaluation of single genes and genome-scale screening in tissues, including mammary²⁷, intestinal²⁸ and salivary^{99,100} epithelium. The application of CRISPR–Cas9 (clustered, regularly interspaced short palindromic repeats–CRISPR-associated protein 9)-based genome editing¹⁰¹ further provides the potential for the rapid introduction and functional correction of disease mutations¹⁰².

Tissue-specific genetics

MDCK (Madin–Darby canine kidney) cell acinar formation in 2.5D culture provided an early model of a generic epithelium that was readily accessible to genetic manipulation^{50,58}. The molecular machineries that guide polarity initiation, the specification of an apical membrane and lumen formation were identified in this system and provided a conceptual foundation for our current understanding of polarity in more complex tissues^{58,103–106}. Analogous studies in 2.5D cultures of MCF-10A mammary epithelial cells enabled the detailed dissection of the role of growth factor receptors and polarity proteins in normal development and cancer^{107–110}. Both systems have facilitated high-resolution, real-time studies of the role of oncogenes in disrupting normal epithelial cell behaviours^{111,112}.

Nevertheless, these cell lines lack important features of epithelial tissues; for example, MCF-10A cells lack tight junctions⁸⁷.

Ultimately, the molecular pathways that have been defined in cell line-based assays need to be validated in more physiological contexts; however, many genes are required during early mammalian embryonic development, which prevents the study of their role in later developmental processes. This challenge has been overcome by the use of tissue-specific promoters to induce gene deletion or overexpression at later stages. However, many promoters are mosaically expressed within organs, which leads to complex mixtures of wild-type and genetically modified cells. Depending on the phenotype, this heterogeneity can greatly complicate the interpretation of gene function. This challenge can be overcome by combining *ex vivo* gene manipulation and 3D culture of primary tissues. Knockdown of *p63* using siRNA showed that this transcription factor (which is essential for neonatal survival) is required for both proliferation and differentiation in regenerating organotypic epidermis, and distinct contributions are made by different *p63* isoforms¹¹³ (FIG. 4a). Depletion of *p63* leads to tissue hypoplasia, defects in epidermal stratification and the induction of simple epithelial markers¹¹³. In genetic mosaic epidermis, retrovirally delivered LacZ and haemagglutinin epitope-tagged keratin 14 labels were used to distinguish between wild-type and *p63*^{-/-} populations and to show that control of differentiation by *p63* is cell-autonomous¹¹³. Additional regulators of epidermal differentiation, such as the long non-coding RNA *TINCR*, have been identified by further combining human organotypic skin cultures with transcriptome sequencing, RNAi, protein micro arrays and RIA-Seq (RNA interactome analysis, followed by deep sequencing)¹¹⁴.

Incorporation of fluorescent reporters simplifies the interpretation of mosaic tissues, as it enables real-time comparisons between genetically modified cells and wild-type cells; for example, deletion of epithelial cadherin (*E-cadherin*) in a large fraction (>80%) of mammary epithelial cells in tissue organoids does not prevent the initiation of new epithelial ducts²⁷. This result suggests that *E-cadherin* is dispensable for mammary morphogenesis; however, real-time imaging using Cre biosensors showed that these new ducts are elongated by *E-cadherin*-expressing cells²⁷. *E-cadherin* is in fact required for the maintenance of epithelial architecture and branching morphogenesis²⁷. Importantly, this is consistent with *in vivo* findings, and therefore, the combination of *ex vivo* gene manipulation and fluorescent biosensors enables the rapid evaluation of gene requirement in primary tissues.

Real-time genetic analysis

Real-time tracking can be used to determine the effects of genetic modifications on specific cell behaviours; for example, studies using fluorescent labelling, siRNA knockdown and time-lapse imaging identified the signals that are required for salivary gland clefting^{29,92,115}. Gene expression analysis revealed increased levels of BTBD7 in epithelial cells located within clefts²⁹ (FIG. 4b). Moreover, it was shown that BTBD7 promotes cleft progression by inducing *Snai2* (also known as *Slug*) expression and reducing *E-cadherin* levels²⁹. Live-cell imaging has also been used in embryonic whole-lung explants to demonstrate the requirement for the polarity protein Scribble in lumen morphogenesis in the mammalian lung but not in bud bifurcation¹¹⁶; the depletion of Scribble in lungs leads to the

mislocalization of junction proteins and misalignment of distal epithelial cells. Importantly, the finding that Scribble is required for epithelial cell–cell contacts and lumen morphogenesis is consistent with the *in vivo* Scribble (*Scrib*) loss-of-function phenotype (that is, small, misshapen lungs, reduced airway number and defects in epithelial architecture)¹¹⁶. Thus, genetic manipulation, together with live-cell imaging in 3D culture, identified the specific genes that have a key role in salivary gland clefting and lung morphogenesis. In the future, this approach will aid in identifying the genes that are involved in other cellular processes in additional organs.

A major advantage of 3D culture is the ability to observe and follow the same cells over time. This approach was used in the mouse kidney to identify the cells that require the receptor tyrosine kinase (RTK) RET during branching morphogenesis⁹⁴. Imaging in embryonic whole-organ chimeras showed the dynamic exclusion of epithelial cells that lack RET (*Ret*^{−/−} cells) from the tips of elongating ureteric buds⁹⁴ (FIG. 4c). The authors then increased RET signalling by deleting Sprouty1 (*Spry1*), which is a repressor of RTK signalling. In chimeras with wild-type cells, *Spry1*^{−/−} cells are enriched in the ureteric bud tip domain. Importantly, normal Wolffian duct cells display heterogeneous RET signalling *in vivo* and therefore probably compete on this basis for contribution to the ureteric bud⁹⁴. Taken together, these results reveal a crucial role for RET signalling, specifically in the cells that are most involved in tube elongation.

Inducible gene expression

Recent advances also enable inducible gene expression and precisely timed molecular analyses in 3D cultures²⁷; for example, expression of the transcription factor *Twist1* in mammary epithelial cells induces rapid dissemination of cells into the ECM. Surprisingly, *Twist1*-expressing disseminated cells retain epithelial gene expression, and dissemination requires E-cadherin²⁷ (FIG. 4d). The fraction of cells within a tissue that expresses a gene can also regulate tissue architecture. Mosaic, but not ubiquitous, expression of an activated form of the GTPase *HRAS* induces multicellular protrusions in MCF-10A aggregates¹¹⁷. In a separate study, activated *HRAS* was sufficient to drive tissue overgrowth when expressed in myoepithelial or luminal mammary epithelial cells¹¹⁸. However, inhibitor studies demonstrated the involvement of different RAS effectors, which showed that different pathways were used in the two cell types to induce proliferation downstream of the same oncogene¹¹⁸. Taken together, these studies show that 3D culture enables elucidation of the cellular and molecular effects of gene activity in distinct cell populations within a tissue.

The microenvironment and cell behaviour

The examples in the previous section highlight biological insights from the study of isolated epithelial tissues *ex vivo*. However, *in vivo*, epithelial cells are surrounded by connective tissue, which contains immune cells, blood vessels, fibroblasts and ECM-bound signalling molecules (FIG. 5a). These components can regulate adjacent epithelial and neuronal tissues and contribute to disease progression, particularly in cancer^{119,120}. However, it is difficult to specifically alter either stromal cell or ECM composition *in vivo*. By contrast, 3D culture systems enable the precise manipulation of components of the microenvironment and the analysis of how they affect the structure and function of a cell or tissue^{69,121,122}.

Epithelial–stromal interactions

The ability to add or remove stromal cells in 3D co-cultures has been particularly exploited in cancer invasion studies. It was known that macrophages regulate breast cancer cell invasion and metastasis *in vivo*¹²³. To study the underlying molecular mechanisms, various immune cells and immune cell-derived soluble factors have been added to 3D cultures of breast tumour organoids¹²⁴. Macrophages, T cells that express interleukin-4 (IL-4) or exogenous IL-4 can each promote tumour invasion¹²⁴. Similarly, squamous cell carcinoma invasion into collagen I is strongly promoted by co-culture with fibroblasts¹²⁵, which interact with both cancer cells and the ECM¹²⁵. Importantly, if the fibroblasts are allowed to remodel the ECM and are then removed, their tracks within the matrix are sufficient to promote invasion¹²⁵. Fibroblasts can therefore create lasting, proinvasive changes in the tumour microenvironment¹²⁵. In breast cancer, fibroblasts use Hippo signalling to promote matrix stiffening and invasion¹²⁶. In oesophageal cancer, fibroblasts promote invasion via hepatocyte growth factor (HGF) signalling⁸⁹ and alter the differentiation state of cancer cells¹²⁷.

The vasculature is another potent source of regulatory signals. Recent studies have implicated thrombospondin 1 (TSP1), which is an angiogenesis inhibitor, in normal epithelial differentiation and tumour growth. A clonal 3D co-culture model was used to show that lung but not liver endothelial cells can direct lineage specification in distal lung stem cells¹²⁸, and TSP1 promotes differentiation into an alveolar fate following injury¹²⁸. In a different co-culture model, 3D niches that are composed of lung or bone marrow stroma mixed with endothelial cells were used to show that TSP1 regulates tumour dormancy¹²⁹ (FIG. 5b). This study revealed that TSP1 induces breast tumour cell dormancy in mature endothelium, whereas transforming growth factor- β 1 (TGF β 1) and periostin promote tumour cell growth in neovascular tips, which lack TSP1 (REF. 129). Neovascularization therefore promotes disseminated tumour cells to develop into macrometastases.

3D models of the perivascular niche have also been used to study interactions between brain endothelial cells and cancer stem cells (CSCs) in glioblastoma¹³⁰. Vascular networks within 3D-engineered scaffolds increase CSC maintenance, growth and migration via paracrine IL-8 signalling, and co-implantation of endothelial cells and CSCs *in vivo* promotes tumour formation via IL-8 receptor signalling in CSCs¹³⁰. Importantly, conditioned medium that has been collected from 3D cultures of endothelial cells promotes the maintenance of stem cell markers in CSCs, whereas conditioned medium from 2D cultures does not¹³⁰. Taken together, these data suggest that the tumour stroma is a potent regulator of primary tumour growth and metastasis.

Mesenchymal components can also regulate the regenerative capacity of an organ. In the salivary gland, removal of the parasympathetic ganglion decreases branching morphogenesis and depletes the pool of keratin 5-expressing progenitor cells¹³¹. Morphogenesis and proliferation of these progenitors can be rescued by activation of muscarinic receptors with an acetylcholine analogue¹³¹. Parasympathetic innervation therefore maintains progenitors in an undifferentiated state, and this signalling axis may enable therapeutic intervention to promote organ repair¹³².

Epithelial–epithelial interactions

Tumours are increasingly recognized to contain cancer cells with different genetic and phenotypic characteristics¹³³. 3D cultures of cancer cells have revealed subtype-specific differences in invasive behaviour and in the capacity of fibroblasts to promote invasion^{134,135}. Interestingly, cell lines that have greater migratory capacity are observed to lead collective invasion of less migratory cell lines via direct contact between cancer cells¹³⁴. A recent study developed an assay to prospectively identify the most invasive cells within primary mouse and human tumours by embedding tumour-derived organoids into 3D collagen I gels³⁰. Cancer cells that express basal epithelial markers were found to lead collective invasion in mouse breast cancer models as well as in patient samples, in 3D culture and *in vivo*, across breast cancer subtypes³⁰. Cancer cells that express basal markers directly adhere to, and lead the collective invasion of, cancer cells that express luminal markers³⁰. Thus, interactions between epithelial cancer cells in different differentiation states are crucial for collective invasion. These techniques can be readily generalized to any epithelial malignancy and enable unbiased identification of the cells that are most responsible for invasion and systemic spread. Interestingly, interactions between normal basal and luminal epithelial cells have also been shown to regulate tissue architecture and polarity in the mammary gland^{136,137}.

ECM composition

In both 2.5D and 3D culture, cells from the same source can be explanted into micro-environments that have varied ECM composition or mechanics. In the MDCK kidney acini model, the composition of the ECM regulates the efficiency and mechanism of lumen formation¹⁰⁴. In collagen I gels, cell polarization is slow and lumen formation requires apoptosis¹⁰⁴. By contrast, in Matrigel, cell polarization is rapid and apoptosis is dispensable for lumen formation¹⁰⁴. Direct comparisons of mammary epithelium in different microenvironments showed that the composition of the ECM regulates invasive and disseminative behaviours of both normal and malignant tissues⁶⁶ (FIG. 5c). Importantly, the consequences of gene deletion at the tissue level also depend on the ECM, as deletion of placental cadherin (*P-cadherin*) results in mammary epithelial hyperplasia in Matrigel and increased dissemination in collagen I (REF. 66). Finally, the organization of the ECM can modulate tissue architecture. Radially aligned collagen I fibres promote breast cancer invasion⁷⁰ and correlate with poor patient outcomes¹³⁸. Interestingly, aligned collagen I fibres also promote directional growth of normal epithelial cells^{67,68}, which suggests that structural cues can pattern normal morphogenesis.

Mechanical cues

The mechanical properties of the microenvironment also change during development and disease; for example, epithelial tumours increase in rigidity owing to both ECM stiffening and increased cytoskeletal tension¹³⁹. 3D culture experiments showed that stiff matrices induce integrin clustering, cytoskeletal tension and focal adhesion assembly¹³⁹. Increased cytoskeletal tension, whether induced by matrix stiffness or by ERK signalling, promotes malignant progression¹³⁹. Matrix stiffness can also contribute to cell fate specification, as mesenchymal stem cells commit to distinct lineages when cultured on gels with varied

rigidity¹²². Soft matrices promote neurogenic differentiation, intermediate matrices promote myogenic differentiation and rigid matrices promote osteogenic differentiation¹²².

Recent studies have focused on identifying the signals that cells respond to within these microenvironments; for example, lysyl oxidase-mediated collagen I crosslinking increases ECM rigidity and promotes metastatic progression via stimulation of PI3K signalling⁶⁹. Another study reported the effect of rigidity on cell behaviour by crosslinking poly(ethylene) glycol (PEG) networks within Matrigel scaffolds¹²¹. High rigidity alone suppresses the growth of both normal and neoplastic tissue but does not induce invasion or dissemination¹²¹ (FIG. 5d); however, the addition of adhesive peptides to the PEG network promotes the dissemination of both normal and tumour cells¹²¹. Collectively, these studies show that stromal cells, ECM composition and microenvironmental mechanics can independently regulate cell and tissue function¹⁴⁰.

Stem cell-derived tissues and therapeutics

3D culture can be used to define the necessary and sufficient components to replicate the structure and function of an organ. This approach, which is sometimes termed synthetic tissue biology¹⁴¹, can guide our understanding of normal development and generate replacement tissues¹⁴²; for example, single LGR5⁺ epithelial cells that have been isolated from the small intestine are sufficient to generate organoids with crypt–villus architecture, without mesenchymal cells⁶. However, the efficiency of generating intestinal organoids from LGR5⁺ cells is greatly increased by co-culture with Paneth cells¹⁴³. Organoid cultures can also be passaged indefinitely to produce sufficient cells for transplantation^{37,38,144}. In principle, stem cells from the injured organ of a patient could be used to generate organoids to repair the damage. Supporting this concept, a recent study induced experimental colitis in mice and introduced LGR5⁺ organoids into the colon¹⁴⁴. The organoids engrafted and formed crypts with barrier function¹⁴⁴. In cases in which the disease state is caused by a known mutation, combinations of patient-derived iPS cells with genome-editing techniques, such as CRISPR–Cas9, could enable the generation of replacement cells and tissues in which the mutation has been corrected¹⁰². This strategy has been validated in organoids derived from patients with cystic fibrosis, in which correction of the disease-inducing mutation in the cystic fibrosis transmembrane conductor receptor (CFTR) restored channel function in 3D culture¹⁰².

3D cultures of ES cells or iPS cells can also assemble into specific tissues and organs. A striking example is the formation of the optic cup from ES cell aggregates, with the stratified neural tissue containing all six cell types in their appropriate spatial arrangement⁴⁴. Improved culture conditions have enabled the routine production of both rod and cone photoreceptors and cryogenic storage of sheets of stratified human neural retina⁴⁵. Adaptation of these techniques for the mouse retina enabled transplantation and functional engraftment into retinal degenerative mice¹⁴⁵. This therapeutic approach has also been used for endocrine glands: mouse iPS cells were first differentiated into immature pancreatic cells and then differentiated into islets in 3D culture¹⁴⁶. Following transplantation into hyperglycaemic mice, these islets incorporated into the pancreas and improved blood glucose levels¹⁴⁶. A recent study further showed that co-culture with mesenchyme increases

the efficiency of expansion of human endodermal progenitors more than 10^6 -fold and preserves the capacity of the progenitors to give rise to glucose-sensing, insulin-secreting cells following transplantation *in vivo*¹⁴⁷. Similarly, transient expression of two transcription factors enables the generation of thyroid follicular cells from ES cells in 3D culture, and following transplantation into athymic mice, these cells rescue plasma levels of thyroid hormones¹⁴⁸. Taken together, these studies reveal that 3D culture is a crucial method to generate replacement tissues for therapeutic purposes¹⁴⁹.

An additional strength of these approaches emerges when studying organs that diverge considerably between mice and humans. ES cell- and iPS cell-based methods enable the real-time analysis of anatomically complex human organs such as the brain. A recent study developed cerebral organoids from human iPS cells in 3D culture and replicated the anatomical organization of multiple human brain regions, including cerebral cortex⁴⁷. In turn, organoids derived from iPS cells that had been isolated from a patient with severe microcephaly displayed premature neuronal differentiation and reduced amounts of neuroepithelium, which shows that even complex disorders of the central nervous system can be modelled using 3D culture⁴⁷.

Future directions

A major goal of biomedical science is to translate our understanding of the fundamental principles that govern biological systems to improve patient outcomes. 3D culture can function as an integration point for modelling human disease in experimental systems and also yields precise answers to biological questions. Achieving the full potential of these methods will involve the expertise of scientists and clinicians and may require new funding mechanisms to sustain the interdisciplinary work that is required.

We anticipate that recent advances in 3D culture will accelerate our understanding of the cellular and molecular basis of mammalian organogenesis, both via the study of primary mouse tissues and in artificially generated human organs. Equally important, real-time analysis is expected to yield insights into the dynamic cellular basis of disease processes, especially when robust mouse models have been developed. This combined knowledge of normal development and pathobiology should enable the identification of molecular signals that promote tissue regeneration, restore tissue function and resist disease progression.

A particularly exciting new development is the ability to explant the living tissue of a patient into 3D culture to obtain individualized predictive or prognostic information, which is the ultimate goal of personalized medicine (BOX 1). It is now possible to culture primary human cells or tissues using excess material from surgery^{30,37,73,102}. These human cell cultures could be used to test novel therapeutic strategies, to guide the selection of therapies for a specific patient or to grow replacement cells or tissues from the cells of the patient. The success of this therapeutic approach will require a high degree of standardization and reproducibility across experiments. In addition, patient tissue in 3D culture could be analysed for its dynamic molecular responses to standardized cellular and molecular cues, such as targeted therapies¹⁵⁰.

The development of robust vascular networks in 3D culture^{10,11} should enable the generation of larger and more complex tissue constructs¹⁵¹, improve transplant efficiency¹⁰ and catalyse a transition in 3D culture from the self-organization of simple tissues to the synthetic assembly of multicomponent organs. This transition in complexity should enable an increased focus on the recapitulation of organ function^{152,153}. Recent efforts in this direction suggest that functional properties of an organ can be replicated in microfluidic assays that do not closely model the normal *in vivo* tissue architecture⁷⁸. Finally, the successful functional incorporation of transplanted organoids into mouse colon¹⁴⁴, retina¹⁴⁵, thyroid¹⁴⁸, liver^{38,152} and pancreas^{146,147} suggest that organ function can be restored in human patients by the transfer of functional units of tissue rather than by whole-organ transplantation.

Acknowledgements

The authors apologize to the many scientists whose outstanding work could not be cited owing to space limitations. A.J.E. and E.R.S. were supported by a Research Scholar Grant (RSG-12-141-01-CSM) from the American Cancer Society. A.J.E. was also supported in part by funds from the National Institutes of Health National Cancer Institute (NIH-NCI) (U01 CA155758), by a Jerome L. Greene Foundation Discovery Project, by a grant from the Mary Kay Ash Foundation (036-13), by funds from the Cindy Rosencrans Fund for Triple Negative Breast Cancer Research and by a grant from the Breast Cancer Research Foundation.

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Box 1 Therapeutic applications of three-dimensional culture

Predictive assays

The vast number of potential cancer drugs and drug combinations cannot be serially evaluated in individual patients. This challenge has motivated efforts to develop chemotherapy-sensitivity and -resistance assays in which the therapeutic response of the tumour of a patient could be directly assessed^{154,155}. Although no current assays are ready for routine clinical practice^{154,155}, emerging techniques based on three-dimensional (3D) culture and microfluidic systems can potentially provide personalized information on response to therapy (see the figure, part **a**). For example, *ex vivo* slice cultures of tumours have been used to identify heterogeneous responses to small-molecule compounds such as PI3K inhibitors¹⁵⁶. Similarly, primary glioblastoma specimens show variable cell death rates and DNA damage responses following treatment with the chemotherapeutic drug temozolomide after irradiation¹⁵⁷. Primary samples can also be challenged with molecular therapeutic agents and assayed for downstream signalling responses¹⁵⁰. Validation of the predictive value of such assays will require clinical trials that test whether assay-guided clinical management results in better patient outcomes than the standard of care^{154,155}.

Prognostic assays

Prognostic assays could be developed to measure functionally based outputs, such as morphological criteria (for example, the extent of invasion into a defined microenvironment³⁰) (see the figure, part **b**) or molecular criteria (for example, expression of components of the MAPK pathway¹⁵⁰). These assays can potentially provide information that is independent of that derived from static phenotypic analyses that have been conducted on formalin-fixed paraffin-embedded sections. If such assays correlate with patient outcomes, they could provide a means to generate individualized prognoses based on tissue from the patient.

Preclinical therapeutic testing

Organotypic models of disease can provide efficient proof-of-principle tests of novel molecular approaches to disease management (see the figure, part **c**).

Genome editing. Primary intestinal organoids that are derived from patients with cystic fibrosis carry mutations in the gene that encodes the cystic fibrosis transmembrane conductance regulator (CFTR) (for example, *CFTR* F508)¹⁰². Forskolin induces rapid swelling in organoids from healthy individuals but not in organoids from patients with cystic fibrosis. Correction of the *CFTR* F508 mutation by CRISPR–Cas9 (clustered, regularly interspaced short palindromic repeats–CRISPR-associated protein 9) in patient-derived stem cells that express Leu-rich repeat-containing G protein-coupled receptor 5 (LGR5⁺) results in the restoration of CFTR function and in forskolin-induced swelling in the organoids¹⁰². Notably, CFTR function improves more with gene editing than with small-molecule drugs¹⁵⁸.

Rational therapeutic design. The survival of a cancer cell in the presence of a therapeutic agent can depend on its 3D localization within a tissue. Dual inhibition of PI3K and

mTOR in ovarian cancer spheroids leads to the death of inner matrix-deprived cells but the upregulation of pro-survival programmes in matrix-attached cells¹⁵⁹. Combined inhibition of PI3K, mTOR and BCL-2 eliminates both matrix-deprived and matrix-attached cells by apoptosis in tumour xenografts and in primary ovarian and breast cancer samples from patients.

Epithelium

A type of animal tissue derived from ectoderm or endoderm that lines all cavities and body surfaces and consists of one or more layers of polarized, tightly connected cells.

Connective tissue

A type of animal tissue derived from mesenchyme that provides structural and nutritional support and connectivity among other tissues; it consists of individual cells, ground substance and fibres.

Extracellular matrix (ECM). The non-cellular component of tissues that provides both structural support and signalling cues to cells; it is composed of a network of proteins such as collagen, fibronectin and laminin.

Mesenchyme

Loosely organized, undifferentiated cells derived from embryonic mesoderm that give rise to the connective tissues of the body and the lymphatic and circulatory systems.

Induced pluripotent stem cells (iPS cells). Adult somatic cells that are genetically reprogrammed to generate embryonic-like pluripotent stem cells.

Basement membrane

An organized thin layer of extracellular matrix proteins that separates the epithelium from the surrounding connective tissue.

Stromal cells

The connective tissue cells of an organ (for example, fibroblasts), which support the function of the parenchymal cells of the organ.

Tissue stem cells

Adult stem cells that can give rise to some or all of the specialized cells of the tissue or organ from which they originate.

Matrigel

A gelatinous basement membrane matrix derived from Engelbreth-Holm-Swarm mouse sarcoma cells; it promotes cell differentiation and models the in vivo microenvironment of many tissues.

Self-organization

In tissues, the spontaneous formation of a highly ordered structure from a population of cells in the absence of pre-patterns.

Stratified epithelium

An epithelium that is composed of two or more layers of cells; it is often found in locations that require increased protection, such as exterior body surfaces.

Microfluidic systems

Devices that comprise submillimetre channels, pumps and valves that enable controlled, reproducible analysis of small samples of cells in nanolitre or picolitre volumes.

Asymmetric divisions

Cell divisions that result in two daughter cells with different fates, such as localization into distinct epithelial cell layers with unequal inheritance of polarity proteins.

Cre-lox

A site-specific recombination tool that uses the enzyme Cre recombinase to induce deletions, translocations or inversions in segments of genomic DNA that are flanked by loxP sites.

CRISPR-Cas9

(Clustered, regularly interspaced short palindromic repeats–CRISPR-associated protein 9). A genome-editing tool that uses the microbial RNA-guided Cas9 nuclease to make targeted changes in the DNA of eukaryotic cells.

Hypoplasia

Abnormal tissue or organ development owing to a deficient number of cells.

Hyperplasia

Abnormal tissue or organ development owing to an excess number of cells.

Tumour xenografts

Human tumours that are implanted into immunocompromised animal hosts.

Figure 1. Cellular inputs to organotypic cultures

a Whole-organ and organ-slice cultures. Tissues that develop during embryogenesis, such as the salivary gland, kidney and lung, are commonly explanted as whole organs; for example, explants of metanephric kidney that have been isolated from the embryonic urogenital ridge will undergo vigorous branching morphogenesis in three-dimensional (3D) culture. Tissues that develop postnatally, such as the mammary gland, intestine, brain and aorta, can be sectioned into tissue ‘slices’ owing to their large size. **b** Tissue organoid cultures. Primary organs can be harvested and processed by mechanical disruption and enzymatic digestion into tissue fragments (known as tissue organoids). The native stromal cells and extracellular matrix are typically removed, which enables isolated culture of the epithelial tissues. The resulting organoids contain diverse epithelial cell types organized in their normal spatial configuration and are typically explanted into commercial extracellular matrices, such as Matrigel or collagen I; for example, mammary epithelial organoids will undergo branching morphogenesis in 3D Matrigel. **c** Stem cell organoid cultures. Stem cells can be used to generate organoids that model the architecture and cellular composition of a larger organ. Both embryonic and adult induced pluripotent stem (iPS) cells have been used to generate organoids of the kidney, lung, intestine, liver, optic cup and brain; for example, embryonic stem (ES) cells that are cultured in the presence of Matrigel and differentiation factors will aggregate and self-organize into optic cup-like structures. Alternatively, single tissue stem cells that have been isolated from an adult organ can be used; for example, Leu-rich repeat-

containing G protein-coupled receptor (LGR5) - expressing (LGR5⁺) tissue stem cells that are embedded within Matrigel will generate many tissues of the digestive tract. **d** Primary cell cultures. Primary keratinocytes from the skin and oesophagus have been cultured on cell culture inserts to organize into stratified epithelium. In addition, primary epithelial cells from the salivary gland, lung, kidney and pancreas, as well as endothelial cells, have been used in two and one-half-dimensional (2.5D) or 3D culture.

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Figure 2. The major categories of cell culture

a Two-dimensional (2D) culture. Cells are typically cultured directly on a highly rigid substrate such as glass or plastic. The medium can be supplemented with extracellular matrix (ECM) proteins to induce a more differentiated cell state; for example, addition of laminin I will induce the differentiation of mammary epithelial cells. **b** Two and one-half-dimensional (2.5D) culture (also known as drip culture). Cells are cultured on top of a thin, organized layer of ECM, and diluted ECM proteins (such as laminins) are present in the medium. This format is ideal for imaging and antibody staining and is sufficient for epithelial acinar formation (for example, in MCF-10A and MDCK (Madin–Darby canine kidney) cell lines). 2.5D cultures have also been used to generate endothelial networks. The mechanical or structural properties of the ECM layer can be varied, and microfluidics can be

used to generate flow-over gradients. **c** Three-dimensional (3D)-embedded culture. Cells are cultured within a gel of ECM proteins. Cells are uniformly exposed to an organized ECM and can further remodel and restructure the ECM over time; for example, mammary tissue organoids will undergo branching morphogenesis in 3D Matrigel. This format requires that the ECM solution is cell-compatible both in liquid and in gel form, and it enables the incorporation of different ECM components, multicellular tissues and stromal cells. If constructed within microfluidic devices, these cultures can be subjected to in- or through-gel gradients. ECMs can also be precisely patterned in 3D. **d** Mechanically supported culture. Cells, organ slices or whole organs are cultured on a tissue culture insert that is either submerged in medium or maintained at an air–liquid interface. Histologically realistic epithelial tissues can be constructed in stages, with initial assembly of keratinocytes into an epithelial cell layer on a submerged culture insert, followed by exposure of these cells to an air–liquid interface to induce the formation of stratified epidermis. Stromal cells can be co-cultured with the epithelial cells or added to a separate compartment within the culture dish to study paracrine effects without direct physical contact between cell types. Slices of large organs, such as the brain, can be cultured on these inserts.

Figure 3. The cellular basis of epithelial tube elongation

a Schematic diagram showing epithelial bud initiation and tube elongation. Although the process is conceptually similar across the various organs, it was unclear whether tube elongation was accomplished by conserved cellular mechanisms. **b** Mammary epithelium elongates from a mammary placode into a surrounding fat pad starting at 3 weeks after birth. Branching morphogenesis involves transitions from simple to stratified to simple epithelium. The terminal end bud initiates and elongates as a multilayered structure at the growing front and eventually repolarizes into a simple bilayer⁶² (inset). **c** Salivary gland epithelium

develops embryonically from a single stratified bud that undergoes successive clefting and extracellular matrix remodelling to form a branched network with simple architecture. At the single bud stage (at embryonic day 13.5), the epithelium already contains a morphologically distinct outer layer of columnar cells, which form the acinar epithelium of the gland, and many inner rounded cells, which form the ductal epithelium¹⁶⁰ (inset). **d** Kidney branching morphogenesis initiates embryonically when the Wolffian duct evaginates into the surrounding metanephric mesenchyme as the ureteric bud. The epithelial bud transitions from simple to pseudostratified to simple architecture⁹⁴ (inset). **e** Lung development occurs embryonically. Avian lung maintains simple organization throughout branching morphogenesis and initiates new buds via apical constriction⁸⁵ (inset). **f** Salivary gland epithelium requires fibroblast growth factor 10 (FGF10) for branching morphogenesis, and heparan sulphate increases the affinity of FGF10 for its receptor. Specific sulphation patterns of heparan sulphate regulate FGF10-mediated morphogenetic events, such as proliferation, end-bud expansion and duct elongation⁹⁵. **g** In branching ureteric bud tips, pre-mitotic epithelial cells delaminate into the lumen to undergo cell division while maintaining a thin basal process at the site of origin. One daughter cell (blue) reinserts at the original site and the second daughter cell (green) inserts at a position one to three cell diameters away⁹⁶. **h** In the chick lung, treatment with the proliferation inhibitor aphidicolin does not block bud formation, which shows that cell proliferation is dispensable for bud initiation⁸⁵. **i** During mouse lung development, domain branching is characterized by a localized increase in cell division within the incipient bud relative to adjacent trunk cells. Within the bifurcating bud at the end of the tube, there is both an enrichment of proliferation relative to the trunk and a polarization of the plane of cell division within the future cleft region⁹⁷. Arrows indicate the orientation of cell division.

Figure 4. Genetic regulation of cell behaviours in mammalian tissues

a siRNA-mediated knockdown of *p63* showed that this transcription factor is required for both proliferation and differentiation in regenerating organotypic postnatal epidermis. Depletion of *p63* in all cells leads to tissue hypoplasia, defects in epidermal stratification and differentiation, and loss of simple epithelial markers¹¹³. Mosaic mixtures of control cells and *p63* siRNA-treated cells leads to a cell-autonomous failure of differentiation in the *p63* knockdown cells¹¹³. **b** Fibronectin was known to accumulate within the forming clefts in the salivary gland¹¹⁵. Gene expression analysis showed that fibronectin binding induces expression of *Btbd7* in epithelial cells within the clefts²⁹. BTBD7 regulates cleft progression by reducing cell–cell adhesion and promoting the formation of transient intercellular gaps²⁹.

c Labelled cells within chimeric embryonic kidneys compete for contribution to the ureteric bud, depending on their individual level of RET signalling⁹⁴. In a chimeric Wolffian duct, labelled epithelial cells that lack the receptor tyrosine kinase (RTK) RET (*Ret*^{-/-} cells) are excluded from the tips of elongating ureteric buds in favour of wild-type cells⁹⁴. By contrast, cells that are depleted of Sprouty1 (*Spry1*^{-/-}), which is a repressor of RTK signalling, have increased levels of RET and accumulate at the ureteric bud tip domain instead of wild-type cells. **d** Tet-inducible *Twist1* expression leads to loss of tissue polarity and the rapid dissemination of otherwise normal mammary epithelial cells²⁷. Disseminated cells retain epithelial gene expression (for example, cytokeratin 8), localize epithelial cadherin (E-cadherin) and β -catenin to the membrane and require E-cadherin protein to disseminate as single cells²⁷. Part **b** of the figure from Onodera, T. *et al.* Btbd7 regulates epithelial cell dynamics and branching morphogenesis. *Science* **329**, 562–565 (2010). Adapted with permission from AAAS. Part **c** of the figure adapted from *Dev. Cell*, **17**, Chi, X. *et al.*, Ret-dependent cell rearrangements in the Wolffian duct epithelium initiate ureteric bud morphogenesis, 199–209, Copyright (2009), with permission from Elsevier.

Figure 5. The role of the microenvironment in regulating epithelial function

a Schematic overview of different components of the tissue microenvironment, including immune cells (for example, macrophages), blood vessels, fibroblasts and extracellular matrix (for example, collagen I). The components and properties of the microenvironment can be readily modified in three-dimensional (3D) culture. **b** Co-cultures of lung or bone marrow stroma mixed with endothelial cells were used to generate a 3D organotypic microvascular niche. The angiogenesis inhibitor thrombospondin 1 (TSP1) induces breast tumour cell dormancy in mature endothelium, whereas transforming growth factor- β 1 (TGF β 1) and periostin promote tumour cell growth in neovascular tips, which lack TSP1 (REF. 129). **c** Direct comparisons of the same tissue in different microenvironments (that is, Matrigel or collagen I) shows that the composition of the ECM regulates invasive and disseminative behaviours of both normal and malignant mammary epithelium⁶⁶. **d** The mechanical properties of the microenvironment can affect cell and tissue function. High

rigidity (which is achieved by crosslinking poly(ethylene) glycol (PEG) networks within Matrigel scaffolds) suppresses the growth of both normal and neoplastic tissue but does not induce invasion or dissemination¹²¹. The addition of adhesive peptides promotes dissemination of both normal and tumour cells¹²¹. Part **b** of the figure adapted from REF. 129, Nature Publishing Group. Part **c** of the figure adapted from Nguyen-Ngoc, K.-V. *et al.* ECM microenvironment regulates collective migration and local dissemination in normal and malignant mammary epithelium. *Proc. Natl Acad. Sci. USA* **109**, E2595–E2604 (2012).

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Table 1

Cellular and molecular techniques for three-dimensional culture

Organ	Cellular input	Culture format	Refs *
Mammary gland	Cell line (for example, MCF-10A)	2.5D culture	51,111,161
	Whole organ	Mechanically supported	15,162
	Tissue organoid	3D-embedded culture	5,27,66,72,163
	Primary cells	2.5D culture	57,164
Salivary gland	Embryonic whole organ	Mechanically supported	92,99,115
	<i>Ex vivo</i> epithelial-mesenchymal recombination	Mechanically supported	100,165
	Tissue organoid	3D-embedded culture	33,63,95
	Primary cells	3D-embedded culture	166,167
Kidney	Cell line (for example, MDCK)	2.5D culture	58,105,168,169
	Embryonic whole organ	Mechanically supported	96,170,171
	<i>Ex vivo</i> epithelial-mesenchymal recombination	Mechanically supported or 3D-embedded	172,173
	Tissue organoid	3D-embedded culture	34,64,174
	Stem cell organoid (ES cells)	2.5D culture	175
	Stem cell organoid (ES cells and iPS cells)	Mechanically supported	176,177
	Primary embryonic cells	Mechanically supported	55,56
Lung	Normal or neoplastic lung slice	Mechanically supported	17,156,178
	Embryonic whole organ	Mechanically supported	14,116,179
	Tissue organoid	3D-embedded culture	35,179
	Stem cell organoid (tissue stem cells)	3D-embedded culture	128,180
	Primary cells (human alveolar cells)	2.5D culture	59
	Primary cells (foetal pulmonary cells)	3D-embedded culture	181
Small intestine	Cell line (for example, Caco-2)	2.5D culture	182,183
	Organ slice	Mechanically supported	18,19
	Tissue organoid	3D-embedded culture	6,28,36,102,184
	Stem cell organoid (LGR5 ⁺)	3D-embedded culture	6,143
	Stem cell organoid (iPS cells)	3D-embedded culture	185
Colon	Organ slice	Mechanically supported	19,20
	Tissue organoid	3D-embedded culture	36,37,144,184,186
	Stem cell organoid (LGR5 ⁺)	3D-embedded culture	37,144
Liver	Whole organ and organ slice	Mechanically supported	14,153,178
	Tissue organoid	3D-embedded culture	38
	Stem cell organoid (LGR5 ⁺)	3D-embedded culture	38
	Stem cell organoid (iPS cells)	2.5D culture	152
Stomach	Tissue organoid	3D-embedded culture	39
	Stem cell organoid (LGR5 ⁺ and Troy ⁺)	3D-embedded culture	39,187
Pancreas	Embryonic whole organ	Mechanically supported	14,84

Organ	Cellular input	Culture format	Refs [*]
	Tissue organoid	3D-embedded culture	40
	Stem cell organoid (LGR5 ⁺)	3D-embedded culture	40
	Primary pancreatic ductal cells	3D culture	65,188
Oesophagus	Primary cells (oesophageal keratinocytes)	Mechanically supported	54,89,127
Skin	Cell line (for example, HaCaT)	3D-embedded culture	189
	Primary cells (epidermal keratinocytes)	Mechanically supported	52,53,73,114
Prostate	Tissue organoid	3D-embedded culture	41
	Primary cells (human prostatic epithelium)	3D-embedded culture	190
Optic cup	Stem cell organoid (ES cells)	3D-suspension or 3D-embedded culture	44,45
Brain	Organ slice	Mechanically supported	21,22
	Stem cell organoid (ES cells)	3D-suspension culture	46
	Stem cell organoid (iPS cells)	3D-embedded culture, spinning bioreactor	47
Blood vessels	Primary cells (for example, HUVEC)	2.5D culture	191,192
	Primary cells (for example, HUVEC)	3D-embedded culture	193
	Organ slice (aorta)	3D-embedded culture	23
	Primary cells (for example, HUVEC)	3D bioengineered platform	11,12,151,194

^{*} Where possible, the original reference for the approach is included and is supplemented with additional recent references that highlight major technical advances. 3D, three-dimensional; ES, embryonic stem; HUVEC, human umbilical vein endothelial cell; iPS, induced pluripotent stem; LGR5, Leu-rich repeat-containing G protein-coupled receptor 5; MDCK, Madin-Darby canine kidney.