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Reverse-engineering organogenesis through feedback loops between model systems

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Abstract

Biological complexity and ethical limitations necessitate models of human development. Traditionally, genetic model systems have provided inexpensive routes to define mechanisms governing organ development. Recent progress has led to 3D human organoid models of development and disease. However, robust methods to control the size and morphology of organoids for high throughput studies need to be developed. Additionally, insights from multiple developmental contexts are required to reveal conserved genes and processes regulating organ growth and development. Positive feedback between quantitative studies using mammalian organoids and insect micro-organs enable identification of underlying principles for organ size and shape control. Advances in the field of multicellular systems engineering are enabling unprecedented high-content studies in developmental biology and disease modeling. These will lead to fundamental advances in regenerative medicine and tissue-engineered soft robotics.

Introduction

Genetic model systems such as the fruit fly (*Drosophila*) [1], worm (*Caenorhabditis elegans*) [2], and the zebrafish (*Danio rerio*) [3] have yielded insights into many of the mechanisms of development and disease over the last century [4] (Figure 1). A key feature of these model organisms is the ability to quickly perform functional genomic studies using a wealth of genetic tools. For example, the GAL4/UAS binary expression system and inexpensive, publicly available transgenic animals for genomic screening are widely used in *Drosophila* (Figure 2 A) [5]. These inexpensive tools enable biochemical signaling pathways to be perturbed in specific subsets of cells. Additionally, fluorescently-tagged biosensors and optogenetic tools [6] (Figure 2 D) provide dynamic readouts and control of cell signaling under genetic perturbations [7–9].

As an example, *Drosophila* has proven valuable in dissecting the genetic basis of many human diseases. Around 75% of the genes involved in human diseases have orthologs in

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Drosophila [12], leading to the development of *Drosophila* models for heart disease [13], diabetes [14] and Alzheimer's [15], among others. *Drosophila* has also been widely used as a model for epithelial-derived cancers [16]. Tumors can be generated by over-or ectopically expressing oncogenes spatiotemporally in a micro-organ of interest [17]. Genome-wide screens can identify genetic components that inhibit tumor development using inexpensive genetically-encoded RNAi stocks [18]. Pharmacological libraries have also been screened using in vivo tumor models [17]. Similarly, worms, zebrafish and mice play important roles in studying organogenesis. Genetic model systems enable inexpensive preclinical drug screens for a variety of human diseases.

Counterpart genetic studies of organ development in mammals have lagged behind model systems like *Drosophila* for several reasons. These include the lack of appropriate 3D models for organogenesis amenable to live imaging studies and comparable inexpensive genetic tools. Mammalian organs are much larger than micro-scale dimensions, which complicates sample processing, imaging, and analysis. Recently, human organoids, embryoid bodies, and gastruloids (Box 1) are serving as new platforms in the emerging field of synthetic developmental biology for investigating morphogenesis and the biophysical basis of developmental diseases [19,20].

Human-derived organoids provide functional organ models for mammalian systems [21]. Organoid cultures have a defined 3D spatial organization and demonstrate cell-cell communication that leads to emergent tissue functions. Combined with modern gene editing techniques, organoids promise to translate the advantages provided by *Drosophila* and other genetic model systems to human developmental studies. Further, human organoids have many direct clinical applications in regenerative medicine [22,23]. However, significant challenges remain in scaling up and scaling out human organoids as yields are poor and variable [24]. These challenges require significant efforts to reverse-engineer the underlying principles of organoid size control - a systems-level property that is regulated by extrinsic, environmental parameters and intrinsic genetic factors [25]. The question of organoid size control, much like organ size control, is largely unresolved and poorly developed in the field. However, it has significant implications for bioprocess optimization of organoids.

Here, we compare a selected set of synergistic efforts investigating the underlying basis of multicellular development utilizing human organoid and insect micro-organ approaches (Figure 1). A key shared feature between these models is a similar length scale. A cross-comparison strategy that includes investigations in both vertebrate and invertebrate systems are leading to unexpected, transformational discoveries that transcend the species context. These discoveries can be leveraged to solve outstanding problems in tissue engineering, developmental biology, and human disease. They can also inspire tissue-engineered approaches in soft robotics [26] and cell-based biosensors [27].

Mammalian organoids

A key advantage of human organoid studies is the high relevance to human health and developmental research. A recent example can be found in the use of brain organoids to examine ZIKV induced microencephalopathy in the infant brain [28]. The brain has always

proven difficult to study due to the difficulty in obtaining brain tissue without damaging the test subject. However, advances have been made in recent years with producing and culturing brain organoids [28–33]. Qian et al. [28] exposed forebrain organoids to ZIKV. This work demonstrated that the virus preferentially infects neural progenitor cells (NPCs) over neurons. Yet, the use of brain organoids to understand disease mechanisms has limitations. Cerebral organoids are created from the spontaneous differentiation of pluripotent stem cells (PSCs), which can lead to a high degree of heterogeneity between organoid batches. This complicates analysis and necessitates analyzing a large number of organoids to reach quantitative conclusions [31]. A better understanding of the factors controlling PSC differentiation into more uniform cerebral organoids could mitigate this challenge. In addition to the brain, an ever-increasing repertoire of organoids are being deployed as models for many of the organs in the human body [34].

Both 2D or 3D human cell cultures can be induced to self-assemble into organoid structures when presented with specific chemical [24] or topographical [35] cues. The presence of specific growth factors has long been established as a key mediator of tissue specific differentiation and morphogenesis [24]. More recently, micropatterned substrates have been demonstrated to induce organoid self-assembly. In a report by Shen et al. [35], a fabricated substrate consisting of randomly aligned nanoscale fibers directed self-organization of human airway epithelial cells into tissue-like structures containing a lumen. This example represents an important demonstration that the topographical environment alone can direct morphogenesis. The cell signaling pathways activated due to exposure to these structures remain to be characterized.

Mechanical properties of the substrate have also been implicated as important factors for self-assembly in addition to chemical cues and topology [22,36]. Takebe et al. [22] recently demonstrated a generalized method for generating organ buds from endothelial cells and mesenchymal stem cells (MSCs) [22,37]. They induced aggregation of an endothelial/MSC mass that self-assembled into organ buds by tuning the stiffness of the hydrogel culture substrate. The aggregation and subsequent assembly were highly dependent on the mechanical properties of the gel. This work highlights the importance of the defining cell interactions between multiple cell types and the mechanical environment on inducing self-assembly of larger structures.

The induction of tube formation in intestinal epithelial cultures has been recently reported by Sachs et al. [36]. This was accomplished by embedding proliferating intestinal organoids in a contracting collagen gel. This allowed cells to align and fuse into macroscopic hollow tubes composed of multiple cell types [36]. Without contraction of the culture gel, fusion into macroscopic tubes did not occur. This highlights the importance of dynamic mechanical environment in directing self-assembly.

Other recent work has also shown that both the size and density of 2D or 3D cell cultures are determinates of cellular self-assembly. Arora et al. [24] found that approximately 13% of cultured hindgut spheroids formed intestinal organoids in culture. They demonstrated that spheroids with a diameter greater than 75 μm were enriched 1.5-fold for the potential to form organoids. Further, those with an inner mass of cells were enriched 3.8-fold. This work

represents an important first step in establishing cell culture protocols for high-throughput production of organoids. However, it remains a compelling question why some spheroids never go on to form organoids, regardless of size or cellular density.

Despite these exciting advances, the question of how to robustly control organoid size and shapes is poorly understood. Organoid studies have unveiled key developmental differences between model systems. For example, recent work with stem cell derived retinal epithelia from both mouse and human cells demonstrated dramatic size variation consistent with species size differences [38,39]. Yet, both organoid models are smaller than in vivo organs. The analysis of the relative levels of biochemical signaling pathways active during optic cup formation is needed to better understand the size regulation. Such information could help define how cells sense when to stop growing and unlock the critical question of organ size control.

Species specific developmental differences highlighted by recent organoid studies can reveal conserved principles of organogenesis. For example, a basic unit operation of morphogenesis is the ability of an epithelial sheet to fold into curved shapes [40]. Recent reports have demonstrated the induction of folding in human brain organoid cultures by deletion of PTEN to stimulate sustained cell-cycle re-entry as observed in the developing human cortex [41]. Interestingly, deletion of PTEN in mouse brain organoid cultures does not lead to folding [41]. This highlights the different developmental mechanisms that can work to shape organs.

Although organoid advances have yielded many novel insights, there are some key issues and challenges to be overcome. Mammalian cell culture requires controlled physiological conditions (37 °C and 5% CO₂). Genetic manipulations are also more challenging. Modifying mammalian genomes was an expensive proposition until the development of low-cost gene-editing technologies like CRISPR-Cas9 (Figure 2 B) [42]. Even today, the genetic resources for mammalian systems are less extensive and more expensive than simpler genetic models. Further, reliable and inexpensive protocols for generation of organoids from 2D or 3D cell cultures have yet to be established. Additionally, the use of organoids in developmental and disease research is not ethically neutral. The issues of biobanking and donor consent for human-sourced cells are often overlooked [43,44]. In many cases, the tools to answer a specific hypothesis exist in simpler model systems. Further, model systems continue to provide a track record of identifying new biological findings highly relevant to human health [45].

Insect micro-organs

A chief advantage of model systems is the ability to study organ development within the full in vivo context. For example, the *Drosophila* wing imaginal disc has served as a prototype organ for quantitative biology and is ideal for addressing systems-level questions related to complex signaling pathways *in situ* [46]. Studies in the *Drosophila* wing disc first demonstrated compartmentalization and cell fate mapping through lineage restriction [47,48]. Cell competition, a process in which slowly dividing cells are eliminated by more proliferative neighbors, was also first observed in *Drosophila* [49]. Cell competition has

since become a central theme for human cancer research [50] (Figure 3). Importantly, the hypothesis of mechanical feedback on organ size control has also been advanced through both computational and experimental efforts with *Drosophila* wing discs (reviewed in [25]).

Many genetic tools exist in *Drosophila* which allow selective modification of protein expression with high spatiotemporal control [5]. Such tools, including three complementary binary expression systems, allow for the study of cellular interactions and signaling in an intact organ environment (Figure 2 A) [51]. Knockdown of genes through RNA interference (RNAi), in addition to over- or ectopic expression, can provide key insights for functional genomics (Figure 2 A, C) [51]. Fluorescently-tagged proteins can also be used to observe downstream impacts of these perturbations in real time. The FLP/FRT system can be used to create clonal populations of genetically perturbed, fluorescently-marked cells to better understand neighbor interactions and cell communication [51]. Additionally, fluorescent sensors for second messengers such as calcium (Ca^{2+}) allow for imaging fast responses to exogenous stimuli [52]. These allow for examining the impact of physiological signals on morphogenesis, regeneration, and cellular growth.

Insect micro-organ and cell cultures can also be used in developing innovative biosensors and new biomechanical devices. Invertebrate cells have proven valuable in the creation of bioactuators for soft robotics [53], an emerging field with many potential applications. For example, insect muscle cells have been used to create actuators which operate at room temperature [53,54]. Additionally, earthworm muscle tissue has been used to create an ambient temperature biological pump [26]. These examples demonstrate invertebrate tissues are continuing to advance synthetic biology applications.

Drosophila imaginal discs and cell lines are invaluable tools for pilot studies of tissue and organ culture studies that complement in vivo analysis [55]. Insect cells do not require the precise environmental conditions mammalian cells need to survive. However, the question of organ size control is unsolved in this system. Culture time for maintaining growth of *Drosophila* organs is limited to around 12 hours ex vivo [56]. This timeframe does not allow for the full course of morphogenesis. The development of culture conditions that recapitulates long-term post-embryonic micro-organ growth remains an exciting challenge in the field. Recent years have witnessed a resurgence in efforts to develop new media formulations [57].

Key species-specific differences in the growth of micro-organs have also been revealed through the study of insect models. For example, studies in the Lepidoptera *Precis* have revealed that the chemical signaling requirements for robust wing disc development are different from those found in *Drosophila* [58]. The identification of these specific developmental contexts would go un-noticed if focus were limited to a single model system.

Recent work in *Drosophila* has also used genetic mechanisms to perturb the mechanical environment of the tissue. This has proven the importance of the ECM in retaining the BMP homolog DPP during organ development [59]. DPP is a morphogen essential for the growth and patterning of the wing [60]. ECM removal resulted in DPP leaking into the surrounding body cavity yielding smaller wing discs overall compared to discs with intact ECM. This

study highlights the importance of the ECM in the maintenance of proper signaling levels during development. Interestingly, genetic modulation of the ECM to increase tissue compression did not affect adult wing size [59]. However, the authors do not rule out secondary effects of this ECM manipulation, which may negate the effects of increased compression on wing size [59]. Overall, much remains to be discovered regarding how ECM remodeling occurs during organ growth.

Insect cell cultures have also demonstrated the potential for organoid generation, but little active research has been done recently. Currie et al. showed that isolated cells from *Drosophila* imaginal discs could also grow out to form epithelial vesicles in primary cultures [61]. Further, when stimulated with 20-hydroxyecdysone, these vesicles assembled into cuticular structures including bristles and trichomes. Insect organoids present a largely untapped model of organ growth that presents many parallels to mammalian organoids. Also, insect organoids could lead to tissue constructs with applications in soft robotics. In the future, they could provide critical insights for devising general strategies to scale up organoid generation.

Synergistic tool development for organoid and micro-organ studies

Proper organ function is also directly linked to the ability to signal between different organs within the body [62]. Advances in microfluidic designs have allowed for simple “body on chip” systems. Microfluidic systems let researchers examine cells cultured from different organs in connected and defined architectures [63,64]. A recent example from Cornell demonstrated a device culturing cardiac, liver, muscle, and neuronal cells in connected chambers [65]. This system could recapitulate known side effects to 5 different drugs. Such efforts have yet to be widely applied to organoids and micro-organ cultures.

In vivo studies in *Drosophila* have proven an important tool in understanding inter-organ communication. Recently, great strides have been made in the *Drosophila* tract, the brain, fat body, and several other micro-organ systems [66]. For example, a recent study examining infection of the *Drosophila* gut found that this local stress was sufficient to trigger global antimicrobial peptide responses in the fat body, a key immune organ [67]. The fusion of micro-organs and fluidic devices would enable more detailed dissection of inter-organ signaling during development. Microfluidics allows for the isolation of specific subsets of organs in culture providing greater experimental control.

Many strategies developed for perturbing cells and micro-organs can be applied across model systems through organoid cultures. Substrate topography and stiffness have been well-established as important forces driving cellular organization in organoids [35,37]. However, in many of these experiments, the topography and mechanical forces exerted on cultures do not vary dynamically with growth. This contrasts with dynamic developmental environments. A recent device developed for mechanical loading of tissues with a deformable membrane (Figure 2 E) was used to investigate the effects of mechanical loading on intercellular Ca^{2+} responses in the *Drosophila* wing disc [7]. This platform holds the potential to investigate the effects of non-constant or cyclic mechanical loads on organ development. It also enables examination of cellular behavior throughout the course of

organogenesis, and cellular sensitivity in healthy versus disease backgrounds. Combined with recently developed techniques to bond hydrogels to the surface to microfluidic devices [68], such a strategy could also be employed to explore the effects of tension on organ cultures.

Conclusions

Human organoids and insect micro-organs provide key bridges between 2D cell cultures and end-point studies of whole organ morphologies. Organoids are teaching us much about the basic operations of organ assembly. However, organoids do not provide the complete picture of *in vivo* organ development. They often lack key physiological contexts such as vascularization or inter-organ communication. Many open questions need to be addressed, such as:

- What factors lead to heterogeneity in transitioning from a spheroid to an organoid?
- What are the mechanisms controlling the final size of micro-organs and organoids?
- What are the differences in size control mechanisms across model systems?
- How is an extracellular matrix formed in an organoid and remodeled during growth in a micro-organoid?
- How can vasculature and innervation be induced in ex vivo cultured organoids?

Many technological advances are still required for organoids to realize their full potential. Standardized culture protocols must be developed to robustly generate consistent organoids. In parallel, advanced *ex vivo* culture and imaging methods need to be developed for genetic model systems to continue as invaluable complementary platforms. Further, the identification of design principles for tissue engineering and organogenesis requires insights from many model systems. For example, the powerful genetic tools available to *Drosophila*, and other model systems, allow researchers to address many open questions regarding how the developmental context of an organ effects cell behavior and biochemical signaling. Design principles that transcend model systems can be tested and explored *in silico* using multiscale computational modeling (Figure 2 F) [10,69]. Techniques created and ideas explored across model systems will lead to significant advances in the field of multicellular systems engineering, disease modeling, and soft robotics.

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Box 1**Terminology**

- Aggregate - A cluster of cells that adhere to each other.
- Embryoid body - 3D aggregate of pluripotent stem cells.
- Gastruloid - Embryoid bodies that recapitulate early stages of gastrulation.
- Micro-organ - fully functional model organs on the scale of ~1 mm or less.
- Organ bud - a tissue mass existing early in organogenesis, prior to blood perfusion.
- Organoid - 3D cell aggregates that assemble into organ-like structures ex vivo and demonstrate a degree of organ function.
- Spheroid - Free-floating cell aggregate that maintains a spherical shape with growth

Highlights

- Organoid technologies are enabling new investigations specific to human development
- Identifying general organogenesis design principles requires a diversity of systems.
- Advances in genetic and fluidic tools are needed to advance organogenesis research.
- Applications include organ models, screening, and tissue-engineering solutions.

Figure 1.

Human organoid cultures and insect micro-organs, such as the wing disc, provide complementary routes to elucidate principles of organogenesis. Human organoid cultures provide insight into how chemical and mechanical cues influence cell self-assembly and organization. Insect micro-organs provide insight into how genetic perturbations influence developmental processes, cell behavior, and morphology. Together they provide unique opportunities to better understand conserved developmental processes while providing a diverse set of tools for biosensors and implantable technologies.

Figure 2.

Representative tools to probe organogenesis. (A) Publicly available stock lists and binary expression systems allow for spatiotemporal control of genetic expression in *Drosophila*. Illustrated here is the GAL4/UAS system. (B) CRISPR/Cas9 allows for gene editing. A guide RNA leads the Cas9 enzyme to a specific site and initiates a double strand break in the DNA. This is then repaired using the internal machinery of the cell to insert or delete DNA sequences. (C) RNAi allows for post-transcriptional silencing of protein expression using small interfering RNA to degrade mRNA. (D) Optogenetics allows for spatial control of gene activation by specific wavelengths of light. (E) Fluidic tools allow for the detailed mechanical perturbation of living tissues. Adapted from [7]. (F) Computational modeling can help rapidly test hypotheses that connect tissue mechanics to biochemical signaling pathways and morphogenesis [10,11].

Figure 3. Several key developmental findings first observed in *Drosophila*. (A) Compartments. (B) Fate mapping. (C) Cell competition. (D) The roles of morphogen signaling and mechanical feedback in organ growth control.