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Review

Programming Morphogenesis through Systems and Synthetic Biology

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Mammalian tissue development is an intricate, spatiotemporal process of self-organization that emerges from gene regulatory networks of differentiating stem cells. A major goal in stem cell biology is to gain a sufficient understanding of gene regulatory networks and cell–cell interactions to enable the reliable and robust engineering of morphogenesis. Here, we review advances in synthetic biology, single cell genomics, and multiscale modeling, which, when synthesized, provide a framework to achieve the ambitious goal of programming morphogenesis in complex tissues and organoids.

Highlights

Stem cell-derived multicellular systems and organoids have opened new opportunities to emulate and understand human development *in vitro* and provide novel patient specific tissue surrogates for disease modeling.

Single cell sequencing technologies and spatial tissue analysis provide a wealth of information on cellular fate and function and offer invaluable opportunities to decipher developmental processes.

Mammalian synthetic biology utilizes designer synthetic gene circuits to program cell fate and functions towards a desired outcome.

Engineering morphogenesis is an emerging area of science that integrates engineering principles with developmental biology to control and guide collective cell behaviors.

Self-Organizing Multicellular Systems

The need for novel human-based cellular systems is vital due to shortages of donor organs, ineffective drug development pipelines, and a scarcity of predictive individualized human models. Animal models have thus far been the tools of choice to investigate developmental processes. However, fundamental differences in the biology between humans and, for example, mice [1], limit the translation of lessons learned from animal models to humans. The discovery of a method to generate a complex tissue akin to the optic cup from pluripotent stem cells *in vitro* [2], prompted a surge of studies involving **organoids** (see Glossary), **gastruloids**, and other forms of complex **self-organizing** tissues [3–5]. Self-organizing systems (reviewed in [3]) provide a new platform to understand developmental biology by emulating embryological process *ex vivo* (also known as 'building to understand') [6,7].

Organoids, a prominent example of self-organizing cellular systems, have been grown in 3D from human adult or pluripotent stem cells. Several examples of human organoids have been developed that can emulate structures and functions associated with human organs such as gut, kidney, liver, lung, and brain [8]. Organoids display the exciting potential to model key aspects of human development and disease processes, as well as advance efforts towards precision medicine and human disease modeling. However, stem cell-derived organoids often lack subsets of stromal cells, immune components, or a vascular system, and often fail to differentiate fully into mature phenotypes [9]. Current *in vitro* **morphogenesis** models frequently lack spatiotemporal control, robustness, and reproducibility that are exhibited *in vivo* by natural biological systems such as developing organs [10]. For example, soluble factors added to culture media affect the entire tissue with minimum spatial control. These limitations impede the development of organoids and negatively impact their utility in biology and medicine. Hence, the rapidly evolving fields of stem cell engineering and organoid technology face key challenges to systematically understand, control, and direct local and global morphogenetic events towards desired fates.

The collective properties of multicellular systems including final cellular composition, tissue identity, and patterning arise from individual cell behavior. Recent technological advances in single cell analysis provide the chance to examine and link cell state to models of self-organization, morphogenesis, and tissue-level behaviors. Additionally, using engineering

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approaches can offer unprecedented capacities to exert biological control, probe the underlying design principles of multicellular systems, and generate miniature organs with phenotypes closer to native tissues. An integrative approach that combines cellular engineering with high-resolution tissue analyses and *in silico* models provide an opportunity to rationally program morphogenesis and advance generation of human-based multicellular systems.

Genetically Guided Morphogenesis by Transcription Factors (TFs)

A substantial portion of macromolecular players in developmental processes in complex animals are genomically encoded in the form of genetic regulatory codes [11]. These developmental **gene regulatory networks (GRNs)** control system-wide spatial positioning of specific cellular functions, progressive pattern formation, and emergence of organ forms and functions [12]. The ability to map and to manipulate GRNs can be exploited to program and guide morphogenesis *in vitro* in a predictable fashion. In fact, several past studies have used TFs to generate a homogeneous cell population from stem cells or somatic cells [13–17]. More recently, TF-based engineering has successfully been applied to generate complex human tissues (Box 1). TF-triggered morphogenesis can elicit spontaneous production of signaling cues that are required for tissue development and spontaneous morphogenesis. For instance, engineering heterogeneous levels of GATA6 TF induced self-vascularizing fetal liver tissue from human-induced pluripotent stem cells (Box 1) [18]. Additionally, cell fates triggered by TFs could override the effect of medium [18,19]. Therefore, while traditional tissue engineering relies on engineering extrinsic cellular microenvironment, engineering tissues through manipulating the intrinsic genetic programs of cells can provide a new complementary approach for advanced **organogenesis in a dish**.

Box 1. TF-Triggered Complex Tissues

The most straightforward approach towards genetically guided morphogenesis is to activate key TFs that have been identified as integral for the development of a given tissue. In fact, a few past biological studies have used this simple form of genetic circuit for engineering complex tissues as outlined below.

Thyroid: endocrine cell types of the thyroid are generated from uniquely double positive NKX2.1^{+}/PAX8^{+} cells during the development of the gut tube. Antonica and colleagues directly expressed NKX2.1 and PAX8 using a doxycycline-inducible switch for the target genes in mouse embryonic stem cells. After growing embryoid bodies for 4 days, the cultures were induced with 3 days of doxycycline which yielded NKX2.1^{+}/PAX8^{+} cells capable of developing 3D thyroid follicle-like structures with the addition of thyroid-stimulating hormone into the medium [77].

Fetal liver: during embryogenesis, tissues are developed through codifferentiation of multiple progenitor cells via reciprocal cell–cell interactions. Taking advantage of this design principle, human induced pluripotent stem cells (hiPSCs) have been engineered with different copy number of an inducible GATA6-expressing circuit [18]. By transient and heterogeneous induction of GATA6 in hiPSCs, formation of all three human germ layers, symmetry breaking and codifferentiation of endoderm and mesoderm tissues are triggered. The cultures are kept in pluripotency medium for the first 5 days and subsequently transferred to basal medium without addition of growth factors. After 14 days, cultures form complex 3D self-vascularized fetal liver-like tissue with different subsets of stromal niches. During the course of study, GATA6-engineered cells progressively self-organize into intricate patterns and self-produce signaling cues necessary for tissue morphogenesis. This study proposes genetic heterogeneity and co-development can act as two design principles for engineering multicellular self-organizing systems.

Thymus: Bredenkamp and colleagues used forced expression of Foxn1 in mouse embryonic fibroblasts to generate Foxn1-induced thymic epithelial cells (iTECs) and tested their ability to support T cell development. Transplantation of aggregates formed from iTECs mixed with PDGFR-$\alpha\beta^{+}$ mesenchymal cells and CD45^{+}Lin^{-} immature thymocytes into nude *Foxn1*$^{-/-}$ mice generated functional thymus tissue capable of supporting generation of CD4^{+} and CD8^{+} T cells [78].

Pancreas: Chen and colleagues demonstrated the potential for renewable sources of pancreatic β cells from conversion of intestinal crypts by transiently expressing β cell enriched TFs Pdx1, MafA, and Ngn3. This approach resulted in rapid conversion of intestinal organoids into a tissue with glucose responsive, insulin producing 'neoislets' [79].

Glossary

Aptamer: nucleic acid or peptide molecule that binds a particular molecular target with high affinity and specificity.
Aptazyme: aptamer domain fused to a ribozyme domain. Upon the binding of the aptamer, catalytic action of the ribozyme region is activated.
Fluorescent *in situ* hybridization (FISH): technique used for visualizing specific sequences of nucleic acids within cells based on complementary base pairing of a florescent or radioactively tagged DNA probe with a target sequence.
Forward engineering: starting with high-level model that establishes the goals and functions of a system before its physical design or construction.
Gastruloid: stem cell-based model that emulates spatiotemporal aspects organization and differentiation of gastrulation.
Gene regulatory network (GRN): network of interacting genes that collectively controls particular cellular functions.
Genetic circuit: partly analogous to electronic circuits, it is used in synthetic biology to define an integrated assembly of genetic parts that produces a genetic function.
Morphogenesis: defined broadly here as events that collectively encode tissue pattern and shapes that are controlled by cell fate decisions, migration, cell–cell interaction and physical cues.
Morphogenetic engineering: here it refers to engineering morphogenesis using genetic techniques.
Organogenesis in a dish: *in vitro* approaches directed towards generation of complex tissues and organ-like structures via emulating aspects of developmental processes.
Organoid: self-organizing, 3D, multicellular system grown from stem cells *in vitro* that contains several organ-specific cell types with associated tissue structures and functions.
Reverse engineering: decomposition of a system into its functional parts in order to guide understanding of performance objectives and functions of individual components.

Genetically Guided Morphogenesis via Integration of Systems and Synthetic Biology

Design principles of how collective morphogenetic behaviors arise from genetically encoded GRNs of cells are inherently complex and incompletely understood. Advances in **systems biology** such as spatiotemporal single cell analysis and computational modeling provide the possibility to **reverse engineer** *in vivo* developmental processes to delineate the choice of TFs or other candidate pathways to interrogate *in vitro*. Subsequently, *in vitro* **morphogenetic engineering** can be carried out by overexpressing target genes or devising more sophisticated designer gene circuits that are interfaced with the candidate cellular GRNs. For instance, layered gene circuits and cascades can be exploited for stepwise controlled cellular differentiation. **Synthetic biology** is an emerging field that offers a compendium of toolsets germane to engineering of such **genetic circuits**. The designer gene circuits can provide spatiotemporal control over cell differentiations, migration, and cell–cell communications, and eventually *in vitro* organogenesis. Hence, they provide tools capable of steering *in vitro* morphogenetic processes (refer to Box 2 for explanations). Here, we review advances in single cell genomics, multiscale modeling, and mammalian synthetic biology. We believe the integration of these

Self-organization: loosely defined as spontaneous formation of ordered structures via local interactions of less organized parts with no centralized ordering or external template.

Synthetic biology: interdisciplinary field of study for engineering biology that entails (re)design and construction of existing or novel biological systems using well-characterized standardized genetic parts.

Systems biology: interdisciplinary field of study that aims to develop an integrated understanding of biological systems using quantitative measures of individual components, their interaction dynamics and computational modeling.

Box 2. Morphogenetic Engineering 101

A simple TF-based switch is an open loop genetic circuit without any feedback control. This simplest form of genetic circuit, is either constitutively active or can be inducible by a small molecule such as doxycycline. Engineering these simple circuits have already shown exciting biological potentials with generation of tissue patterns, functions and developmentally-relevant states [18,77–79]. A single TF-induced cellular system made through overexpression of one TF may be thought as minimal morphogenetic units that, if characterized properly, will advance our biological understanding of morphogenesis and can improve our synthetic potentials. These units can be assembled in a plug-and-play format for generation of advanced tissues or organoids. For instance, TF-angiogenic or TF-innervating units can be incorporated in current organoids to achieve additional tissue complexity. While open loop circuits are invaluable, in natural biological systems feedback loops and sensors provide robustness and maintain dynamic function within a fuzzy cellular context. In this line, Del Vecchio and colleagues have argued that engineering such feedback control mechanisms can augment cell fate programming (also known as synthetic genetic feedback controller) [80]. Additionally, when building a multicellular system, engineering feedback loops can control size and improve cellular composition. Additionally, feedback-driven morphogenesis has been suggested before [81] and enables development of artificial tissue homeostasis [82]. Synthetic biology can provide designer genetic circuits with features such feedback regulations, temporal controls and cascades. Its contribution to morphogenesis of complex cellular systems can be two folds: First, it can help to decode molecular algorithms of cells to understand how they cooperate to encode a collective cell behavior (e.g., by using gene circuits to record cellular memory or to rewire existing GRNs). Second, it utilizes designer synthetic gene circuits to provide ability to spatiotemporally control and program multicellular systems. In both cases, modern systems biology approaches such as single cell genomics and multiscale modeling can generate working models that can identify set of key GRNs or display behavior of molecular networks. The model can guide the synthetic biology efforts while the synthetic biology toolbox alters the parameters and connections to experiment and improve the model's accuracy. Hence, the integration of both parts remains pivotal to understand and engineer multicellular self-organization and morphogenetic functions (Figure I).

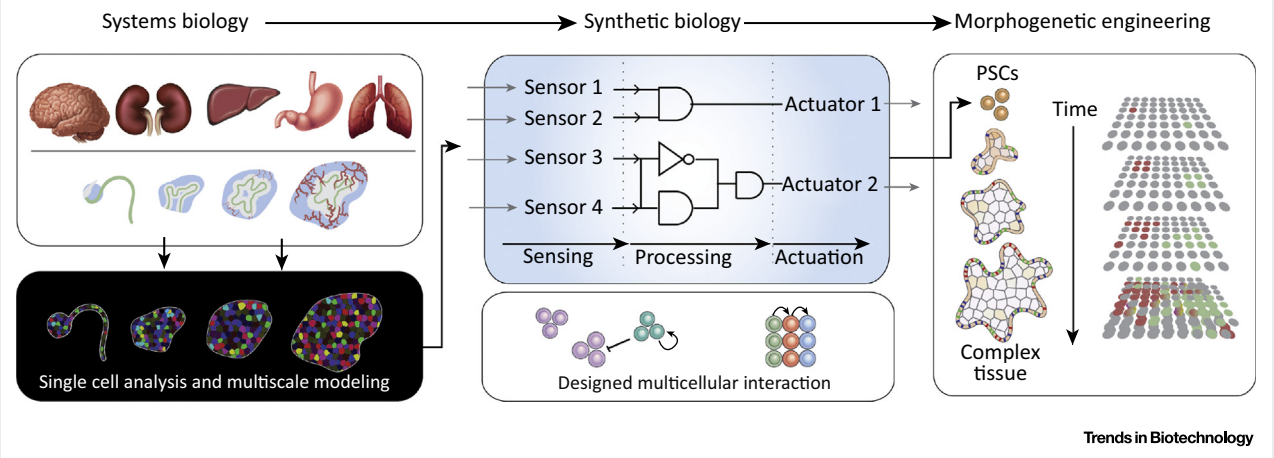

Figure I. Integrating Systems and Synthetic Biology for Morphogenetic Engineering. Abbreviations: PSCs, pluripotent stem cells.

areas can establish a synergistic pipeline for coherent design and engineering of human tissue morphogenesis *in vitro* (Box 2).

Single-Cell Analysis: Blueprint for Decoding Genetic Regulatory Codes

Single-cell RNA sequencing (scRNA-seq) has garnered much interest in recent years. Unlike bulk sequencing, which is prone to masking differences in cells due to averaging, single-cell approaches can capture the inherent heterogeneity between cells [20]. Data generated from these methods, when coupled with spatial and temporal information, can serve as a substrate for a bottom-up understanding of self-organizing processes such as morphogenesis. In this section, we describe several early examples of how single cell analytics can yield quantitative information on cell state, cell type, and localization that will be required for understanding self-organizing morphogenesis. The theoretical utility of the combination of data was explored by Pettit and colleagues when they developed a clustering method capable of incorporating both expression and spatial information to identify known and novel cell types. Inspired by image segmentation techniques, their method, which relies on a hidden Markov random field model, clustered cells in *Platynereis dumerilii* brain more in a spatially coherent, and seemingly more biologically accurate manner as compared to traditional clustering algorithms that do not consider spatial information [21].

A barrier to the broader application of techniques to integrate spatial and transcriptional data is that readily accessible methods to isolate and extract RNA from thousands of single cells requires tissue dissociation and subsequent loss of spatial information. Low-throughput methods that involve manual isolation, such as laser capture microdissection, can maintain some spatial information, but these methods do not generate data of sufficient scale for systems biology studies [22–24]. Likewise, *in situ* hybridization and *in situ* sequencing methods currently do not scale well either in terms of the number of cells, or the number of genes assayed (Box 3). However, it is possible to computationally infer the localization of single cells by integrating scRNA-seq with data from *in situ* methods (Figure 1).

One such approach is Seurat [25], which combines gene expression profiles in the form of scRNA-seq data and *in situ* hybridization data from a small set of landmark genes to infer spatial localization. In this method, the tissue of interest is spatially discretized into domains, or bins, that hold a binary value for each landmark gene, representing if it is expressed in that domain or not. Using this binary spatial map, statistical models are built for gene expression in each bin. Finally, the landmark gene expression of each cell is compared to the statistical models, and Seurat assigns a probability of the cell originating from each bin. Seurat successfully mapped 851 single cells in a developing zebrafish embryo using a discretized reference map of 47 genes. Additionally, Seurat has been used with unsupervised analysis of scRNA-seq data, identifying rare subpopulations of cells, such as spatially characterizing prechordal plate progenitors, endodermal progenitors, and primordial germ cells [25]. In a separate but analogous approach, Achim and colleagues utilized **fluorescence *in situ* hybridization (FISH)** expression atlases and scRNA-seq data to spatially characterize cells in the brain of the annelid *P. dumerilii* [26].

Recently, Halpern and colleagues applied an equivalent approach in the mouse liver, combining single-molecule FISH (smFISH) of just six highly expressed landmark genes, diverse in expression along the lobule axis ('zonation'), with parallel scRNA-seq. This resulted in the spatial expression profiles, or zonation profiles, of all liver genes; 50% of which were found to be significantly zonated, many of which peak at intermediate lobules [27]. Traditionally, hepatocytes are classified into one of two roles: periportal or pericentral. This study provided

Box 3. *In Situ* Molecular Profiling

The development of FISH was a first step in deriving spatial information of RNA data [83]. Since then, advances in image detection and the commercialization of fluorescent probes have paved the way for techniques such as smFISH, which utilizes the hybridization of fluorescent probes to target single mRNA molecules. Variants of this approach have focused on improvements to sensitivity and taken advantage of high throughput DNA synthesizers to create large numbers of singly labeled probes to cut down on false positives and negatives from nonspecific binding [84], allowing for a cleaner and more accurate signal.

Other smFISH variants have focused on methods to multiplex different mRNAs. Lubeck and colleagues developed seqFISH, in which transcripts are fixed in cells and subjected to rounds of hybridization of the same probe sequences with different fluorophores. Samples are imaged and probe stripped after each round, creating a sequential barcode of fluorescent spots that is theoretically capable of identifying F^N unique mRNAs, where F is the number of available fluorophores and N is the number of rounds of hybridization [85]. SeqFISH has since been used to localize cell types in the hippocampus [86].

To achieve the resolution required to map RNA to specific cellular compartments, Chen and colleagues developed expansion FISH (ExFISH). This method relies on expansion microscopy, in which RNA molecules are linked to an expandable gel. The gel is expanded, and RNAs undergo traditional FISH, allowing for super-resolution imaging [87].

Recently developed sequencing-based approaches that capture spatial information have involved the use of *in situ* sequencing without RNA extraction. Ke and colleagues demonstrated the feasibility of sequencing chemistry within fixed cells and tissues with gap-targeted and barcode-targeted sequencing approaches that utilize padlock probing, rolling-circle amplification (RCA), and sequencing-by-ligation chemistry to analyze up to four-base-pair fragments in mRNA. Specificity was demonstrated through the gap-targeted approach by sequencing a different motif of β-actin transcript in human and mouse fibroblasts that differ by a single nucleotide variation and finding that 208 of 215 reads mapped to the correct cell type. Using the same approach, sensitivity was demonstrated by studying codon 12 of KRAS in KRAS mutant A549 and wild-type ONCO-DG1 cell lines and finding that mutations of a single cell could be detected at a 1: 1000 spike-in ratio. Additionally, the barcode-targeted approach was utilized for multiplexed *in situ* profiling of 39 transcripts in breast cancer tissue [88].

The *in situ* sequencing and hybridization techniques described above have since progressed from barcode and gap-targeted approaches. Fluorescent *in situ* RNA sequencing (FISSEQ) begins with the reverse transcription of RNA in fixed cells and the incorporation of aminoallyl dUTP. The amine-modified cDNA amplicons are fixed to the cellular protein matrix and circularized before undergoing RCA and crosslinking in the presence of aminoallyl-dUTP. The resulting RCPs are single-stranded DNA nanoballs consisting of tandem copies of the cDNA sequence that is then sequenced via SOLiD sequencing by ligation. In human primary fibroblasts, FISSEQ was capable of demonstrating differential RNA expression and localization using 30 base pair reads in a wound healing assay [89,90].

evidence for a nonbinary spatial heterogeneity in the liver, revealing different roles for hepatocytes located between the two extremes. A more accurate representation of the division of labor will give rise to a more biological realistic metabolic network model. This method has also demonstrated the feasibility of applying such inference techniques in mammalian organs.

Paramount to understanding the underpinnings of self-organization will be to uncover cell–cell interactions. Camp and colleagues turned to scRNA-seq and established receptor–ligand pairs to determine the extent of cell crosstalk during liver bud development. In their screening method, a potential cell–cell interaction network was created based on an expression cutoff of receptor–ligand pairings between each cell–cell combination. Predictions from the screening were validated by knocking down receptor–ligand pairs and determining the effect on differentiation, as well as by utilizing chemical inhibitors to test for inhibition on liver bud development. These experiments demonstrated the importance of endothelial cell and hepatic endoderm crosstalk in differentiation and implicating a role for vascular endothelial growth factor (VEGF) signaling in liver bud development. The cell–cell interactions predicted by this type of receptor–ligand analysis represent one method of uncovering potential crosstalk between cells during the self-organization process [28].

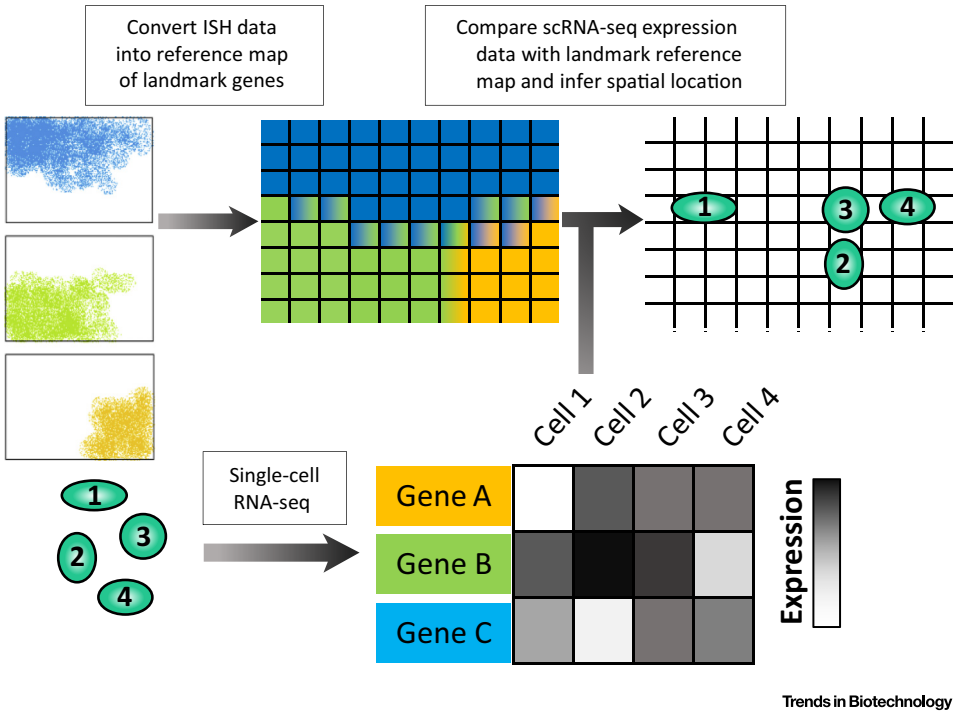

Figure 1. Inferring Location from scRNA-seq. A generalization of the spatial inference strategies presented by Satija and colleagues [25], Achim and colleagues [26], and Halpern and colleagues [27]. *In situ* hybridization data is used to create a spatial reference map of landmark genes. In parallel, scRNA-seq is performed on dissociated cells. Landmark gene expression from scRNA-seq data is compared to the reference map, and location of each dissociated cell is inferred. Abbreviations: scRNA-seq, single-cell RNA sequencing; ISH, *in situ* hybridization.

In addition to yielding quantitative information on cell state, cell type, and localization, single cell analytics can also be used to reconstruct or map developmental GRNs, which will be necessary to model and perform dynamic simulations of cell state transitions, and to design synthetic circuits for improved organoid engineering. Li and Luo and coworkers developed Sinova, a bioinformatics pipeline utilizing scRNA-seq expression data that were able to spatiotemporally reconstruct growth plate development, identifying and reconstructing previously unknown molecular cascades. This included the prediction of previously unknown TFs, such as the Kruppel-like factor (Klf) and Fos families, and the reconstruction of GRNs across different developmental stages [29]. Buganim and colleagues utilized single-cell technologies to profile 48 genes at different time points during reprogramming. This work validated key gene–gene relationships [including a role of Sox2 in activating octamer-binding TF (Oct)4 through preservation of high levels of genes such as nuclear receptor subfamily 5 group A member 2 (Nr5a2)], demonstrated that pluripotency activation is possible with a combination of factors distinct from Oct4, Sox2, Nanog, Klf4, and c-Myc (such as Lin28, Sall4, Esrrb, and Dppa2), and thus revealed novel reprogramming factors [30]. Camp and colleagues utilized scRNA-seq to compare the cellular compositions of human cerebral organoids with fetal neocortex and reconstruct the gene regulatory networks and cell lineage underlying the differentiation process; ultimately finding that the organoid system represents a fairly accurate model of human corticogenesis [31]. Similarly, Gouti and colleagues mapped the GRN underpinning *in vivo* and *in vitro* neural–mesodermal differentiation and described how retinoic acid and Wnt signaling interact with the GRNs to balance neural and mesodermal differentiation [32]. Many other studies that map developmental GRNs have been reviewed by Kumar and colleagues [33]. As a

collective, these studies demonstrate the promise and ability of utilizing single-cell profiling to aide fate engineering.

To date, a spatially aware, cell type composition tool has yet to be developed, and utilizing spatial and composition information to improve cell fate engineering remains a goal in the field. Such approaches will be limited by data collection methods. In the landmark gene expression maps discussed above, for example, biologists are limited to systems in which a subset of genes can generate a highly resolved map. Ultimately, however, spatial, temporal, and regulatory information gleaned from these single cell analyses will not only elucidate specific developmental design elements, but will also be incorporated into multiscale models and simulations that will unravel suitable design paths for *in vitro* engineering of organogenesis.

Multiscale Modeling

Multiscale computational approaches provide a method of faithfully describing self-organization. Because cell–cell and cell–environment interactions are critical determinants of cell fate decisions and because the these interactions are highly heterogeneous in nature, multiscale approaches that model systems from the molecular and cellular level are particularly apt. These models evolve through time based on a set of biophysically inspired rules, ultimately capturing interactions between cells and their environment that generate emergent phenomena. For example, Uzkudun and colleagues have used multiscale models and simulations to map GRNs that explain proximodistal patterning of the limb bud [34]. In general, there are many diverse approaches to performing multiscale modeling; below we discuss agent-based modeling and its application to complex cell population phenomena.

Agent-based models discretize the system being modeled into autonomous entities, each of which act according to a set of rules at each discrete time step throughout a simulation [35]. In such a framework, each agent is fully characterized by a set of inputs, outputs, possible states, and variables stored in its memory. Additionally, an agent must have a set of transition functions that bring the agent from one state to the next; a set of functions that map an input and memory variable to an output and updated memory variable; as well as an initial state and initial memory. At each time step, each agent receives an input signal, and depending on its current memory, it may change states, releasing an output and updating its memory. In a biological setting, this could be paralleled to a cell that receives a chemical or spatial cue and transitions phenotypes based on a set of biological rules producing some form of output. Of course, an agent in biology is not limited to the cell, and for a complex system, a single model often includes many distinct types of agents.

Because this family of models is easily adaptable to biological systems, several groups have implemented them in various settings. Agent-based models have been used to simulate the initiation frequency and location of sprouting angiogenesis [36], leukocyte trafficking in the microvasculature [37], and Nanog and Rex1 heterogeneity in mouse embryonic stem cell (mESC) cultures [38] among others.

In particular, to simulate sprouting initiation, Walpole and co-workers spatially divided each cell into eight membrane nodes and a single centroid node, which were connected by a series of links. Adjacent cells were connected by intercellular links. Implementing rules for VEGF and delta-like protein (DLL)4 signaling, the model was able to accurately predict tip cell location and sprouting frequency [36]. To simulate differences between fluctuations in Nanog and Rex1 expression levels in mESC cultures due to noise and due to deterministic oscillations, Herberg and colleagues built a model of self-renewal, considering cell signaling between individual cells

which stored information on TF dynamics. Combining this method with experimentally observed results showed that the bistability of Nanog levels is due to deterministic regulation sustained by fibroblast growth factor 4/extracellular signal-regulated kinase (Fgf4/Erk) signaling, and changes between the two stable states are helped by random noise [38].

Cell-based models, exemplified by cellular automata (CA) and cellular Potts (PC) models, can be considered as a subcategory of agent-based models in which there is only a single agent type (Box 4). Cell-based models have proven useful to explore complexity in several developmental contexts. For example, Krupinski and colleagues built a cell-based model to describe early morphogenesis of the mammalian embryo. In their model, blastomeres are elastic spheres that interact mechanically with each other, with each blastomere containing a specific genetic network that defines its mechanical properties. Cell division is modeled as a discrete event that conserves total cell volume during that time step. Cells divide either randomly, with content split symmetrically, or along the direction of a polarized vector, with content split asymmetrically. Their multiscale model was subsequently described by equations pertaining to mechanical motion and to genetic network dynamics [39]. In another application of cell-based models, Leung and colleagues developed an agent-based CP model simulating the development of the genital tubercle. Their model restricted the cells to a 2D lattice with dynamics governed by signaling networks encompassing sonic hedgehog, FGF10, and androgen [40].

The history of agent-based (and cell-based) models can be traced back to the 1940s when von Neumann, Ulam, and Conway first introduced CA. Regardless of terminology, all of these multiscale models represent powerful yet underutilized approaches capable of simulating self-organizing processes. The goal of simulation will be to gain insight on the effect of manipulation on system parameters, not only to help elucidate the complexities of self-organization, but also to generate more information that can be used in further experimental and computational studies.

In the context of self-organization, agent-based modeling is appealing for several reasons. First, it is a discrete model, which, unlike a continuous approach, is able to account for the heterogeneity in a system. Because the system is composed of autonomous agents, individual agents can serve different functions, follow distinct sets of rules, and have different properties,

Box 4. Cell-Based Models

As agent-based models, cell-based models discretize the system to be modeled into individual cells or subcellular components. These models come in two types – on-lattice and off-lattice. In the first, cells or cellular components are constrained to the coordinates of a grid. In the second, any cell or cellular component's position is represented by any real number.

Two popular on-lattice or lattice-based models are CA and CP models (Figure I). In CA, cells are assigned to a single site on the lattice and each site holds at most one cell. In CP models, each cell spans several sites on the grid. The simplicity of CA allows for fast simulation of large numbers of cells, whereas the CP model offers a more realistic representation of cell shape. The lattice structure of these models offer ease in determining which cells are neighboring, which are just any pairs in adjacent lattice sites. However, the lattice results in a low degree of spatial flexibility, and processes requiring cell movement or cell division require shifting the rest of the tissue [91]. Unlike agent-based models, in which multiple types of agents can interact with each other and the environment directly, cells in on-lattice cell-based models are thus typically static and homogeneous, while the environment moves through them.

Off-lattice models can offer more flexibility in cell shape and location, and thus these models are often used in mechanical studies of cell populations. Vertex models are one specific category of off-lattice cell-based approaches for characterizing aspects of self-organization in the context of cell mechanics. These models represent the shape of the tissue of interest by a set of vertices that are typically defined as the points of contact between three or more cells. In defining vertices in such a way, cellular interfaces and volumes can be determined based on the location of the vertices. Models can be 2D or 3D, and either apical or lateral. The motion of each of the vertices is determined by equations describing internal and external forces acting on the vertex. Vertex models are easily adapted to biological systems influenced by cellular mechanics. As such, they have been used to study a variety of processes in developmental biology, most notably epithelial morphogenesis [92–94]. Recently, a modified vertex model was used to study the formation of respiratory appendages in *Drosophila* oogenesis [94].

The benefits of implementing a vertex model include the ability to describe the shape of a tissue based on changes in cell shape and rearrangements. However, as with any discrete approach, a vertex model could become cumbersome with a large number of parameters. In this case, continuous approaches may offer an easier solution, despite generalizing the characteristics and behavior of the tissue and losing details of mechanics at the cellular level.

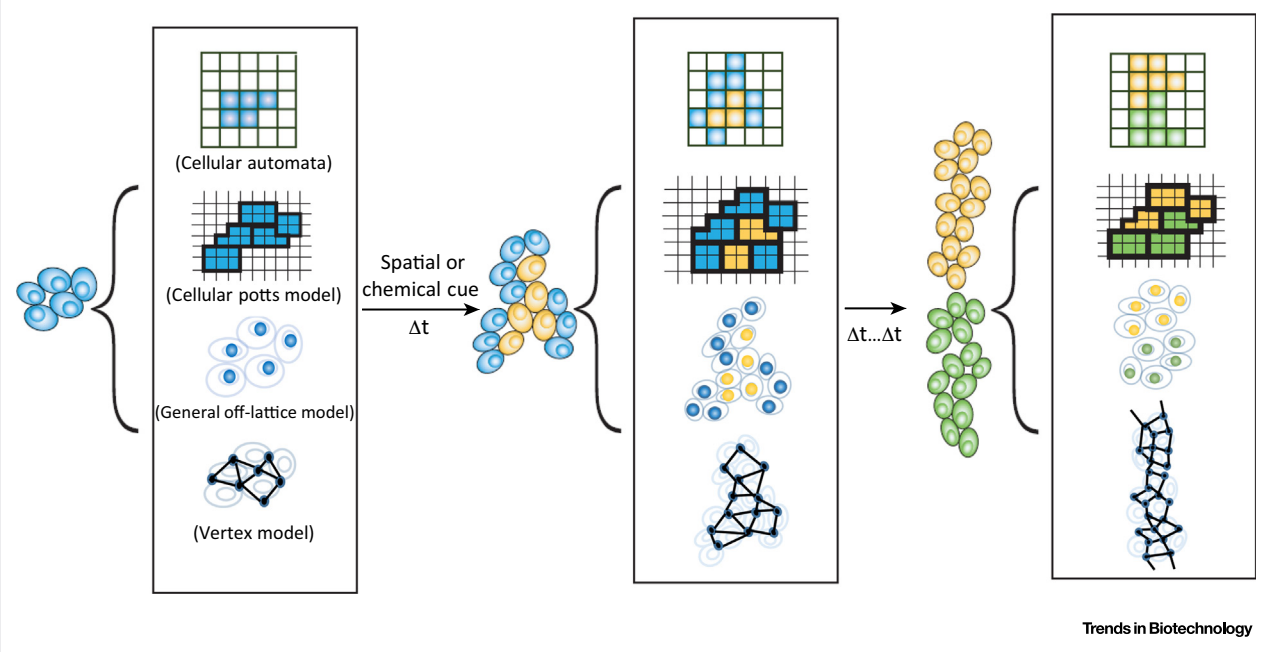

Figure I. Examples of How Cell-Based Modeling Can Be Used to Represent Self-Organizing Processes. Phenotypic changes, as well as mechanical changes, can be modeled by taking discrete steps through time. At each step, changes in spatial or chemical cues drive changes in the system based on a set of predefined rules.

implying that individual agents have unique responses to system inputs. As an example, this type of model can account for spatial considerations, in which a cell located closer to a cue will respond differently, and not just to a different degree, than a cell located further away. Second, the agent-based model is an interaction-based model in that interactions between agents and the environment drive the simulation at each time step. This allows for the consideration of lower-level detail, giving a more biologically faithful representation of the system as long as the set of rules and parameters does not become cumbersome. Finally, multiple agent-based programming languages exist that can be adapted to model biological systems including NetLogo [41] and AgentSpeak [42].

Synthetic Biology for Morphogenetic Engineering

Understanding how individual signaling elements assemble into a morphogenetic complex behavior in time and space remains as a challenge in decoding tissue development. A synthetic biology approach provides a build to understand methodology to answer such questions. It additionally provides a toolbox to guide tissue self-organization towards final desired states. In fact, pattern formation in nonmammalian systems has been engineered using synthetic biology-based strategies [43].

A simple genetic circuit in synthetic biology is composed of sensors, processors, and actuators. Sensors recognize cellular or environmental cues (inputs). Processors determine the type of response. Actuators produce a defined response (output). These elements can shape

genetic circuits that can be interfaced with cellular states or microenvironmental contexts and initiate cell fate programming, cellular communications, and program collective tissue behaviors such as morphogenesis. Ultimately, the goal is to define a set of rules and morphogenetic modules in a plug-and-play format to create defined morphogenetic behaviors. A limited set of such modules was described before including ones for cell proliferation or death, adhesion, migration and sorting [44].

An ability to couple molecular inputs relevant to a cell state or an environmental context (state sensor) to a user-defined output is vital for spatiotemporal control in multicellular systems. Several sensors have been developed to identify biological (e.g., a protein) or nonbiological (e.g., light) signals [45–48]. For example, through promoter engineering and signal amplification using a positive feedback loop, an integrative framework for selective and robust sensing of TFs were achieved [49]. These synthetic promoters can act as a valuable tool to spatially limit the function of genetic circuits in cell type of interest. miRNA levels can differentiate between a wide range of cell types and have been used as classifiers for cell types of interest [50]. By incorporating miRNA target sites in a transcript of interest, a library of miRNA-responsive sensors (Figure 2A) has been constructed [51]. For instance, tissue-specific miRNA sensor-encoding Bim apoptotic proteins were engineered to enrich for cell type of interest such as cardiomyocytes after stem cell differentiation [52]. To achieve programmable integration of multiple inputs, Xie and coworkers [53] generated a classifier gene circuit that integrates information from six endogenous miRNA profiles to differentiate two cell lines with ON:OFF ratios of 8–10-fold. In fact, during mammalian development cell death plays a key role in sculpting the organs and select for fittest cell population [54]. Therefore, miRNA-based cell death circuits can be used to guide *in vitro* tissue formation or reduce aberrant cell fates within developing organoids.

Artificial RNA switches such as RNA **aptamers** and **aptazymes**, are another class of genetic sensors that binds molecules of interest and control gene expression in mammalian cells [48,55]. To this end, Wnt- and nuclear factor (NF)-κB-responsive RNA aptamers were produced which could link activation of underlying pathways to target gene induction. However, engineering aptamers with high affinity to a molecule of interest is not always feasible, which hampers the modularity of this approach. Other seminal studies have developed sensors that can interface an external environmental cue to the transcriptional program of a cell. A modular extracellular sensor architecture (MESA) is a customizable example that acts via ligand-induced receptor dimerization, a protease action, and release of a cognate transcriptional modulator [45]. Combinatorial usage of MESAs can provide a platform for multiparametric environmental sensing [56].

Engineering cellular computation and signal processing enables development of synthetic gene circuits that integrate and convey multiple inputs towards desired cellular outputs. Often, Boolean logic gates have been used to illustrate multi-input–multi-output network topologies in cells [57,58]. Saxena and co-workers developed TF-based gene switches and cascades with differential sensitivity to vanillic acid as an input signal. They could recreate temporal dynamics of the major TFs pancreatic and duodenal homeobox (Pdx)1, neurgenin (Ngn)3, and MafA for pancreatic lineage differentiation. Such stepwise genetic activation in progenitor cells could produce glucose-sensitive insulin-secreting β-like cells [59]. Similar stepwise transcription-factor-triggered cascades can be exploited to encode co-development of cells and structures in a complex tissue. Through composing genetic circuits, complex network topology can also be achieved and may include auto feedback regulatory loops, genetic cascades, bistable expression networks, time-delay circuits, as well as oscillators (Box 2) [60].

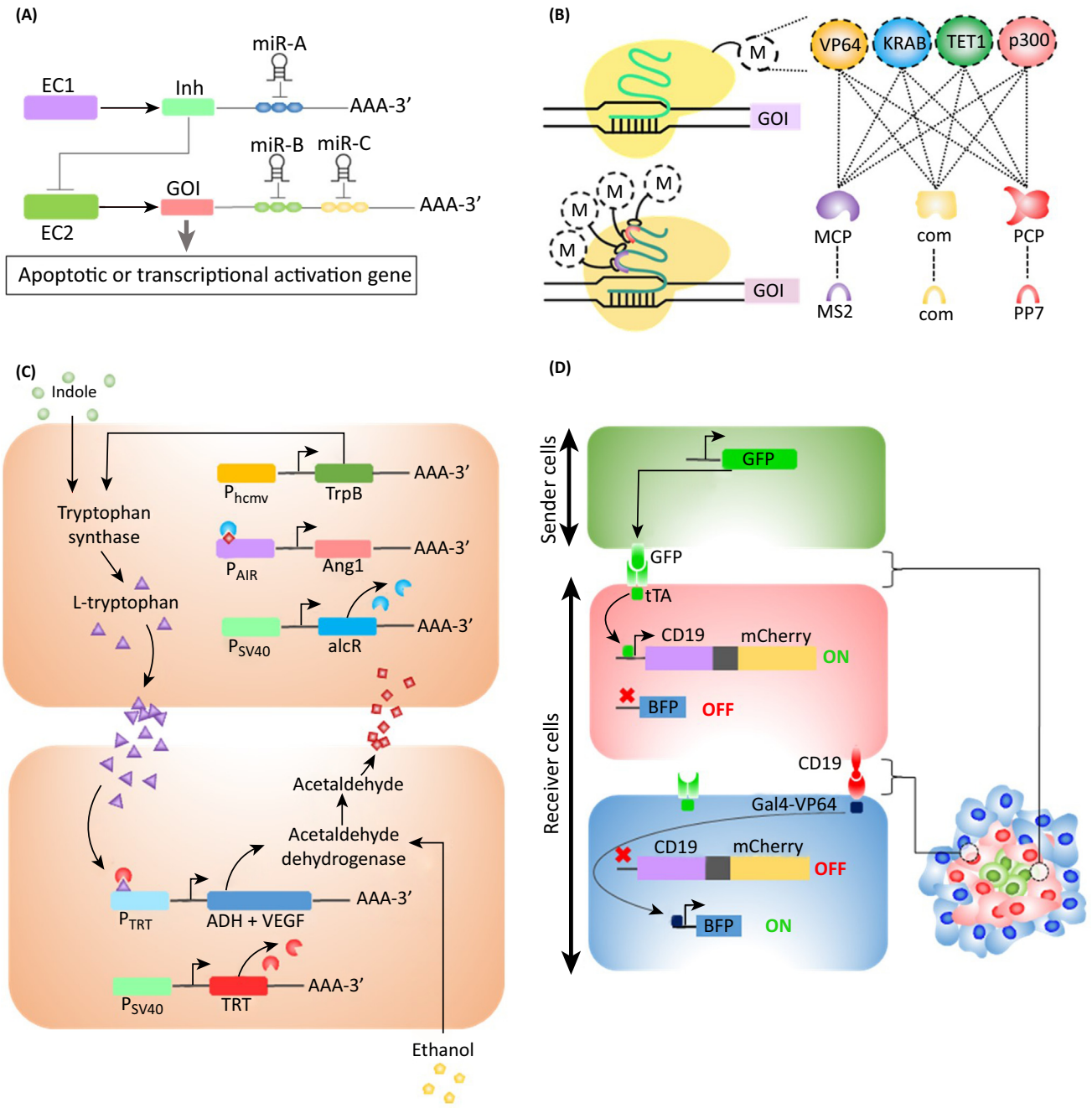

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Figure 2. Synthetic Biology Tools for Programming Cells and Tissue. (A) Complex layered miR classification circuits incorporating GOI can be engineered via cell-specific, high and/or low miR sensors. The circuit requires high levels of miR-A and low levels of miR-B and C for transcription of the GOI. (B) dCas9 system can be used to implement multiplexed activation and repression of GOIs by various effector proteins, including transcriptional activators (VP64) and repressors (KRAB), DNA demethylases (TET1), and chromatin remodelers (p300), either tethered directly to the Cas9 protein, or fused to an RNA-binding domain such as MCP, Com, or PCP. M, modular effector domain [66]. (C) Bacchus and colleagues engineered sender/receiver and processor cells [71]. The genetic circuit interactions shown were successfully utilized to generate a two-way communication system that controls the permeability of an endothelial cell layer by VEGF and Ang1 expression. (D) Morsut and colleagues developed the synNotch system [73], which incorporates modified Notch signaling pathway components into cells to create multilayered self-organizing ring patterns. Sender cell displays GFP on plasma membrane that interacts with anti-GFP synNotch on receiver cell. SynNotch releases tTA and expresses

(See figure legend on the bottom of the next page.)

The advent of precision genome targeting tools provides us with additional set of transcriptional modulators that enable orthogonal gene activation and repression [61,62]. These systems include zinc finger proteins, transcription-activator-like effectors (TALEs), and the clustered, regularly interspaced, short palindromic repeats (CRISPR)-Cas9 system [62]. Deactivated Cas9 (dCas9) that cannot cleave DNA but retains DNA binding function has been engineered with potent transcriptional modulator domains and chromatin remodelers to achieve effective epigenetic and transcriptional programming tool [62] (Figure 2B). Layered dCas9 synthetic circuits can be built to generate a functional cascade [63]. Additionally, guide RNA (gRNA) engineering using different types of aptamers provided an orthogonal platform for recruitment of different proteins such as activator or repressor domains at defined genomic loci [64–66] (Figure 2B). By extending gRNAs to include modified riboswitches that recognize specific signals in Wnt, NF-κB, and oncogenic p53, CRISPR-Cas9-based signal conductors have been generated [67]. This circuit enables transcriptional regulation of endogenous genes in response to inflammatory (NF-κB) or developmental (β-catenin) and oncogenic cues. Programmability of Cas9 and multiplexing of several orthogonal gRNAs offer possibility for combinatorial activation and repression of different GRN targets.

Cell–cell communication and migration are key steps to encode ordered patterns during morphogenesis. Engineering tunable cell–cell communication with short or long range function can program collective cell behavior and induce coordinated functions in mammalian systems. Recent studies have created both soluble and insoluble synthetic intercellular communications and developed strategies to control mammalian cell motility [68,69]. Engineering G protein-coupled receptor resulted in directing mammalian cell motility to a bio-inert drug-like small molecule, clozapine-*N*-oxide (CNO) [70]. A synthetic multicellular two-way communication system was developed using l-tryptophan and acetaldehyde as communicating soluble signals (Figure 2C) [71]. The sender–processor–receiver format can be used to emulate several soluble signaling interactions of interest and enables tissue level computation. Cachat and colleagues used the differential adhesive strengths between homotypic and heterotypic E-cadherin and P-cadherin interactions to drive cell sorting when expressed in mammalian cells [72]. Through cadherin-mediated phase separation they could promote mammalian cells to generate intricate 2- and 3D patterns *de novo.* In an another study, Notch signaling was exploited as a platform to link recognition of an arbitrary extrinsic cellular input to intrinsic transcriptional outputs in an orthogonal manner [73]. Morsut and colleagues designed this platform by replacing extracellular sensor module and intracellular transcriptional module of Notch signaling with diverse ligand binding or TF domains (Figure 2D). This synthetic Notch system (synNotch) was used to generate cellular patterns around GFP expressing sender cells. SynNotch is a novel toolset to engineer user-defined tissue level computing, however it lacks amplification signals typically observed in intracellular signaling events. This means one ligand molecule (input) can presumably activate one intracellular signaling molecule (output). Additionally, other modes of cell–cell communication such as exosomes can be engineered to establish exchange of protein or RNA cargos between sender and receiver cells [74].The diverse exosomal inputs signals (e.g., miRNAs) opens myriad of possibilities for transcriptional regulation of morphogenesis or broadcasting cellular fitness or tissue composition during *in vitro* tissue development.

both CD19 and red fluorescent reporter proteins. CD19 triggers anti-CD19 synNotch receptor on the adjacent cell and induces BFP. B, C, and D adapted from [66], [71], and [73], respectively. Abbreviations: ADH, alcohol dehydrogenase; AlcR, acetaldehyde-dependent transactivator; Ang1, angiopoietin 1; BFP, blue fluorescent protein; Com, phage Com protein; com, Com RNA binding locus; EC, engineered DNA circuit; GFP, green fluorescent protein; GOI, gene of interest; Inh, EC2 inhibiting gene; M, modular effector domain; MCP, MS2 coat protein; miR, miRNA; P_{AlR},acetaldehyde-responsive promoter; PCP, PP7 coat protein; P_{hCMV}, human cytomegalovirus immediate early promoter; P_{SV40}, simian virus 40 promoter; P_{TRT}, L-tryptophan-inducible promoter; TrpB, *Escherichia coli* tryptophan synthase β-subunit; TRT, L-tryptophan-dependent transactivator; tTA, tetracycline-controlled transactivator; VEGF, vascular endothelial growth factor.

Concluding Remarks and Future Perspectives: Reading and Writing of Human Tissue Complexity

Organoids and other self-organizing tissues have shown promise to replicate fundamental facets of human tissue development. At least for therapeutic human translation or disease modeling it is critical to engineer human tissues that can closely mimic their natural counterparts. With this biological design objective in mind, different strategies have been proposed to advance our current approaches relevant to 'organogenesis in a dish' [75]. Organ-on-a-chip engineering approaches have focused on probing cell and tissue microenvironments using microfluidic devices and tissue scaffolds with smart biomaterials and controlled mechanical properties [76]. The approach relies on presentation of defined physiochemical and mechanical cues from outside the cells. Hence, it necessitates knowledge and tools to recapitulate composition and spatiotemporal dynamics of developmental cues presented to the cells, at a given time in morphogenesis.

Synthetic biology-based tissue engineering focuses on engineering cells from inside via genetic circuits with defined functions. It provides a **forward engineering** approach to generating tissues and organs via genetically programing cell behavior and subsequently tissue morphogenesis. However, with a large space of parameters, finding a best set of candidate genes and pathways for forward engineering might become a challenge (see Outstanding Questions). Yet, the developmental program of mammalian tissues already offer an unmatched library of GRNs that evolved through many years of evolution. If reverse engineered properly, the information can aid synthetic biology for rational engineering of morphogenesis. Additionally, via assembly or rewiring of genetic networks in one or multiple cell populations, a synthetic biology practice can help in multiscale decoding of organizational principles central for the final integrated cellular behaviors (i.e., self-organization and morphogenesis). By integration of systems and synthetic biology one can envision new generation of programmable tissues and designer organoids that can closely recapitulate human development, shed light to our own engineering design principles, or deliver user-defined organ functions. Combined with other engineering approaches such as organ-on-a-chip engineering they can shape the future of modern tissue engineering.

Outstanding Questions

What is the best strategy to deliver large genetic circuits to mammalian stem cells without encountering gene silencing or perturbed function over time?

The predictability of synthetic gene networks within the mammalian cells remains poor. How can unintended interactions with host cells and limitations of cellular resources among engineered gene circuits be identified and eliminated?

What computational strategies can be used to identify a core set of tissue parameters and cellular GRNs that are amenable for engineering a given morphogenetic behavior?

How can the balance between engineering defined function and emergence of new behavior be maintained? Can we exploit emergent behaviors to improve morphogenetic engineering?

How can synthetic morphogenetic circuits be developed with the robustness of naturally occurring genetic circuits, which have the advantage of multiple feedback systems and redundant parts?

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