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Current Opinion in Chemical Biology

Engineering cell–cell communication networks: programming multicellular behaviors

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Cell–cell communication governs the biological behaviors of multicellular populations such as developmental and immunological systems. Thanks to intense genetic analytical studies, the molecular components of cell–cell communication pathways have been well identified. We also have been developing synthetic biology tools to control cellular sensing and response systems that enable engineering of new cell–cell communication with design-based regulatory features. Recently, using these molecular backgrounds, synthetic cellular networks have been built and tested to understand the basic principles of multicellular biological behaviors. These approaches will provide new capabilities to control and program desired biological behaviors with engineered cell–cell communication to apply them toward cell-based therapeutics.

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Current Opinion in Chemical Biology 2019, **52**:31–38

This review comes from a themed issue on **Synthetic biology**

Edited by **Hirohide Saito** and **Yohei Yokobayashi**

https://doi.org/10.1016/j.cbpa.2019.04.020

Introduction: cell–cell communication as key to program higher order biological behaviors

In multicellular organisms, precise regulation of coordinated, multicellular behaviors based on cell–cell communication is key for higher-order macroscale biological functions. For example, during development, complex tissue structures emerge from small groups of cells. To drive tissue morphogenesis, cells communicate with each other using transmembrane and diffusible proteins to decide cell fate such as differentiation and proliferation and self-assemble three-dimensional, complex structures by controlling the mechanical properties of cells. Other examples are the coordinated response of multiple types of immune cells against pathogens and the higher-order brain functions mediated by neural circuits. These multicellular systems started with simpler ones in primitive multicellular organisms and became more sophisticated systems through long-time evolution. We can learn from the process of evolution how to engineer cell–cell communication networks to build new multicellular behaviors (Figure 1a).

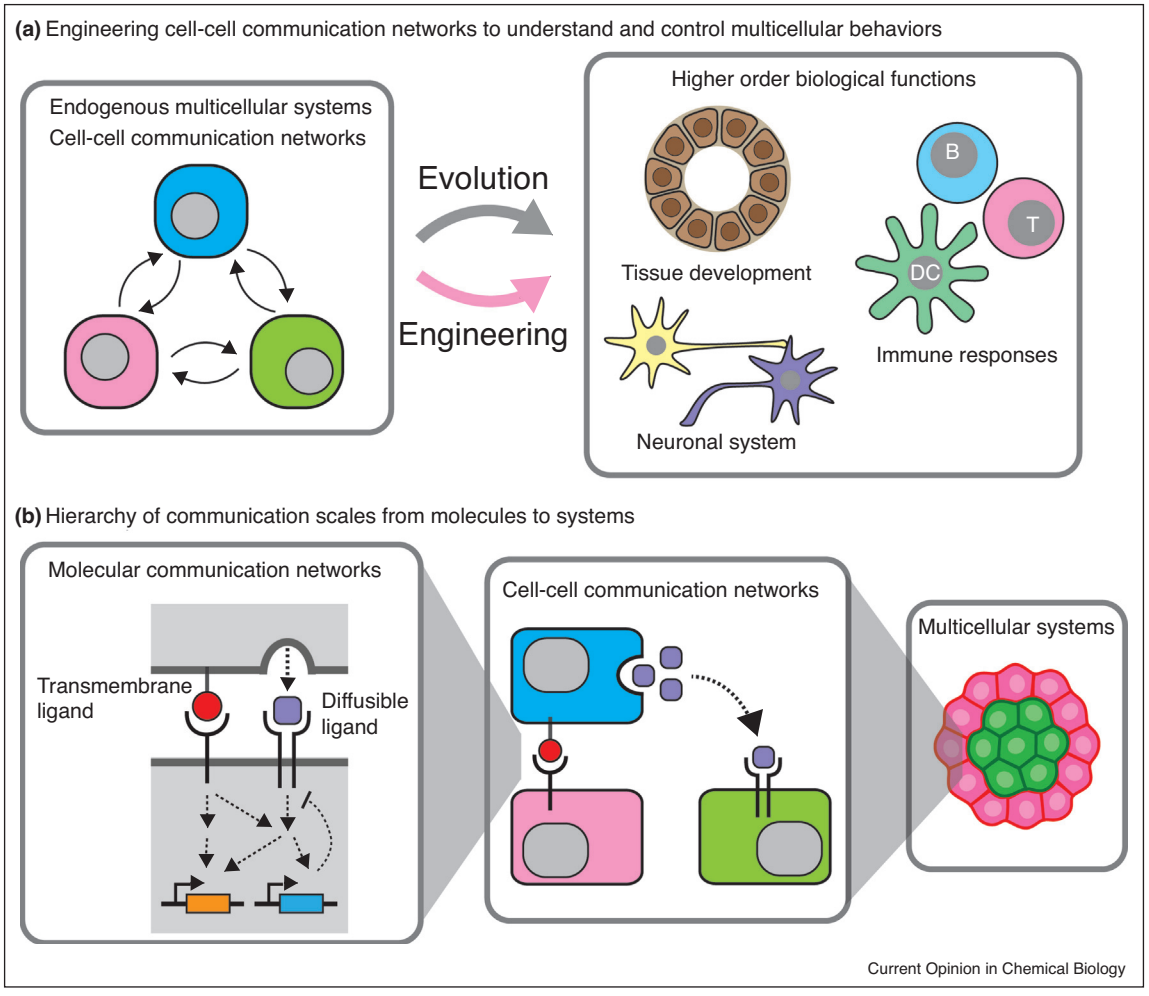
In general, cells can sense environmental cues such as transmembrane ligands and secreted molecules through receptors, process environmental information via intracellular molecular networks, and then make decisions to output-specific behaviors such as gene expression, cytoskeletal changes, and secretion [1]. When one cell senses an output of another cell as an input, cell–cell communication networks are formed, driving collective behaviors leading to functional outputs as a system (Figure 1b).

How are molecular communications and multicellular behaviors linked across different scales? Thanks to recent advances in molecular biology and genetics, the molecular mechanisms of cell–cell communication have been intensely studied. On the basis of this molecular background, we can synthetically construct cell–cell signaling networks to test and understand the basic principles of how the cell–cell communication can drive biological functions [2–4]. Since native signaling pathways form complex networks with multiple inputs and outputs, we have been developing orthogonal cell–cell communication modules to create novel cell–cell signaling with user-defined input and output to achieve desired multicellular behaviors [5–8]. In this review, we briefly describe the molecular background of native and engineered cell–cell communication and discuss how it can be engineered in order to control the behavior of multicellular systems.

Channels for controlling cell–cell communication

Cells can communicate with adjacent cells using transmembrane ligands and receptors. The Notch-Delta pathway is a well-known juxtacrine signaling system [2,9]. Notch receptor and its ligand Delta are both transmembrane proteins that regulate gene expression that mediates cell differentiation. When Notch receptor recognizes Delta on a neighboring cell, Notch undergoes conformational changes and is cleaved by transmembrane proteases, leading to the release of Notch intracellular domain (NICD) into the cytoplasm. The NICD then translocates to the nucleus to drive target gene expression. To engineer novel cell–cell communication channels that can control customized

Figure 1

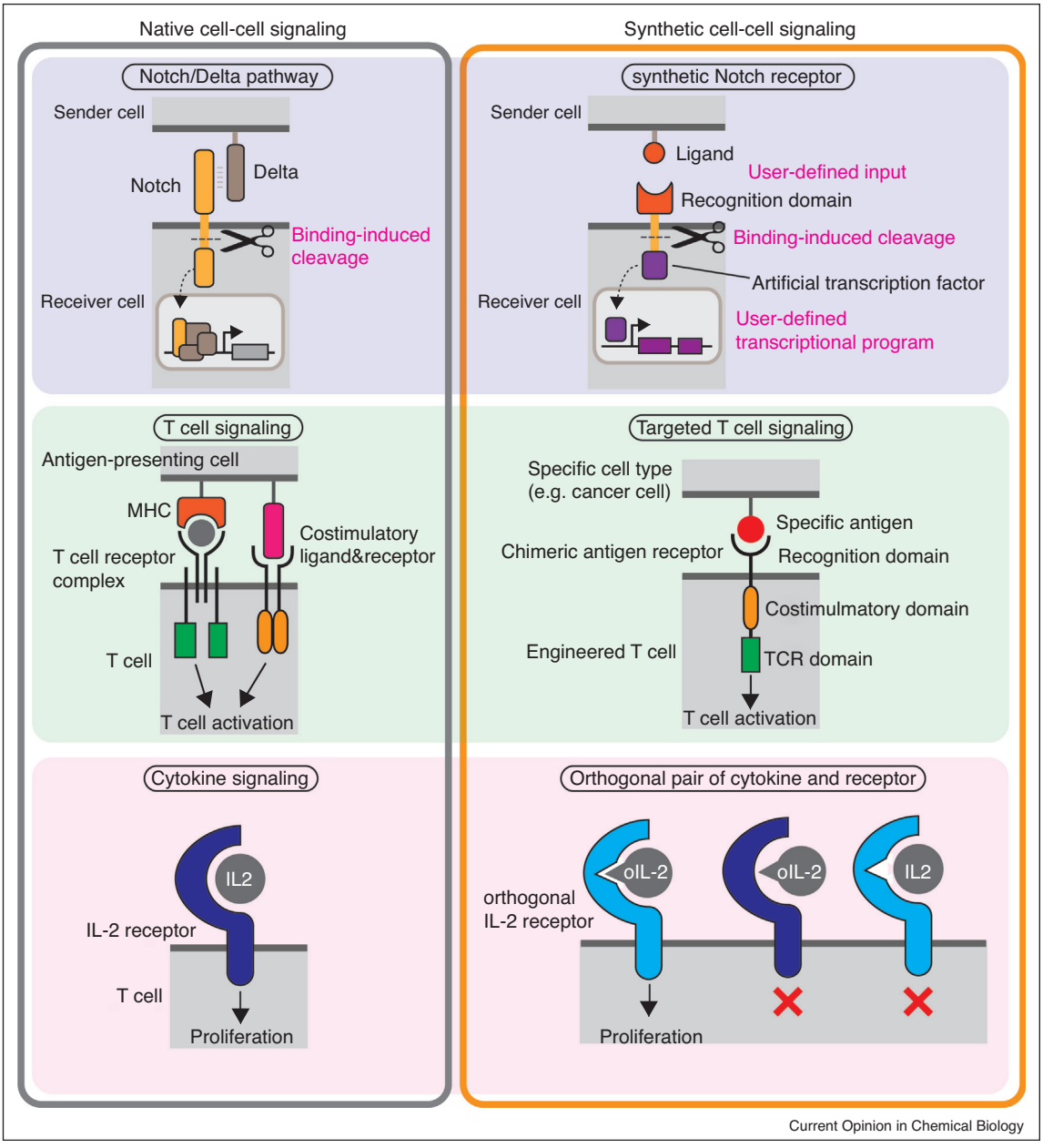


Engineering cell–cell communication networks.
(a) Engineering cell–cell communication networks to understand and control multicellular behaviors. Endogenous multicellular systems contain complex cell–cell communication networks that enable higher-order biological functions such as tissue development, immunological responses, and neural circuits. These elaborate systems in multicellular organisms developed through the evolution of cell–cell molecular interactions. Drawing inspiration from evolution, we can engineer cell–cell communication networks to drive new multicellular biological behaviors.
(b) Hierarchy of communication scales from molecules to systems.
At the molecular scale, receptors sense membrane-tethered and diffusible ligands to trigger intracellular signaling networks. Cells then decide what types of behaviors to output. At the cellular scale, cells sense and respond to other cells' outputs to form cell–cell communication links. At the system level, cellular behaviors are dynamically regulated and coordinated by their interactions, giving rise to macroscale biological functions.

cell sensing and response behaviors, we have developed synthetic Notch receptor (synNotch) (Figure 2) [10]. We replaced the Notch extracellular domain with a specific single-chain antibody to bind a selected ligand of interest, and also replaced the NICD with a synthetic transcription factor, which can drive target transgene expression. Thus, using the synNotch receptor, we can engineer new gene-regulatory interactions between spatially proximal cells: synNotch-expressing 'receiver' cells recognize cognate ligand expressed on neighboring 'sender' cells and in response, specific target genes can be induced in the 'receiver' cells.

In many cases, however, multiple types of receptors and intracellular proteins are involved in signal transduction at the cell–cell interface. For example, when a T cell recognizes an antigen on an antigen-presenting cell (APC), a cluster of T cell receptors, major histocompatibility complexes (MHCs), costimulatory receptors, adhesion molecules, intracellular kinases, and scaffold proteins form an immunological synapse to activate T cell receptor signaling [11,12]. Recently, there has been tremendous interest in engineering immune cells to redirect their therapeutic functions toward cancer treatment [13,14]. To artificially target T cell activities

Figure 2



Channels for native and synthetic cell–cell communication.
Using design principles from native receptor interactions, these synthetic receptors allow for reprogramming of cell–cell signaling. Top row (purple): Native and engineered Notch signaling. (Left) Delta presented on the surface of a sender cell binds to the Notch receptor on a receiver cell, resulting in cleavage of the Notch intracellular domain (NICD), a transcription factor promoting differentiation. (Right) synNotch is based on the binding-induced-cleavage principle and uses the Notch core sequence. However, the NICD is replaced with an artificial transcription factor to drive transcription of user-defined genes, and the extracellular domain is replaced with a recognition module such as a single chain antibody, enabling programing of cell–cell communication with custom molecular input and transcriptional output. Middle row (green): Native and targeted T cell signaling. (Left) T cells use the T cell receptor (TCR) to sense antigens loaded onto the major histocompatibility complex (MHC) expressed on antigen-presenting cells (APCs). APCs can also provide costimulatory signals, and the combination results in T cell proliferation, cytokine secretion, and cytotoxic activity. (Right) To synthetically target T cell activation to a specific tumor cell type, chimeric antigen receptors (CARs) were constructed, replacing the extracellular domain with an interchangeable single-chain antibody, and creating an intracellular signaling domain that combines elements from the TCR and costimulatory receptor. Bottom row (pink): Native and orthogonal cytokine signaling. (Left) T cells sense the activating cytokine interleukin-2 (IL-2) using the IL-2 receptor β (IL2Rβ, dark blue) along with α and γ subunits (not shown). The trimeric receptor in complex with IL-2 results in proliferation. (Right) Ortho-IL2Rβ (light blue) has mutations that prevent binding of IL-2. Ortho-IL-2 (oIL-2) is a mutated IL-2 with poor binding to the native IL2Rβ, but binds to Ortho-IL2Rβ and produces cell proliferation.

against a specific, clinically relevant antigen, chimeric antigen receptors (CAR) have been developed (Figure 2) [15]. Current-generation CARs contain a single-chain antibody in the extracellular region and intracellular domains containing multiple signaling domains from the T cell receptor and costimulatory receptors. CARs can recognize cognate cell surface antigen directly without MHC to activate T cell immunological responses, which allows us to engineer T cells to target any type of cells in principle, including cancer cells expressing specific antigens. The details of how different types of CARs have been developed and tested in mouse models and clinical trials have been reviewed in detail elsewhere
[16–18].

Cells can also communicate without direct cell–cell contact by secreting diffusible proteins. A localized source of a diffusible protein can form a gradient of its concentration, which can provide positional information to surrounding cells. In development, this type of diffusible protein is called morphogen and can induce pattern formation by causing cells to choose different cell fates in response to different amounts of morphogen along the gradient [19,20]. When multiple morphogens interact simultaneously with positive or negative regulation, the resulting reaction–diffusion system can generate various types of cell-autonomous patterns arising independently of a pre-existing pattern [21,22]. In addition to pattern formation by morphogens, many growth factors and cytokines are diffusive to regulate cell behaviors such as proliferation. Recently, engineered orthogonal cytokine-receptor pairs have been developed based on interleukin-2 (IL-2), which promotes T cell proliferation and activation (Figure 2) [23•,24]. The pair of engineered IL-2 and its receptor can interact with one another to activate intracellular IL-2 signaling, but this pair is orthogonal to native IL-2 and IL-2 receptor: the engineered IL-2 does not activate native IL-2 receptor, and engineered IL-2 receptor is not activated by native IL-2. Using the orthogonal cytokine system, we can expand a subset of engineered T cells selectively with minimum effect on endogenous T cell activation, which should be useful to limit side-effects by unintended T cell activation.

Engineering novel multicellular systems

Toward understanding universal principles of tissue organization

Native cell–cell signaling pathways are intermingled, with extensive crosstalk, making it challenging to perturb and analyze individual pathways quantitatively in an *in vivo* context. To isolate a specific signaling pathway for further analysis, we can try to construct minimal cell–cell signaling circuits *in vitro*. Recently, the reaction–diffusion system of Nodal–Lefty has been reconstituted to generate synthetic cell-autonomous pattern formation (Figure 3a) [25•]. Human embryonic kidney cells (HEK293) were engineered to express the components of the Nodal signaling pathway with a luminescence reporter. By further engineering of the Nodal positive feedback circuit with the Lefty inhibitory feedback regulation, a synthetic pattern of Nodal-positive and Nodal-negative domains was spontaneously generated. The reconstituted system revealed that secreted Nodal is confined in the space between the cells and the culture dish, results in a narrower spatial distribution than Lefty, which gives rise to striking patterns characteristic of reaction–diffusion systems [26].

By studying cell–cell signaling circuits that controls cell proliferation, we can ask questions about the design principles that enable homeostasis of interacting cell populations within a tissue. Recently, a two-cell system that exhibits reciprocal growth factor exchange has been reconstructed *in vitro* using murine macrophages and fibroblasts [27•]. The exchange of growth factors between the two cell types recapitulated population stability in cell ratios while maintaining robustness against perturbations in initial cell number or ratio.

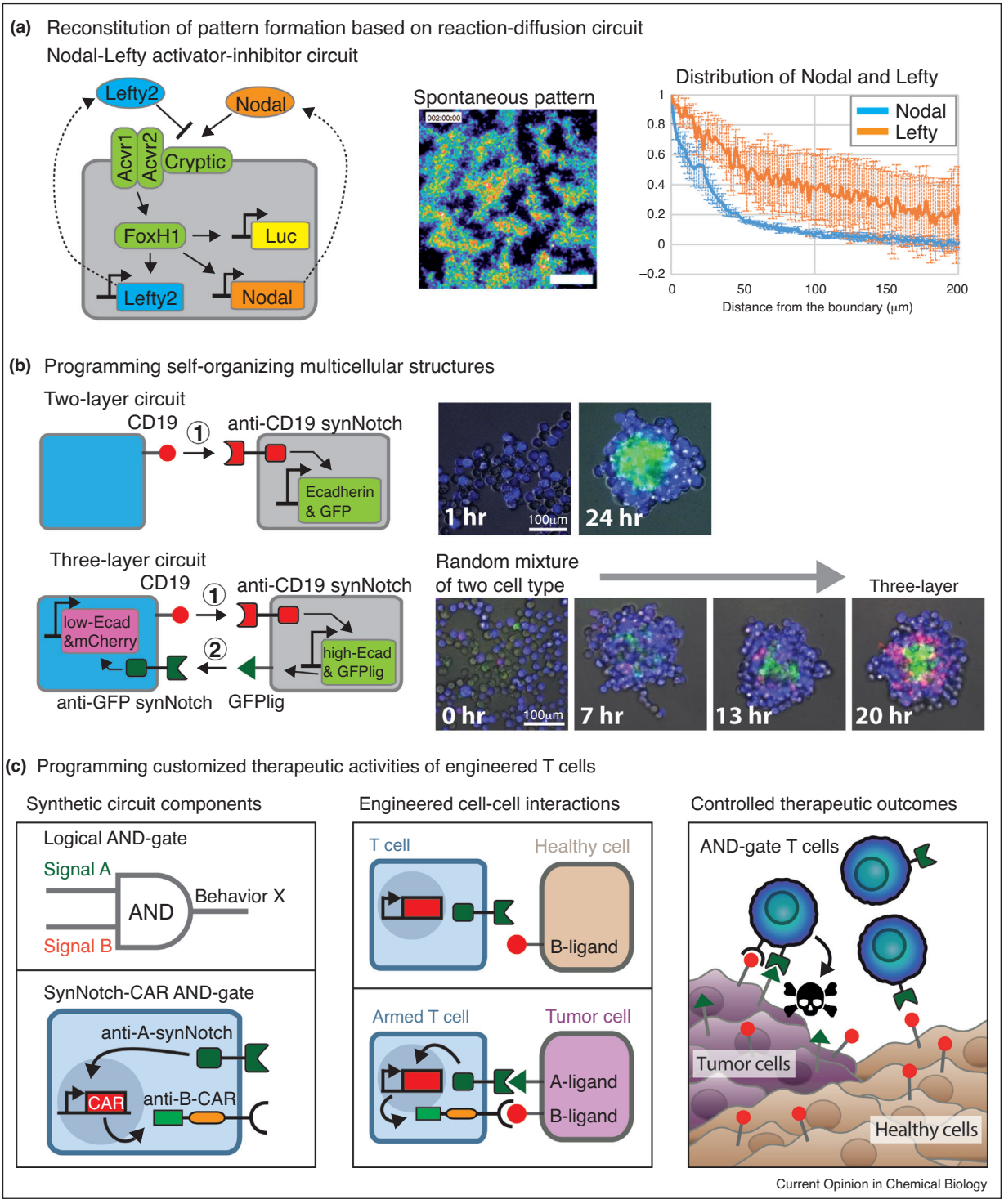
In summary, isolation of natural cell–cell interactions in reconstituted systems is a powerful tool to define the principles of cell–cell signaling circuits that output multicellular behaviors, which may be crucial in future efforts to forward-engineer similar systems for therapeutic purposes, or to find ways to rebuild those compromised by disease states.

Synthetic morphogenesis

One approach to understanding genetically encoded algorithms that can direct individual cells to communicate and autonomously construct complex macroscale structures is to rewrite and test synthetic genetic programs. The modular synNotch platform enables us to design synthetic cell–cell communication programs in which specific cell–cell contacts can control expression of target effector genes. Using this platform, we designed synNotch circuits that control three types of outputs: cadherin-based cell adhesion for spatial cell sorting, fluorescent proteins to identify cell types, and additional synNotch ligands to drive multistep signaling programs [28••].

When cells express different amounts of cadherin, those expressing higher levels bind to each other more strongly and form an aggregate. Conversely, cells expressing lower levels get excluded from this core and form an outer layer [29–31]. We recapitulated this spatial cell sorting by inducing E-cadherin expression through the synthetic cell–cell communication of sender and receiver cells, resulting in self-organization into a two-layer structure (Figure 3b). To increase the complexity of the self-organizing structures, we

Figure 3



Engineering multicellular behaviors with synthetic cell–cell signaling.
(a) Reconstitution of pattern formation based on a reaction-diffusion circuit. Sekine *et al.* designed a Nodal–Lefty activator-inhibitor circuit in which Nodal activates its receptor complexes to drive the expression of Nodal, Lefty and Luc reporter. Here, while Nodal induces itself as a positive feedback, induced Lefty works as an inhibitor of Nodal signaling. This system formed a reaction-diffusion circuit with different signaling ranges of Nodal and Lefty, resulting in spontaneous pattern formation. (Adapted from Sekine *et al.* [25•]).
(b) Programming self-organizing multicellular structures. Top: Two-layer circuit. A mouse fibroblast line (L929) was engineered to produce CD19-expressing sender cells and synNotch-expressing receiver cells that induce E-cadherin and GFP. When they were cocultured, the receiver cells were activated by contact with the sender cells, forming a green core aggregate that self-organized into two-layer structure. Bottom: Three-layer

modified the receiver cells to produce a second synNotch ligand (membrane-tethered GFP) in addition to E-cadherin in order to activate a second level of communication. We also modified the sender cells to express an anti-GFP synNotch receptor to recognize the receiver cells' new ligand and, in response to ligand-binding, to express a smaller amount of E-cadherin with a second fluorescent protein, mCherry. This two-step signaling cascade between two types of cells achieved sequentially programmed assembly of three distinct layers. The first signaling interaction led to a two-layer structure being formed. Then, the second signaling process occurred only in the sender cells attached to the GFP ligand-expressing core cells, resulting in expression of mCherry and a low amount of E-cadherin to be sticky toward the core, forming a surrounding layer. The resulting self-organized structure contained three-layers: a GFP-expressing core, an mCherry-expressing middle layer, and an external layer (Figure 3b). We also designed synthetic developmental programs in which different types, rather than amounts, of cadherins were induced in order to generate a wide variety of symmetric and asymmetric three-layer structures [28••]. In these programs, cell–cell signaling controlled cadherin expression to output spatial organization of cells. Through this process, changes of cell positions caused new cell–cell signaling with different neighboring cells. Subsequently, the new signaling partners induced new cadherin induction and cell sorting. By repeating this process, cells self-organized into more complex structures with each increase in cell type [28••].

Overall, these results indicated the flexibility and power of our modular synthetic signaling system to construct self-organizing multicellular structures. Thus, it will be interesting to control more morphogenetic factors in these self-organizing programs in addition to cell adhesion in order to program more sophisticated and functional multicellular structures, for example diffusible morphogens, regulators of cell proliferation, death, or motility, transcriptional factors of transdifferentiation, and so on. Using these systems, we may be able to program therapeutic cells that could, for example, sense-specific signals from damaged tissues to create synthetic tissue patches that secrete growth factors that stimulate host cells for wound-healing [32].

Engineering complex immune cell circuits

The recent clinical successes of CAR-T cells highlight the potential of T cells as a 'hackable' cellular chassis for creating living therapies with customizable behavioral circuitry [33]. To realize such a goal, we must develop technology to engineer cell–cell communication so that therapeutic cells will be able to distinguish 'good' from 'bad' cells with high fidelity, as well as to induce behaviors in themselves and their targets, which will include both engineered and non-engineered cells. Such a technology must encode several new capacities beyond those seen in conventional CARs, including modular combinatorial sensing for precise cell type recognition, quantitative control over continuous variables such as dose–response curves, and the ability to execute engineered genetic programs with limited crosstalk into native signaling channels. Several recent studies have begun to outline some of the possibilities for making information exchange between T cell and target more specific and robust.

Modularity is an important design feature of both biological and engineered systems [34]. CARs are by design modular intramolecularly, with researchers testing different co-stimulatory domains to optimize activation [35], but the fact that they are a one-piece component limits their flexibility [36]. Each new intended target may require a redesign and optimization of the whole. The same is true for any redirection or improvement made to the downstream signaling. To overcome these limitations, Cho *et al.* recently created a split, universal, programmable (SUPRA) CAR [37]. By splitting the sensory and response domains into two molecules, it became simpler to change targets and the performance became more robust to such changes. Accordingly, changes to the signaling component to make quantitative adjustments to the magnitude and nature of activation featured similar advantages in facility and flexibility. This innovation demonstrates the power of applying engineering principles such as modularity to improving communication between target cells, engineered T cells, and therapeutic outcomes.

So-called cancer antigens are often also expressed on healthy cells, making CARs risky due potential cross-reaction, or 'on-target off-tumor' toxicity [38]. SynNotch is a powerful tool for introducing new input/output components into cells, so we recently demonstrated how this component can introduce logical control to improve CAR

(Figure 3 Legend Continued) circuit. First, signaling by CD19 ligand on the sender cells activated GFP ligand expression and a high level of E-cadherin in the receiver cells to form the two-layer structure with a green core and blue outer layer (13 hour). Second, delayed signaling induced GFP ligand activated a low expression level of E-cadherin and mCherry in the sender cells attaching the core, inducing the stepwise formation of three layer structure (20 hour) (adapted from Toda *et al.* [28••]).

(c) Programming customized therapeutic activities of engineered T cells. (Left) Drawing inspiration from logic gates in electrical and computer engineering, the AND-gate requires two external signals to decide to output a given behavior. The synNotch-CAR AND-gate uses synNotch against one ligand to express a CAR for a second ligand. (Middle) An AND-gate component constrains cytotoxic output to targets expressing both ligand types. Without synNotch ligand, CAR is not expressed, making killing impossible despite the presence of the CAR ligand. Targets with both ligands, however, first arm the circuit then become targeted by the CAR. (Right) Favorable therapeutic outcome of killing tumor cells but sparing healthy cells. Because so-called cancer antigens can exist on healthy tissue, adding additional inputs through AND-gates can increase the ability of CAR-T cells to distinguish between 'right' and 'wrong' targets.

safety [39•]. By putting CAR expression under control of synNotch responding to a second antigen, it was shown that the complete circuit functioned as a logical AND-gate, thus cells expressing the circuit only killed tumors expressing both ligands (Figure 3c). Such logical or combinatorial sensing will be crucial in the design of sophisticated T cell therapies capable of 'perceiving' their targets' complex molecular signatures as distinct from healthy cells. Other logic gates such as OR-gates have been engineered in the form of dual-headed CARs that respond to either of two ligands and allow T cells to tolerate some level of tumor heterogeneity or outsmart tumors that tend to switch antigens for immune evasion [40].

Engineering cell communication is not only a matter of recognition of the cognate target, but it also requires fundamentally reconfiguring the response to such interactions. CARs by design directly plug into preexisting T cell activation channels [41]. Taking the concept of cell therapy beyond CARs and cancer will require programming output behaviors in a manner fully 'insulated' from native signaling pathways [42]. We recently showed that synNotch can be used in T cells to couple target cell recognition to custom transcriptional responses independent of native T cell activation pathways, allowing T cells to modify the cell environment in new ways without also engaging T cell activation programs [43]. T cells expressing synNotch without a CAR recognized tumors *in vivo* and delivered payloads such as cytokines or immunotherapeutic antibodies, locally and conditionally.

Conclusion

Cells use sensing and response systems to communicate with each other to achieve complex collective behaviors that are critical for biological function. Engineering of cell–cell communication can help define the basic rules of how cells can be linked together to self-assemble and carry out biological functions, just as chemistry defines the rules that link atoms to build chemical compounds. In addition, modular synthetic biology toolkits such as synNotch will expand our ability to build and explore new cell–cell communication regimes in order to generate desired output functions. The elucidation of these basic rules of cell–cell signaling systems can lead to better understanding of the design principles of control of multicellular behaviors, which will help us to engineer therapeutic cells for immune cell therapy and tissue repair.

Conflict of interest statement

W.A.L. has a financial interest in Gilead Biosciences.

Acknowledgements

We thank the Lim lab members for helpful discussions. We apologize to authors whose relevant work was not cited in this review due to space constraints. The authors are supported by Japan Society for the Promotion of Science (JSPS) Overseas Research Fellowship; Human Frontier Science Program (HFSP) (S.T.), 5P50GM081879; NSF DBI-1548297Center for Cellular Construction; the DARPA Engineered Living Materials program; and the Howard Hughes Medical Institute (W.A.L.).

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- •• of outstanding interest

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