

Stem cell bioengineering: building from stem cell biology

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Abstract | New fundamental discoveries in stem cell biology have yielded potentially transformative regenerative therapeutics. However, widespread implementation of stem-cell-derived therapeutics remains sporadic. Barriers that impede the development of these therapeutics can be linked to our incomplete understanding of how the regulatory networks that encode stem cell fate govern the development of the complex tissues and organs that are ultimately required for restorative function. Bioengineering tools, strategies and design principles represent core components of the stem cell bioengineering toolbox. Applied to the different layers of complexity present in stem-cell-derived systems — from gene regulatory networks in single stem cells to the systemic interactions of stem-cell-derived organs and tissues — stem cell bioengineering can address existing challenges and advance regenerative medicine and cellular therapies.

Cell therapies

Clinical treatments that introduce living cellular material into a patient. They may engraft in the body, leading to long-term replacement of damaged or missing tissue, or stimulate endogenous repair and promote endogenous viability.

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For over half a century, stem cells have captured our imagination for their promise of providing a foundation for strategies to regenerate diseased tissues and organs. A major catalyst was the effort by Till and McCulloch to quantitatively describe fundamental stem cell properties¹. These properties — the ability to ‘self-renew’ or divide to produce more stem cells and the ability to differentiate to give rise to specialized cells — make stem cells an attractive cell source for clinical applications. The potency, or potential, of different stem cell types refers to their ability to give rise to specialized cells of the body and can be organized as a hierarchy, with decreasing potency as stem cells become more committed along a specific lineage trajectory (FIG. 1). Over the past few decades, much progress has been made towards the use of stem cells for clinical applications. Bone marrow transplants, which contain haematopoietic stem cells (HSCs), are routinely conducted to treat patients with multiple myeloma, lymphoma, leukaemia and various autoimmune diseases². Ocata Therapeutics (Marlborough, MA, USA), acquired by Astellas Pharma (Tokyo, Japan), has pluripotent stem cell (PSC)-derived retinal pigment epithelium cell therapies in phase I and II clinical trials for blindness^{3,4}. Asterias Biotherapeutics (Fremont, CA, USA) has a human embryonic stem cell (ESC)-derived oligodendrocyte progenitor therapy for spinal cord injury in clinical trial⁵, with promising early patient data suggesting signs of improved motor function. ViaCyt (San Diego, CA, USA) has also moved to clinical trials for their subcutaneously implanted medical device containing human-ESC-derived β cells to treat type 1 diabetes mellitus⁶. Recently, transgenic stem cells

have been used to functionally correct and regenerate the diseased epidermis of patients with junctional epidermolysis bullosa, a genetic skin disorder^{7,8}. In one of these studies, the authors not only regenerated the entire epidermis of a patient with an extremely severe form of the disease but in doing so also provided evidence that the epidermis is renewed by a stem cell population and not by equipotent progenitors^{8,9} — a longstanding debate in the epidermal stem cell field. There has also been a boom in the development of cell therapies as targeted immune effectors. A notable example is chimeric antigen receptor (CAR) T cells, which are being developed by several biotechnology companies, including Kite Pharma and Novartis.

While stem cells have direct clinical applications as a substrate from which to derive regenerative cell therapies, they also serve as a clinical tool to identify small-molecule-based treatments for diseases that have historically been intractable. Recent advancements in stem-cell-based organoid technology have enabled scientists to mimic the symptoms of diseases such as cystic fibrosis or Alagille syndrome by growing patient-derived cells in a dish^{10,11}. Disease-in-a-dish models such as these can begin to make progress in developing personalized therapeutics for patients with devastating genetic disorders. In fact, patient-derived organoid models of cystic fibrosis have enabled the prediction of patient-specific response potential to drugs in preclinical testing¹².

Clearly, stem cells have broad implications for innovative advancements, both directly by serving as a starting material for cell therapy in disease treatment and

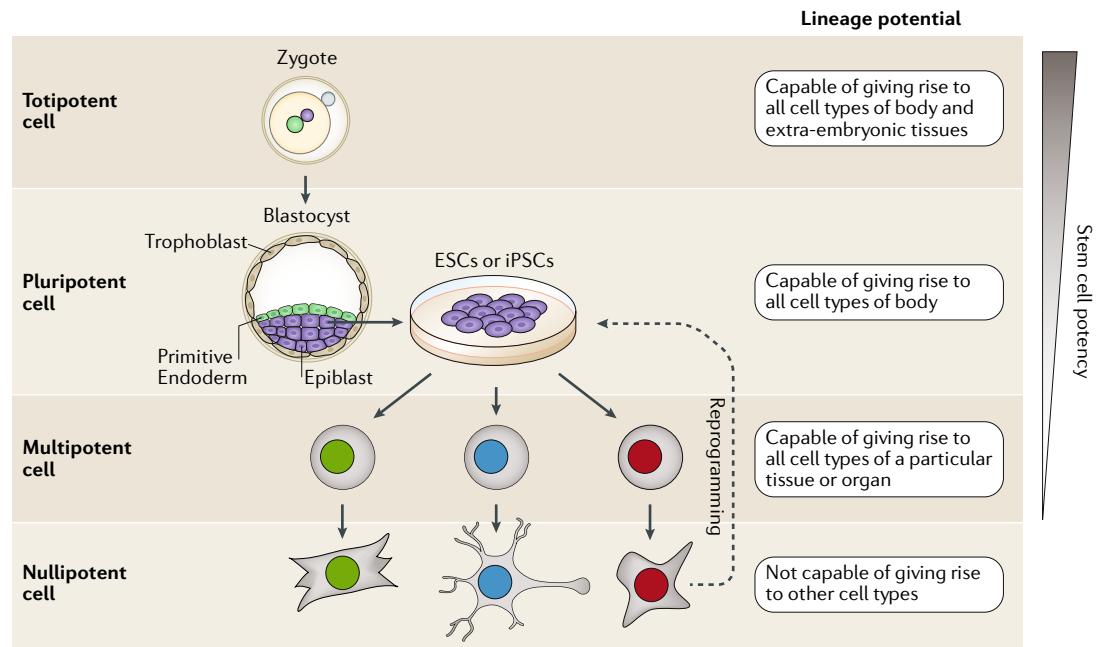


Fig. 1 | The stem cell hierarchy. Stem cells are defined by their ‘potency’, or potential to give rise to specialized cell types. At the top of the stem cell hierarchy sits the ‘totipotent’ stem cell, which arises from the union of the sperm and egg. The resultant fertilized egg is capable of giving rise to all of the cell types in the body as well as the supportive extra-embryonic tissues, including the umbilical cord and placenta. Following a series of well-defined divisions, the totipotent stem cell gives rise to a blastocyst, consisting of a layer of outer cells called the trophoblast, which have committed to producing extra-embryonic tissues, as well as an ‘inner cell mass’, which specifies to give rise to either the epiblast or the primitive endoderm. The epiblast cells are ‘pluripotent’ stem cells and give rise to the fully formed fetus by the end of development. Through a series of differentiation decisions that follow as development progresses, the pluripotent stem cells undergo lineage commitment events and become limited in their potency, giving rise to ‘multipotent’ stem cells, which have a restricted ability to give rise to specialized cell types within a confined tissue system. For example, hematopoietic stem cells are restricted to giving rise to all of the cell types of the blood system. These multipotent stem cells then give rise to the specialized cell types of the body, which are considered to be ‘nullipotent’ as they do not have the ability to give rise to other cell types. ESCs, embryonic stem cells; iPSCs, induced pluripotent stem cells.

indirectly as a source from which tissue models can be derived for disease research and drug development. However, widespread use of stem cells in regenerative therapeutics is hampered by our incomplete knowledge of the rules that govern stem cell fate choices and a lack of technologies that enable translational impact of stem cell biology. Bioengineers fill a unique niche in stem cell research by viewing stem cell populations as dynamic systems that can be described with computational models and strategically manipulated using technologies to produce desirable behaviours. The ultimate goal of stem cell bioengineering is to devise strategies to manipulate stem cell populations (or stem cell ‘systems’) and enable predictable control over the regulation of stem cell fate, whereby a finite set of strategic manipulations to the stem cell population robustly results in desired outcomes. Bioengineering strategies can be applied to acquire a more complete systems-level understanding of the rules that operate within (stem) cells and how those rules guide intracellular and cell–environment interactions that lead to tissue and organ development and function.

Here, we review how bioengineering approaches can be used to unpeel the layers of complexity in stem cell biology to understand the rules that regulate stem cell fate choices as well as the tools that bioengineers employ

to manipulate and control these rules. We then review recent advances that have applied bioengineering strategies to tackle each of these layers of complexity.

The stem cell system

Accelerating the development of stem-cell-derived therapies requires the ability to enforce robust control of stem cell fate choices as well as the spatiotemporal organization of emergent complex tissues and organs that exhibit appropriate biological form and function, while being capable of robustly interfacing with the dynamic *in vivo* environment. In this section, we describe the layers of complexity present in stem-cell-derived systems and how information encoded within these layers flows between them.

Information flow in stem cell systems

Tissues and organs can be viewed as multicomponent biological systems derived from, and maintained by, stem cells. Stem cells receive cues from the microenvironment in the form of both biochemical and biophysical signals (FIG. 2a). Biochemical signals include autocrine signals that are secreted and subsequently received by the same cell as well as juxtacrine and paracrine signals that are received from adjacent or neighbouring cells. Biophysical signals are mediated by cell–cell contact and

Embryonic stem cell (ESC). A type of pluripotent stem cell, derived from the inner cell mass of the developing embryo, that is responsible for giving rise to all of the cells in the developing fetus but not the extra-embryonic tissues.

Organoid
A minimal and miniaturized organ that is developed from a suspension of stem cells in vitro. These stem cells undergo division and self-organization to give rise to a 3D structure that mimics the anatomy of organs in the body. Thus, organoids can serve as models for understanding organ development and for modelling disease states.

Cell fate

A cell's identity based on its expression of genetic, proteomic and epigenetic markers but also in terms of its functional abilities. Cell fate determines a cell's self-renewal ability, proliferative ability, differentiation potential, survival and motility.

Autocrine

A form of cellular signalling in which secreted chemicals bind to receptors on the same cell. By contrast, juxtacrine and paracrine signalling induce responses in neighbouring cells, either through direct contact (juxtacrine) or secreted chemicals (paracrine).

Extracellular matrix (ECM)

A collection of extracellular molecules, including proteins, proteoglycans and polysaccharides, that supports the growth of nearby cells by providing biomechanical and biochemical cues. It enables cell adhesion and cell–cell communication.

Gene regulatory networks (GRNs)

(GRNs). A set of genes and their direct and indirect regulatory interactions with one another. GRNs are akin to decision-making computational circuits that serve to process input signals and generate robust outputs in cell behaviour.

Network motifs

Interaction patterns that recur more frequently than in randomized networks — for example, negative autoregulation (or ‘autorepression’) and the feedforward loop.

Niches

The *in vivo* microenvironments in which stem cells reside that regulate their homeostasis and fate choices.

Morphogenesis

The process by which developing organisms acquire their structure and shape.

by interactions with the shape, topology, compliance and composition of extracellular matrix (ECM) proteins. Depending on the physiological state, stem cells sense specific cues either through receptors that bind ligands or through proteins (for example, integrins) that interact with the neighbouring ECM. These cues are then transmitted through the cells via a cascade of molecular signal transduction events that lead to the production of new signals or result in structural changes. These signals and cues then serve as inputs to the decision-making circuitry present within the stem cell to produce appropriate outputs on the basis of physiological needs. For example, upon injury of the intestinal epithelium, tissue-resident innate lymphoid cells secrete IL-22, a biochemical signal that directly targets intestinal stem cells (ISCs), increasing their proliferation and facilitating the regeneration of the epithelium^{13,14}. Another example is the maintenance of hair follicle homeostasis by type XVII collagen (COL17A1), an ECM molecule that provides crucial biophysical signals for hair follicle stem cell maintenance^{14–16}. Loss of COL17A1 results in ageing and commitment of stem cells towards more specified lineages, causing miniaturization and eventual loss of hair follicles. Age-induced miniaturization of hair follicles can be rescued by the forced expression of *Col17a1* (REF.¹⁵).

Achieving predictive control of stem cell fate requires not only a catalogue of the extensive components of the appropriate signals and modifying elements that regulate stem cell responses (as is currently heavily emphasized in stem cell biology research) but also a system-wide view of the manner in which these signals are presented to stem cells, the correct signalling pathways to be activated, the rules that stem cells follow in making downstream cell fate decisions and the dynamics of this process.

Layers of complexity in stem cell biology

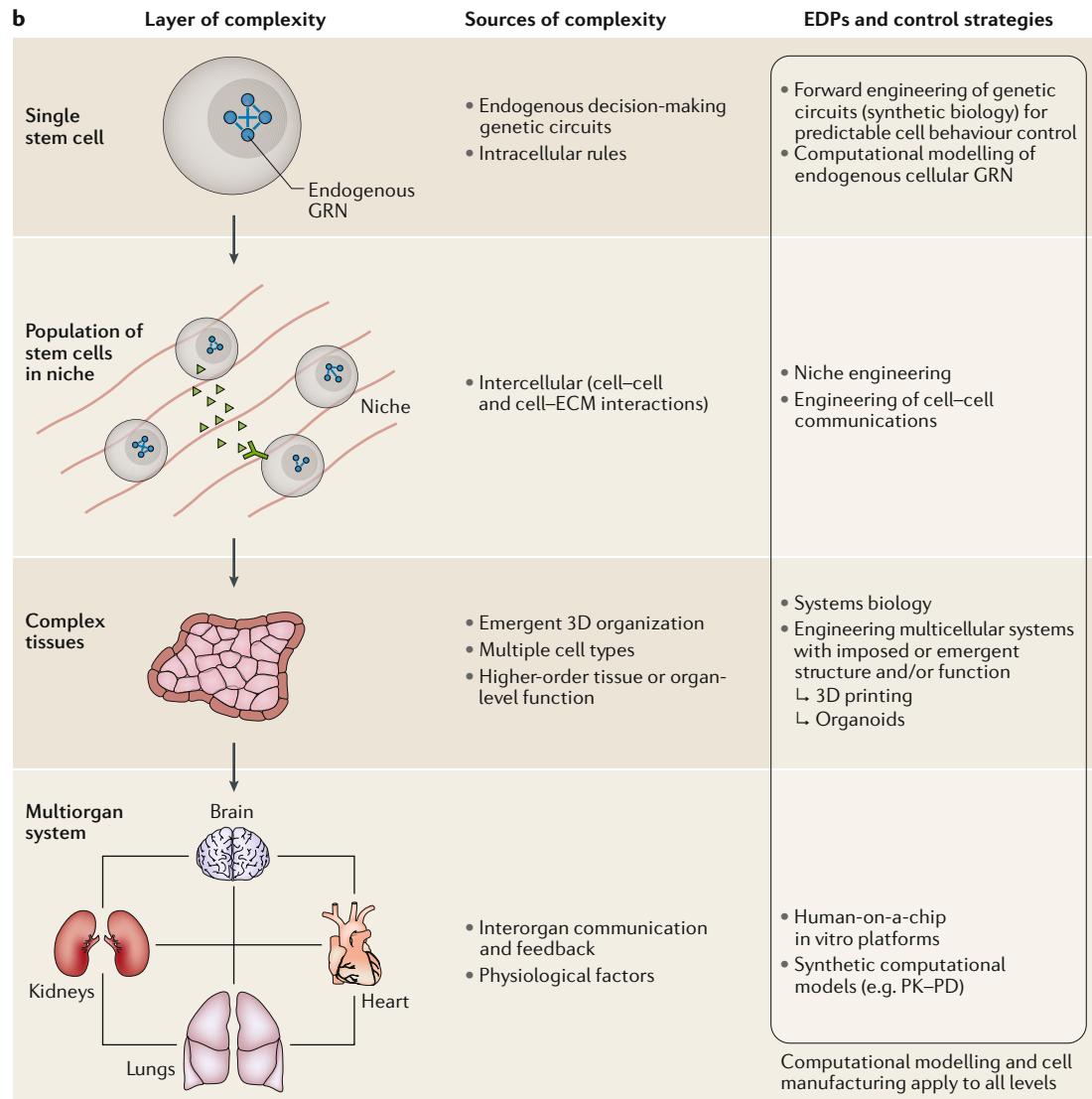
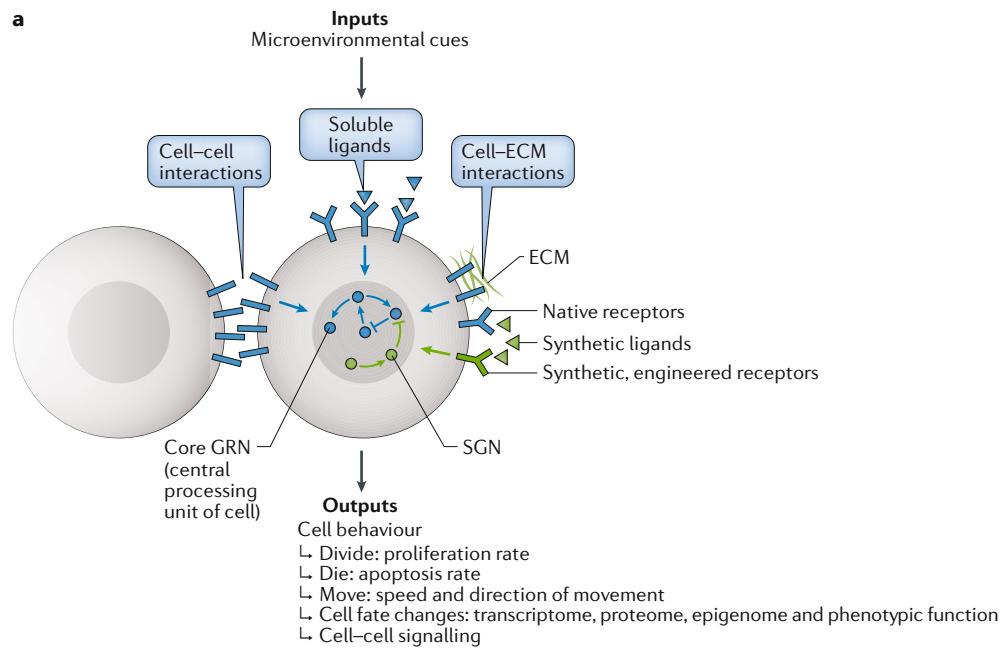
The stem cell and its inner circuitry. At the core of the stem cell system is the individual cell, the functional unit within the population (FIG. 2a). Cells are computational machines that interpret instructions, encoded in genes, which govern cell behaviour. Gene regulatory networks (GRNs) serve as the circuitry that enables cells to interpret and process instructions from the microenvironment (FIG. 2a). Thus, to understand the central rules that govern stem cell behaviour, it is necessary to first uncover the key genes involved in these decision-making circuits (the ‘parts’), how these regulatory players interact with one another and how they are connected to signals in their surroundings (the ‘rules’).

GRNs are a group of molecules that can be considered akin to nodes of a telecommunication network. These regulatory molecules interact with each other, other transcripts and proteins and the DNA to tightly regulate the transcriptional state (and development potential) of stem cells. The cellular GRN can be viewed as an internal mechanism that defines the set of potentially attainable states that a cell can traverse in response to a set of inputs. Additional layers of regulatory control overlay and converge on the cellular GRN to modify, restrict and

direct the subset of states that the cell actually achieves. These realized states dictate the cellular identity and inform the functional outcomes of the cell — such as the decision to maintain stem cell status or to differentiate — driven by the expression or repression of differentiation-associated genes and proteins. Incoming signals to the stem cells, either biochemical or biomechanical in nature, act as inputs to the cellular GRN and modulate the expression of target genes (FIG. 2a). Such inputs represent one form of perturbation that can change the GRN state. Furthermore, epigenetic modifications, such as DNA methylation and histone modifications, serve as a mechanism to both shape and stabilize the structure of the cellular GRN by altering the accessibility of gene promoters¹⁷. For example, the Polycomb group protein repressive system in mice and humans has been shown in PSCs to repress a set of genes associated with differentiation, including *HOX* and *PAX* genes¹⁸. In recent years, the regulatory control of stem cell GRNs and the network motifs present within them have been studied extensively¹⁹, yet a comprehensive understanding of how multiple, often dynamic GRN inputs are integrated to guide cellular decision-making processes remains incomplete.

The stem cell niche. Studies in *Drosophila melanogaster* have helped to consolidate a perspective that stem cells *in vivo* are ‘housed’ in microenvironments called niches²⁰. These niches are composed of a complex combination of factors that include supportive cells that provide appropriate signals to stem cells via cell–cell communication; the surrounding ECM, which can vary widely in terms of composition, geometry and compliance; other sources of mechanical stimuli; and physiological factors such as oxygen and pH²¹ (FIG. 2b). In the fairly accessible tissues of invertebrate model systems, scientists have been able to track individual stem cells and observe their ability to self-renew over the course of the lifetime of an animal^{20,22–24}. Although identifying stem cells and their niches in mammalian tissues has proved more difficult — owing to the larger cell numbers present, the infrequency of resident stem cells and the lack of functional assays in the field to identify them — there have been great strides recently due in large part to new imaging and live-cell tracking technologies^{20,25–31}. These studies have revealed that a remarkable degree of stem cell plasticity can occur *in vivo*, opening the door to efforts to manipulate *in vivo* niche parameters for regenerative purposes.

Stem-cell-derived tissues and organs. Higher-order stem-cell-derived cell populations such as organs and tissues are highly complex and consist of multiple different cell types ordered in a spatially defined manner. These spatially ordered populations emerge from^{32–34} and are maintained by^{29,35–37} intercellular communication between the cells that make up the multicellular system (FIG. 2b). Multicomponent biological systems, such as organs and tissues, form during embryogenesis from stem cells in a self-organized manner governed by underlying developmental rules that give rise to emergent complexity via a process called morphogenesis³⁸.



◀ Fig. 2 | **Layers of complexity in stem cell systems.** Stem cells can be viewed as computational machines, but several layers of complexity confound the ability to uncover the underlying computational rules. **a** | Stem cells receive cues from their microenvironment (cell–cell interactions, soluble ligands and cell–extracellular matrix (ECM) interactions) and generate cell signalling cascades that converge on the endogenous gene regulatory network (GRN; blue) of the cell. The GRN serves as the central processor, converting these input signals into outputs of the cell, which manifest as changes to the behaviour of the cell (decisions to divide, die or move; cell fate changes; and the release of signals to other cells). Synthetic biology approaches enable the addition of desired ligand and receptor interactions as well as downstream synthetic genetic networks (SGNs) within stem cells and their progeny. **b** | Several layers of complexity confound the ability of the stem cell field to uncover the underlying rules that are used by stem cells to convert inputs into outputs. The first layer of complexity is the single stem cell, which relies on its endogenous GRN to make decisions. The second is populations of stem cells and stem-cell-derived cells within their niche. The third layer consists of complex tissue structures that arise as emergent patterns and functions from the underlying rules that govern individual stem cells and their interactions with one another (layers 1 and 2). The fourth layer results from interorgan communication. The complexity that arises from the various layers of the stem cell system are a result of underlying rules, such as GRNs, cell–cell and cell–ECM communication, diverse cell types, emergent higher-order morphology and function and physiological communication and feedback. Examples of bioengineering approaches that have been used to unpack and better control these layers are listed. EDPs, engineering design principles; PK–PD, pharmacokinetic–pharmacodynamic.

The pursuit to fully understand the mechanism that dictates the complex, spatially ordered emergence of tissues from a seemingly homogenous population is an active area of investigation, further invigorated by the recent engineering of early developmental tissues *in vitro*^{25,39,40}. Developmental morphogenesis is an area of research that has always been enriched by theoretical and mathematical models^{41–44}, and new developmental engineering tools are enabling direct testing of the hypotheses generated by these models. Furthermore, intercellular communication not only gives rise to emergent morphogenesis but also enables the functional properties of multicellular adult tissues and homeostasis. For instance, we have used bioengineered cardiac microtissues to demonstrate that the contractile force response of cardiomyocytes is considerably bolstered in the presence of supportive non-myocyte fibroblasts⁴⁵. Taken together, deciphering the interactions between cells in multicomponent organs or organoids is of crucial importance to understanding how form and function are regulated.

Interactions between stem-cell-derived tissues and organs. *In vivo*, stem-cell-derived tissues and organs interface with a complex environment where communication between distal organs via cell-secreted factors (for example, proteins or hormones) has a crucial role in maintaining homeostasis⁴⁶ (FIG. 2b). A salient example includes the physiological effects of insulin, which is secreted by pancreatic β cells to regulate systemic glucose levels. Indeed, any successful regenerative medicine therapy would require the repaired tissue or organ to successfully communicate with the entire physiological system. A detailed understanding of these interactions will benefit not only stem-cell-based therapeutics but also drug development efforts by providing information on the potential systemic responses that can arise. Given the incredible level of complexity involved, this field remains an active area of research.

The engineering approaches

Engineers are skilled at analysing problems and devising solutions that fulfil a set of objectives while recognizing limitations inherent to a system. As such, engineering design follows an iterative series of steps towards the design and optimization of solutions. The engineering design process begins with the framing of the problem, which requires the careful mapping of the system and the identification of simplifying assumptions, as well as the core set of objectives, requirements and limitations that define the design space. Once the engineer has become familiar with the characteristics of the system they are seeking to control, they must predict the impact of imposed perturbations on the system before optimizing these changes to produce a controlled, desired response. In this way, engineers must move along an ‘understand–predict–control’ axis^{47,48} — a fundamental engineering design principle (EDP) (FIG. 3).

Stem cell bioengineers play a key role in overcoming the layers of complexity and uncovering the rules that govern the behaviour of stem cell systems. They do so by first exploring the existing knowledge of stem cell regulation either at the single-cell level (through the use of GRNs) or at the multicellular level, probing the system to uncover novel regulatory interactions and develop solutions that take advantage of these new insights. Next, stem cell bioengineers must develop a framework in which to quantify the impact of perturbations on the behaviour of the system by strategically identifying parameters that are experimentally observable or measurable. Finally, stem cell bioengineers develop designs and test the impact of these strategic perturbations on the stem cell system. The stem cell bioengineering paradigm facilitates the design and implementation of efficient stem cell expansion and differentiation protocols, as well as the development of complex multicellular systems such as tissues and organs. To this end, stem cell bioengineers apply core EDPs to accelerate discovery and translation (FIG. 3). Key components of the stem cell bioengineering toolkit include technologies that enable engineering abstractions of the cellular GRN or the niche and frequently employ computational models.

Modelling stem cell behaviour

In silico computational modelling has a key role in the understand–predict–control axis. Engineering assumptions and estimations facilitate the development of conceptualized and simplified models that distil complexity to a finite set of core principles to capture the behaviour of the stem cell system in an informative manner. These abstracted models, which can rely on empirical components, offer a useful perspective for framing and tackling multiscale and dynamic system complexity. Basic predictive models also enable exploration of the impact of perturbations on the system and optimization of the inputs required to drive the stem cell system to the desired state. Experimental prototyping, using key tools such as tissue engineering, niche engineering or synthetic biology, can then be used to test *de novo* predictions and designs generated by mathematical models. Simultaneously, the degree to which the system behaves in a desirable manner is quantified through key metrics that can be

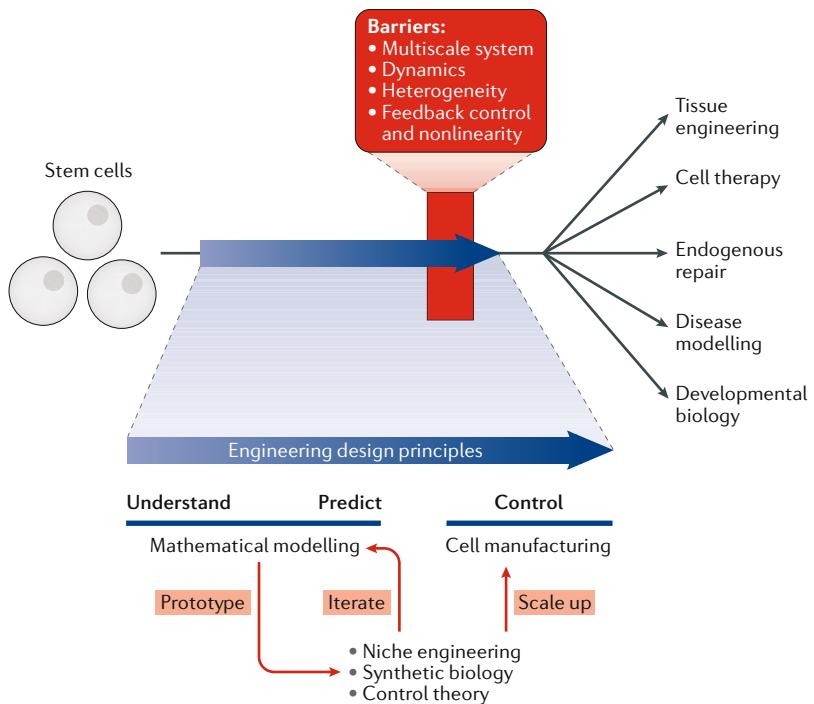


Fig. 3 | Stem cell bioengineers apply engineering design principles to overcome barriers in stem cell research. Owing to their highly dynamic (time-changing) and heterogeneous nature, stem cell systems are faced with barriers that serve to impede advancements in cell therapy, endogenous repair, disease modelling, developmental biology and tissue engineering approaches. Through the application of engineering design principles, stem cell bioengineers provide expertise in cell manufacturing, niche engineering, synthetic biology and mathematical and computational modelling to overcome these barriers. Such expertise applies key approaches that facilitate movement on the ‘understand–predict–control’ axis within the stem cell realm. Mathematical and computational modelling capture the underlying rules within stem cell systems and facilitate progress towards predicting the behaviour of the system in response to perturbations. The predictions of models in general can be prototyped computationally and experimentally via the key tools of niche engineering, synthetic biology and control theory. The process of moving from model to prototype is an iterative one, whereby the experimental behaviour of the prototype can better inform the model assumptions, which in turn affects the model predictions and so on. Ultimately, the key parameters that are observed to drive the behaviour of the stem cell system can be fine-tuned and closely controlled in large-scale manufacturing pipelines for the production and control of stem cells and their derivatives.

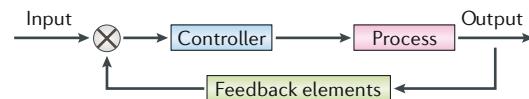
captured through measures of gene expression, population dynamics and functional outputs. The proposed design then undergoes evolution and testing in an iterative manner until an optimized solution is devised that meets the requirements of the system while respecting its limitations. Importantly, engineers can consolidate skill sets from various fields — including cell biology, systems biology, computational modelling and tissue and niche engineering — to uncover novel biological findings and design comprehensive solutions to control stem cell behaviours. For example, Antebi et al. used a mathematical modelling strategy to simulate combinatorial interactions between multiple bone morphogenetic protein (BMP) receptors and ligands to uncover a repertoire of computational functions that result from the promiscuous nature of the BMP signalling pathway⁴⁹. These modelling predictions led to the novel experimental finding that the outcome of BMP-mediated cell signalling was dependent on the

specific expression profile of the various BMP receptors, which enabled messages to be encoded uniquely for different cell types and in a spatially defined manner. Experimental perturbations to the system confirmed these findings, which may provide particularly relevant insights for developmental and differentiation processes governed by BMP signalling in stem cell systems both *in vivo* and *in vitro*. Indeed, computational modelling paradigms that integrate the multiple layers of complexity can enable holistic visualization of fate regulation and are therefore of high value to the field of stem cell bioengineering⁵⁰.

Mathematical models are also powerful tools for bridging our understanding of stem cell systems across different scales. For example, mathematical models can be harnessed to couple molecular cues with macro-scale biomechanical outcomes. Indeed, this approach has been used to uncover the role of BMP signalling in controlling the looping of the small intestine, which results from mechanical buckling following elongation of the intestine⁵¹. Such systems biology approaches, which explore the intersections between the macro-scale physical environment of tissues and the underlying cellular and molecular cues that drive these physical outcomes, provide interesting new insights and paradigms. These approaches may in fact provide opportunities to dissect the intimate connection between the evolution of the underlying molecular and cellular rules that shape developmental patterns and the requirements put forth at the tissue and organ level for organism survival.

Control systems theory

Stem cell fate, the emergence of tissues and their homeostasis are strictly regulated and highly reproducible events despite variations that might occur in the environment. Efforts to understand the regulation of stem cell fate and complex organ-level and physiological behaviour *in vitro* can greatly benefit from principles of control theory that view biological cues as inputs to the stem cell system and the responses as system outputs (FIG. 4). Fate ‘regulation’ in a biological context is in many ways synonymous with ‘feedback control’⁵², a fundamental concept in control systems theory. Systems that contain feedback signals are considered to be ‘closed-loop’ and are able to adjust and adapt in response to deviations from desired output values (FIG. 4a). By contrast, stand-alone ‘open-loop’ systems in which information flow is unidirectional are highly susceptible to environmental noise (FIG. 4b). For example, a simple genetic circuit consisting of an inducer that binds an activator and turns on a gene of interest constitutes an open-loop system in which the inducer is the input and the resultant protein of interest is the output (FIG. 4d). By contrast, if the protein output of the genetic circuit were to feed back and regulate its own expression, either positively or negatively, this would constitute a closed-loop system (FIG. 4c). In control engineering, the error (that is, the difference between the desired and actual system output) is used to identify an appropriate input for the system to regulate or control the system response and minimize the discrepancy between the system output and the desired output (FIG. 4a). Feedforward loops, which prominently occur in natural genetic networks, offer a means of reducing noise within open-loop genetic systems

a Closed-loop control system

Responses:

(i) Input



Output



(ii) Input + noise



Output + error



— Expected input
··· Actual input due to noise δ

— Expected output
··· Actual output due to error ϵ

b Open-loop control system

Responses:

(i) Input



Output



(ii) Input + noise



Output + error



— Expected input
··· Actual input due to noise δ

— Expected output
··· Actual output due to error ϵ

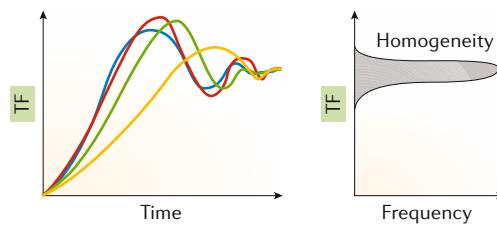
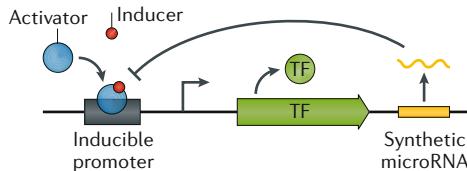
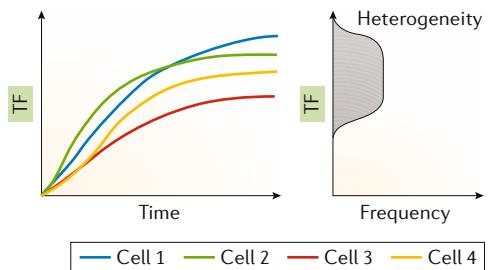
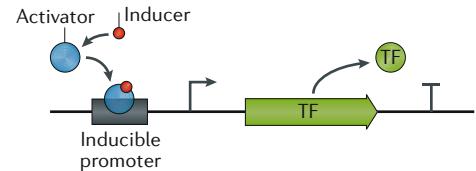
c Closed-loop gene expression control**d Open-loop gene expression control**

Fig. 4 | Feedback regulation in stem cell systems provides robust control of system dynamics. **a** In closed-loop systems, which incorporate feedback control, the output levels from the process can be maintained close to desired levels, making them preferable over their open-loop counterparts in instances when the output of a system needs to be strictly controlled. δ indicates input noise, and ϵ indicates output error. **b** In open-loop systems, the controller processes the input and sends a signal to the device to be controlled (the ‘process’), resulting in an output. This system is highly susceptible to noisy inputs and can result in dramatic shifts from the desired output levels. **c** An example of a closed-loop genetic circuit, whereby an inducible promoter is used to drive the expression of a gene of interest, ‘TF’, as well as a microRNA that negatively regulates the production of TF, is shown. This negative feedback loop reduces heterogeneity in the system. By using this negative feedback-mediated genetic circuit, the expression of the TF can be fine-tuned in the system. **d** An example of an open-loop gene circuit in which an inducible promoter is used to drive the expression of TF is shown. Owing to stochastic effects, random insertion sites of the genetic circuit into the cell’s genome and heterogeneity in endogenous levels of TF, this gene circuit design gives rise to heterogeneity in TF expression.

and are also of use within synthetic control systems⁵³. Analogous to the use of feedback to control the behaviour of machines and devices, feedback in biological systems mediated by biochemical and biophysical signals enables the regulation of biological responses and stabilizes cells in desired states^{19,54,55}. These feedback signals enable communication between all layers of complexity discussed

above, ranging from systemic signals to cues that initiate changes at the GRN level of individual stem cells.

The intersection of control systems theory and synthetic biology provides interesting opportunities to better control the cell state and is discussed in the section below. To this end, open-loop and closed-loop synthetic genetic circuits can be used to either exploit or control

gene expression heterogeneity for cell fate programming applications within stem cell systems.

Recent advances in handling complexity

Through the application of EDPs, stem cell bioengineers have made key contributions to dissecting stem cell regulatory complexity and have started to impose robust control over stem cell systems (TABLE 1). In this section, we will explore how stem cell bioengineering approaches are being used to tackle the different layers of complexity.

Engineering the stem cell GRN

Several key tools have been developed that enable stem cell researchers to probe cell fate with increasing resolution and depth.

Omics analyses, genetic perturbations and computational modelling

Omics technologies have played a particularly important part in this process, leading to the mapping of the cell epigenome, proteome and transcriptome⁵⁶. A number of technologies have also recently made single-cell omics analyses possible and enabled the exploration of biological heterogeneity in both *in vitro* and *in vivo* stem cell systems^{57–62}. The growth of these data sets has facilitated the identification of novel markers to demarcate divergent or rare cells within cell populations^{63–66}, which can be used for targeted characterization of cell types following enrichment steps or as part of a flow cytometry or mass cytometry analysis⁶⁷. These data sets are a rich resource for understanding how gene expression relates to cell fate and serve as the raw material from which to computationally construct GRNs using various approaches (Bayesian networks, Boolean networks, artificial neural networks and ordinary differential equations)^{68,69}. They also serve as a foundation for identifying key ligands and receptors involved in cell–cell communication networks that interface with the core cell GRN⁷⁰.

Gene regulation is a dynamic process involving feedback and multiple components. Attaining a predictive and quantitative understanding of such dynamic processes becomes exceedingly difficult with increasing complexity. Quantitative measurements of cell fate also provide a means of probing the dynamics of gene expression changes in response to extrinsic stimuli or genetic perturbations. Small-molecule, RNA interference or CRISPR-based gene knockout screens provide a means of exploring the impact of removing individual regulatory elements from cells in a high-throughput manner^{71–74}. Combined with chromatin immunoprecipitation (ChIP) data sets, these screens serve as a means of validating GRNs that are constructed from omics data sets by testing the degree of agreement between model predictions of loss-of-function (or gain-of-function) genetic perturbations and experimentally observed results. Computational strategies that provide insights into the logic of the GRN (for example, Boolean models) are invaluable to stem cell bioengineers. Through these approaches, GRNs that capture the decision-making genetic circuitry in cells have been uncovered^{75–77}. GRNs serve as an *in silico* minimal model

to predict the impact of unknown genetic perturbations on cell fate⁷⁸ and can serve as an important tool along the understand–predict–control axis (FIG. 5).

Cell tracking. Several tools have also made substantial contributions in tracking the stem cell lineage both *in vitro* and *in vivo*. Fluorescence reporter-based systems enable easy lineage tracing with spatial readout within cell populations but are limited by the number of available reporters⁷⁹. Molecular barcoding techniques that use variable DNA barcodes or viral integration sites offer high clonal resolution with the caveat that cells need to be sacrificed and removed from their spatial context for sequencing and analysis⁸⁰. More recent developments in lineage tracing technology have aimed to fill these gaps, offering high resolution and full lineage tree reconstruction *in vivo*^{81,82} or without loss of spatial information⁸³. Live imaging also enables lineage tree reconstruction within *in vitro* systems⁸⁴.

Synthetic engineering of biological circuits and functions

While omics analysis and computational modelling help to understand and predict the genetic circuits that encode the behaviour of single cells ('reverse engineering'), engineering approaches are needed to programme these circuits ('forward engineering'). In particular, synthetic biology is a rapidly growing field that employs the use of genetic engineering and molecular biology to design and construct the genetic networks present within cells. The central goal of synthetic biology is to build the genetic programme of a cell in a bottom-up manner by combining genetic parts into modular genetic circuits that have well-characterized behaviours (on the basis of iterative rounds of predictive modelling and experimental prototyping)⁸⁵. These circuits can then be utilized to construct higher-order functions and sophisticated cell behaviours. The synthetic biology approach includes both editing of native or endogenous GRNs within stem cells and the introduction of new synthetic genetic networks (SGNs) that can interact with the native network to drive desired functionality. SGNs process input signals to generate a desired output, and may either function independently from or integrate with the core GRN of a cell (FIG. 2a). Several excellent reviews have explored the history and progress of the field of synthetic biology to date^{85–91}.

Synthetic biology has already made key advances in mammalian cell engineering through the development of circuitry with multiple inputs and outputs and increasingly complex architecture and functionality^{92–95}. This circuitry can integrate with novel synthetic ligand and receptor strategies^{96,97}, enabling engineers to design mammalian cells that sense desired signals from their microenvironment and respond with customizable downstream functions⁹⁷ (FIG. 5). The ability to design SGNs with controllable inputs, processes and outputs makes it possible to drive cell fate changes in PSCs to improve differentiation pipelines^{86,98}. In fact, by using control systems theory, it may become possible to programme SGNs with automated and robust control over cell fate changes for cell programming applications^{99,100}. Naturally, synthetic biology also lends itself to engineering genetic circuits that improve the observability of the

Bayesian networks

Probabilistic models that relate the dependencies of the expression of a set of genes on one another through a directed graph.

Boolean networks

Models of gene regulatory networks that can predict gene expression outcomes given the initial state of genes in the network as well as the derivation of steady-state gene expression status.

Artificial neural networks

Networks composed of nodes, which can be genes, that process and transmit information. The output of each node is a nonlinear function of a sum of its regulatory inputs.

Ordinary differential equations

A mathematical framework capturing gene expression dynamics as a function of the presence of regulators and the rate of change of mRNA and/or protein concentration due to production and degradation.

Reverse engineering

The process of analysing a system to uncover underlying design rules to create representations of the system at higher levels of abstraction (inverse of forward engineering).

Forward engineering

The iterative process by which a system is designed, prototyped, tested and further optimized from a model (the classical engineering design process).

Table 1 | Stem cell bioengineering approaches applied to overcome the layers of complexity within the stem cell system

Approach	Application	Example refs	Example of insight gained
The stem cell GRN			
Omics technologies	Querying intracellular 'parts' that constitute the GRN	57–62	Guo et al. ⁵⁷ developed a single-cell multi-omics technology to analyse chromatin state, DNA methylation, copy number variation and ploidy simultaneously from individual mammalian cells. Such techniques can provide highly valuable information to inform approaches that infer GRNs that regulate stem cell responses and behaviour
Omics technologies	Demarcation of rare and divergent cell types	63–66	Shakiba et al. ⁶³ identified CD24 as a surface marker that allows divergent reprogramming and pluripotent stem cell states to be demarcated. Specifically, CD24 expression distinguished transgene-dependent and transgene-independent reprogramming mouse states, as well as naïve versus primed human pluripotent stem cell states
System perturbation	Knockdown and/or knockout of GRN components	71–74	Shalem et al. ⁷² demonstrated the value of genome-wide CRISPR–Cas9 knockout screens for both negative and positive selection screening in human cells
Computational modelling	GRN construction	19,68,69,75–78	Using a data-constrained computational approach, Dunn et al. ⁷⁵ developed a simplified GRN capable of capturing the rules employed by mouse embryonic stem cells in making early fate decisions
Cell tracking	Fluorescent tracking	31,79	Pilz et al. ³¹ employed <i>in vivo</i> chronic imaging of the mouse hippocampus to provide a comprehensive lineage relationship description of neural stem and progenitor cells giving rise to newborn neurons
Cell tracking	Clone and lineage tracing	80–84	Two recent studies developed synthetic engineered systems that employ CRISPR–Cas9 technology to progressively introduce and accumulate mutations in a predefined barcode region, enabling reconstruction of lineage relationships of cells <i>in vitro</i> ⁸³ and <i>in vivo</i> ⁸¹
Synthetic biology	Synthetic biological circuits in mammalian cells	92–95	Prochazka et al. ⁹⁴ constructed a synthetic circuit with a 'bow-tie' architecture in human cells that enabled detection and integration of multiple cell-type-specific microRNAs to programme multiple proteins in different dynamic modes
Synthetic biology	<ul style="list-style-type: none"> • Synthetic biological functions • Novel ligands and receptors • Cell fate programming 	86,96–98,100	Morsut et al. ⁹⁶ engineered diverse forms of chimeric Notch receptors in which both the intracellular and extracellular modules of the receptor were replaced with heterologous protein domains and demonstrated various applications ranging from driving user-defined biological responses to self-organized patterning in cell populations
The stem cell microenvironment			
Niche engineering	Cell size	122–124	Dupont et al. ¹²⁴ demonstrated that YAP and TAZ can function as the effectors of biomechanical signals from the extracellular environment
Niche engineering	Juxtacrine signalling	125	Chen et al. ¹²⁵ developed a platform that enables controlled cell–cell interactions to study juxtacrine-signalling-mediated cellular responses
Niche engineering	Autocrine and paracrine signalling	126–133,142	Csaszar et al. ¹⁴² demonstrated that a deeper understanding of how paracrine signalling from specialized cells affects stem cell fate choices can benefit clinical translation. The authors engineered a platform to counteract the inhibitory effect of specialized blood cells on self-renewal of HSCs to achieve a rapid increase of output HSC numbers during <i>in vitro</i> culture
Materials engineering	Substrate compliance	136,137	Gilbert et al. ¹³⁶ employed hydrogels with tunable mechanical stiffness properties to identify the appropriate stiffness range that enabled <i>in vitro</i> culture and self-renewal of muscle stem cells
Materials engineering	Dynamic ECM response (degradation)	138,139	Madl et al. ¹³⁹ demonstrated that remodelling of the ECM can be used as an approach to maintain stemness of neural progenitor cells in 3D culture systems
Computational modelling	Predicting autocrine and paracrine signalling	70,126,133,140–142,144	Peerani et al. ¹²⁹ employed computational modelling and micropatterning to identify predictable colony size effects in activation of JAK–STAT signalling in mouse embryonic stem cell colonies
Stem-cell-derived structures			
Tissue engineering	Bottom-up approaches	146–148,152	Kang et al. ¹⁴⁸ demonstrated the ability to 3D print vascularized tissues of various architectures with a variety of different cell types. Such techniques can be of high value to the field of regenerative medicine as they may be able to generate clinically relevant tissues and organs. Rodenizer et al. ¹⁵⁰ reported an <i>in vitro</i> platform called TRACER that recapitulates aspects of complex metabolic and oxygen gradients of 3D tumours
Tissue engineering	Harnessing self-organization	10–12,21,30,39,151–158,162–164	Hughes et al. ¹⁵⁸ demonstrated that mechanical compaction of the ECM due to mesenchymal condensation enables the generation of complex 3D topography at tissue interfaces. The authors develop a framework to engineer complex tissue curvature by spatially patterning mesenchymal condensates
Computational modelling	Predicting self-organization	32–34,39,157,158,167,168–170	Tewary et al. ³⁹ reported an <i>in vitro</i> experimental platform that demonstrated that reaction diffusion and positional information can work in concert to give rise to self-organized, emergent fate patterning of stem cell populations

Table 1 (cont.) | Stem cell bioengineering approaches applied to overcome the layers of complexity within the stem cell system

Approach	Application	Example refs	Example of insight gained
Stem-cell-derived structures (cont.)			
Materials engineering	Synthetic environments to facilitate self-organization	¹⁵⁹	Gjorevski et al. ¹⁵⁹ engineered a designer hydrogel that mimics the dynamic characteristics of animal-derived matrices that are conventionally used to generate organoids. The authors demonstrated the capacity of this synthetic hydrogel to enable organoid formation, overcoming multiple limitations associated with clinical applications of organoids
Genetic engineering	Gene circuits to facilitate self-organization	¹²¹	Guye et al. ¹²¹ employed a genetically engineered heterogenous pulse of GATA6 expression in human induced pluripotent stem cell cultures to rapidly induce the three germ layer fates in a spatially patterned manner. Within 2 weeks, these cultures developed into complex, spatially ordered tissues that recapitulate early developmental stages resembling a liver bud-like phenotype with haematopoietic and stromal cells
Automated and/or high-throughput platforms	Enabling large-scale studies with stem-cell-derived structures	^{153,161}	Czerniecki et al. ¹⁶¹ developed a platform that permits complete automation of high-throughput generation of kidney organoids for genetic and drug screening. They demonstrated proof of concept of high-content screening with a model of PKD and identified a previously unknown role of myosin in PKD
Interorgan and systemic interactions			
Parabiosis	Querying systemic interactions	¹⁷²	In their study, Conboy et al. ¹⁷² established an experimental platform that surgically enforced a shared circulatory system between young and old mice and demonstrated that age-related reduction in regenerative potential of stem and progenitor cells of old mice could be rescued by systemic factors present in the blood of young mice. Platforms such as these can provide valuable solutions to understand how stem cells maintain tissue or organ homeostasis during the lifetime of the organism
Niche and tissue engineering	Human on a chip	^{150,176,178–180,182–184}	Huh et al. ¹⁸⁰ report a method to generate an <i>in vitro</i> model of lung-like behaviour and how it may be adapted to develop other organ-on-a-chip models. Models such as these represent low-cost alternatives to conventionally employed animal models for pharmaceutical, chemical and environmental applications
Computational modelling	Predictive pharmacokinetics and pharmacodynamics	^{181,182,184}	Tsamandouras et al. ¹⁸⁴ developed an interconnected liver and gut organ-on-a-chip model that enabled high-content quantitative data collection and demonstrated that pharmacokinetics for multiple drugs could be tested in a manner that is robust to experimental perturbations. Furthermore, mechanistic analysis of the acquired data from this platform also enabled interrogation of intrinsic parameters associated with the pharmacokinetics processes occurring in the organ-on-a-chip platform

ECM, extracellular matrix; GRN, gene regulatory network; HSCs, haematopoietic stem cells; JAK–STAT, Janus kinase–signal transducer and activator of transcription; PKD, polycystic kidney disease; TAZ, transcriptional co-activator with PDZ-binding motif; TRACER, tissue roll for the analysis of cellular environment and response; YAP, yes-associated protein.

endogenous cell GRN, enabling us to better probe the biology of stem cells¹⁰¹. These insights result in improved models of cell GRNs, in turn better informing the design of SGNs, and this positive feedback loop continues. Furthermore, synthetic biology enables stem cell bioengineers to programme stem cell and stem-cell-derived cells for specific clinical functions, opening up new possibilities in the realm of cell therapy and regenerative medicine¹⁰². The design of T cells with engineered CARs is a prime example of this approach and serves as a case study for future cell engineering technologies that will bring synthetic functionality to stem cell systems for clinical applications¹⁰³.

Bottom-up approaches such as synthetic biology are primed to utilize control theory to inform their design. For example, genetic circuits providing inducible expression of a gene of interest have utility in both open-loop and closed-loop topologies. Closed-loop and feedforward genetic circuits enable the fine-tuning of gene expression while eliminating noise and variability in the system^{53,99}. Of note, endogenous GRNs, such as Hedgehog¹⁰⁴ and tyrosine kinase¹⁰⁵ signalling pathways, incorporate negative feedback. Mathematical and experimental studies have confirmed a role for negative

feedback in attenuating and manipulating the frequency range of noise^{106,107} while increasing gene expression stability¹⁰⁸. Indeed, genetic circuits with integral feedback have been shown to reliably track target gene expression levels while also remaining robust to disturbances^{109,110}. It has been suggested that feedback control can be implemented in genetic engineering approaches that aim to provide controlled overexpression of transcription factors (TFs) for cell fate reprogramming¹⁰⁰. By including both a controllable activator and a negative regulating microRNA, the genetic circuit can provide tunable and homogenous levels of exogenous factor overexpression, thus eliminating sources of variability in cell fate outcomes (FIG. 4c). Positive feedback also has a key part to play in both endogenous GRNs and SGNs. In general, positive feedback can enable a switch-like response, in which small amounts of gene expression are amplified until a threshold level is reached and the cell state toggles¹¹¹. Indeed, positive feedback has been shown to have a role in the endogenous control of bistable cell state changes^{112–114}, which can be further stabilized with the addition of negative feedback¹¹⁵. Furthermore, positive feedback in synthetic genetic circuit design has been shown to lead to bistability¹¹⁶.

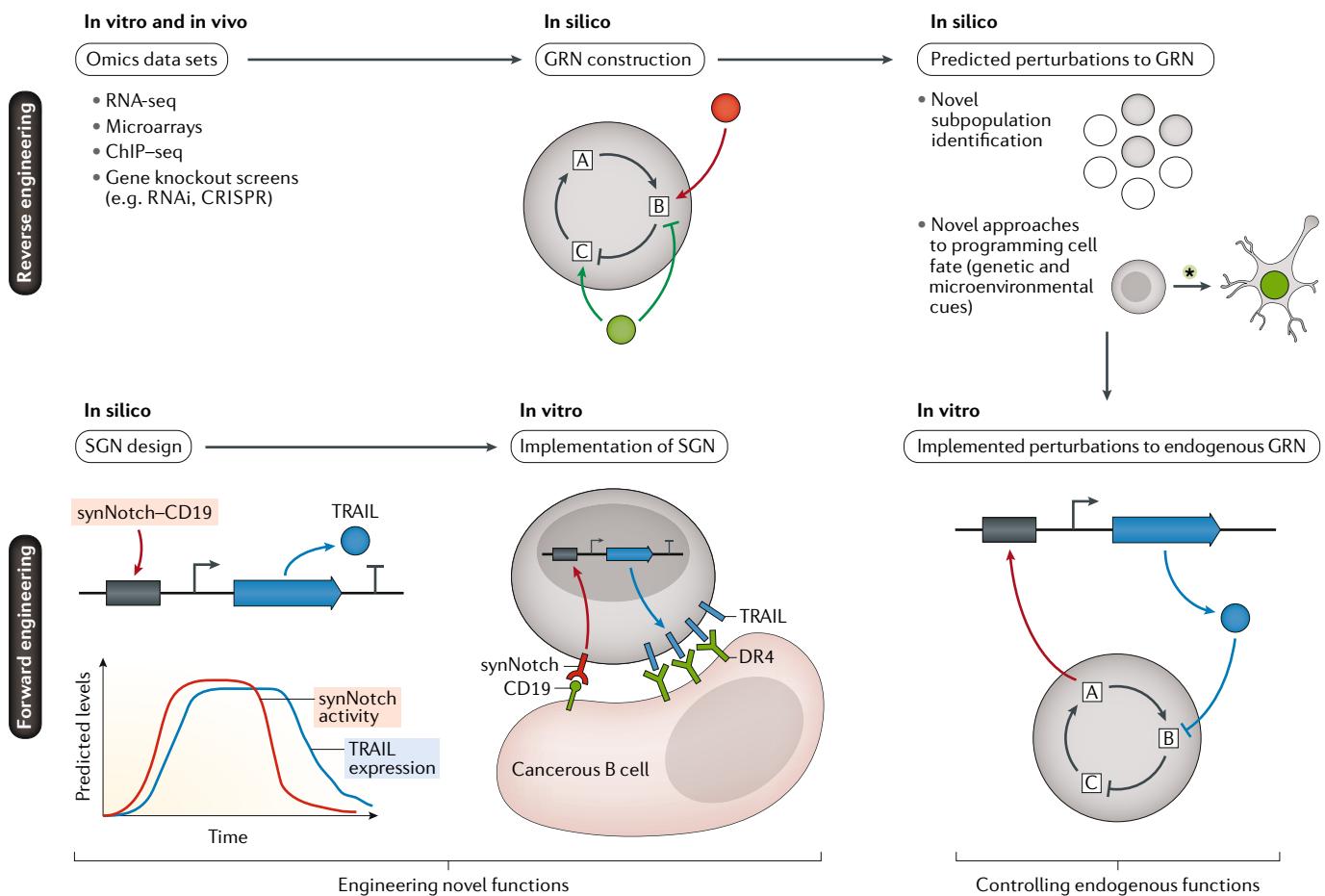


Fig. 5 | Reverse and forward engineering strategies are used to map and manipulate the stem cell gene regulatory network. Using large omics data sets that capture the transcriptional state and protein interactions in stem cell populations, computational techniques can be used to construct and simulate cellular gene regulatory networks (GRNs; represented by A, B and C in the figure) in a reverse engineering approach. These modelling efforts lead to in silico predictions that include the identification of novel subpopulations as well as the anticipated impact of perturbations to key nodes of the GRN, which can be powerful for cell programming applications. These predictions are then experimentally validated by experimentally manipulating the cellular GRN. In parallel, forward engineering approaches seek to design and build synthetic genetic circuits (SGNs) that bestow novel functionality in cells. For example, the synthetic Notch (synNotch) system enables cells to sense desired inputs and respond by triggering a downstream circuit of choice, whether endogenous or synthetic. Synthetic biology approaches also allow for perturbations to the endogenous GRN of the cell, enabling model validation. The green asterisk represents genetic and microenvironmental cues. ChIP-seq, chromatin immunoprecipitation followed by sequencing; DR4, death receptor 4 (also known as TNFRSF10A); RNAi, RNA interference; RNA-seq, RNA sequencing; TRAIL, TNF-related apoptosis-inducing ligand.

By contrast, open-loop genetic circuits generally cannot attenuate stochastic changes in gene expression levels and therefore can be expected to give rise to heterogeneity between cells, although feedforward loops are a notable exception⁵³ (FIG. 4d). Indeed, the role of gene expression variability in cell fate decision-making has become increasingly recognized, providing natural examples for the application of the open-loop circuit topology for stem cell engineering^{111,117}. One such example is photoreceptor expression in *D. melanogaster* eyes, where the stochastic expression of the TF Spineless directs a subset of cells to adopt a yellow fate¹¹⁸. Another example involves the emergence of neuroblasts in *D. melanogaster*, which acquire their fate following a stochastic increase in Delta expression. The Delta-elevated cell subsequently stimulates the Notch pathway in neighbouring

cells and a reinforcing feedback loop ensues, causing the Delta-presenting cell to downregulate Notch signalling and acquire a neuroblast fate while neighbouring Notch-high cells acquire an epidermal fate¹¹⁹. Yet another example involves the key pluripotency TF Nanog. Stochastic fluctuations in Nanog expression can have implications for the state of PSCs, where cells with low Nanog expression are more susceptible to differentiation cues¹²⁰. Clearly, variability resulting from stochastic differences in gene expression has a role in native cell fate commitment processes and may also show utility in systems using synthetically designed genetic circuits. For example, in the context of cell fate programming, open-loop systems are well suited for applications in which stochastic differences in gene expression are desired and can drive cell-to-cell variability¹²¹.

Engineering the stem cell microenvironment

Given the complex nature of cell–microenvironment interactions, bioengineers have taken the approach of studying the individual components of the microenvironment independently. Niche engineering comprises a suite of bioengineering tools and technologies ranging from microfabrication techniques such as micropatterning, tissue engineering and biomaterials to polymer chemistry. These tools enable querying the design principles that regulate stem cell fate by perturbing specific biological parameters. Examples of these modifiable parameters include the shape and size of a single stem cell, autocrine, paracrine and juxtacrine signalling and composition and stiffness of the surrounding ECM.

Niche engineering by micropatterning. Approaches to deconvolve the various potential interactions that stem cells can experience strive to separate individual stem cells from the rest of the population and environment such that the responses to the experimental conditions tested are devoid of any confounding factors. Micropatterning represents one technology that readily enables control of stem cell interactions by defining colony geometry and environmental factors and has therefore been used by stem cell bioengineers for many years. One of the earliest studies that used micropatterning to probe stem cell fate choices investigated the effect of cell size on the differentiation of epidermal stem cells¹²². The authors found that single epidermal keratinocytes maintained stemness when patterned in large islands where the cells were able to spread, whereas cells confined to smaller micropatterned areas increased the expression of differentiation-associated proteins¹²². A similar approach was subsequently taken to investigate the effect of cell size in differentiating human mesenchymal stromal cell (hMSC) populations¹²³. Differentiating hMSCs that were allowed to spread in large micropatterned ‘islands’ acquired an osteoblast fate whereas those that were restricted to smaller islands acquired an adipocyte cell fate, and RhoA activity was attributed to orchestrating these responses¹²³. Later studies have demonstrated the involvement of Yes-associated protein (YAP) and the transcriptional co-activator with PDZ-binding motif (TAZ) as the nuclear effectors of mechanotransduction-associated cell fate decisions¹²⁴. Taken together, these studies reveal the crucial importance of biomechanical signalling in maintaining and regulating cell fate outcomes in populations of progenitor cells.

Micropatterning

Technology that enables transfer of miniature ‘islands’ of extracellular matrix proteins to enforce control of the shape and size of adherent cells either as single cells or cell colonies.

Stemness

The characteristic of a cell that makes it a stem cell. That is, the ability to self-renew and differentiate to specify to different cell types.

enabled the predictive control of endogenous JAK–STAT signalling activation¹²⁴, providing an approach to investigate the dynamics of autocrine and paracrine signalling. Micropatterning has also been demonstrated to increase the efficiency of reversion of mouse epiblast stem cells (mEpiSCs) to mESC fates, an observation that occurs owing to an enhanced responsiveness of the JAK–STAT pathway¹²⁵. It has also been shown that increased local BMP activity, and the crosstalk between the BMP signalling pathway and the JAK–STAT pathway, underlie the increased responsiveness to the effector of JAK–STAT signalling STAT3 (REF¹²⁶). This approach of investigating the niche parameters of mEpiSCs has also been employed for human PSCs (hPSCs), enabling identification of the effects of colony size on maintaining pluripotency or controlling differentiation trajectory^{127–129}. Small colony sizes of hPSCs, which enable rapid fate switching in response to differentiation cues, can be employed to identify lineage decisions made by hPSCs in high-throughput platforms to provide deep insight into the early cell fate decisions made by hPSCs^{130,131}.

Niche design using extracellular matrix proteins and materials engineering. Given that ECM signalling in niches can have a crucial role in influencing stem cell behaviour, identifying the ECM composition of specific niches can rapidly advance our understanding of the appropriate signals needed to control stem cell responses. One study employed an ECM microarray platform that enabled combinatorial screening of a variety of ECM molecules to identify optimal ECM composition for *in vitro* maintenance of primary rat hepatocytes as well as directing mouse ESCs toward an early hepatic fate¹³⁴. Similarly, a second study reported that exploiting the synergistic interaction of NOTCH ligands and cues from an ECM protein called vascular cell adhesion protein 1 (VCAM1) increased the ability to generate both mouse and human T cells during *in vitro* culture¹³⁵. A parallel question to the biomechanical regulation of stem cells is how do stem cells respond to the compliance of the surrounding ECM? Identification of culture conditions to maintain muscle stem cells (MuSCs; also known as satellite cells) *in vitro*, which had been challenging for the field for a long time, represents a prominent example of how the compliance of the surrounding ECM can regulate stem cell fate. A recent study used polyethylene glycol (PEG) hydrogels with tunable stiffness to investigate the effect of substrate compliance in MuSC maintenance¹³⁶. The authors found that the stiffness of the substrate during *in vitro* culture directed the MuSCs to either self-renew or differentiate. Furthermore, they identified an intermediate level of stiffness that paralleled the stiffness of muscle tissues *in vivo* and was able to optimally promote self-renewal of the MuSCs. Notably, although substrate stiffness-induced effects have been clearly demonstrated in MuSCs using PEG hydrogels¹³⁶, a study that employed human keratinocytes as a model system demonstrated that observed stiffness-induced changes in stem cell fate may be affected by the choice of hydrogel¹³⁷. In their study, Trappmann et al. demonstrated that keratinocyte stemness could be regulated on polyacrylamide (PAAm) hydrogels but did not have an

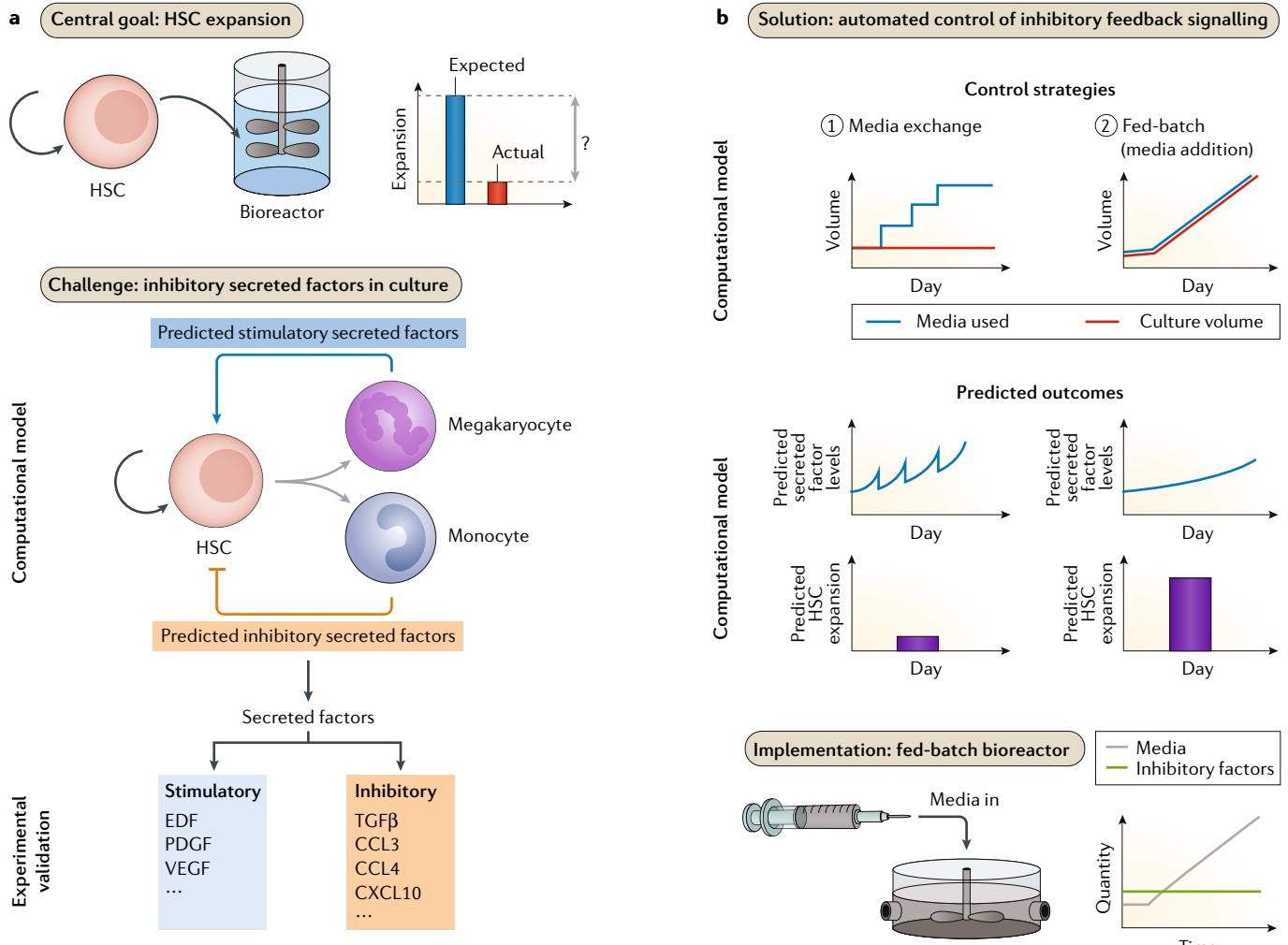
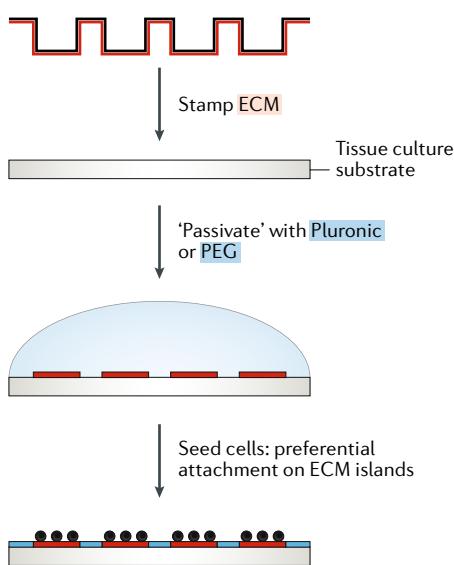
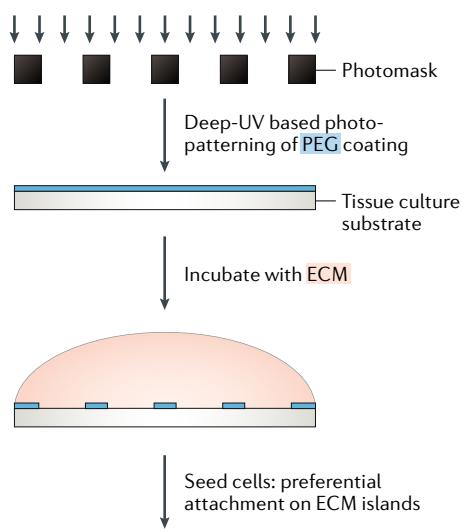
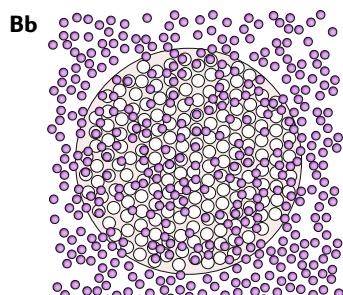
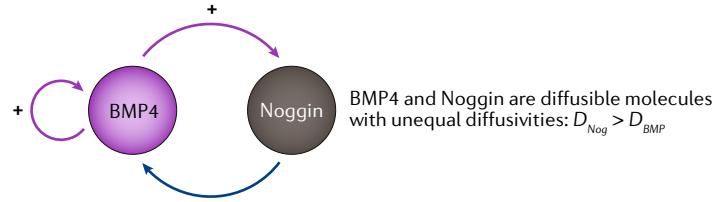
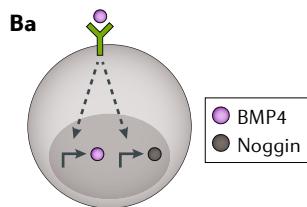


Fig. 6 | A combined computational modelling and bioreactor design strategy enables enhanced expansion of haematopoietic stem cells. **a** Using a compartmental model of haematopoietic stem cell (HSC) differentiation, Kirouac et al. predicted the existence of a negative feedback loop in which mature blood cells inhibit the expansion of HSCs and haematopoietic progenitors through the secretion of inhibitory secreted factors^{140,141}. This model prediction was then validated experimentally and led to the observation that monocytes are the source of these inhibitory secreted factors. **b** Using mathematical modelling, Csaszar et al. simulated the ability of different bioreactor media addition and exchange strategies to successfully dilute the inhibitory secreted factors from HSC expansion culture, leading to the identification of the fed-batch strategy, which was experimentally validated and shown to lead to optimized expansion outcomes¹⁴². CCL, CC-chemokine ligand; CXCL10, CXC-chemokine ligand 10; EDF, endothelial differentiation-related factor; PDGF, platelet-derived growth factor; TGF β , transforming growth factor- β ; VEGF, vascular endothelial growth factor. Part **b** adapted with permission from REF.¹⁴², Elsevier.

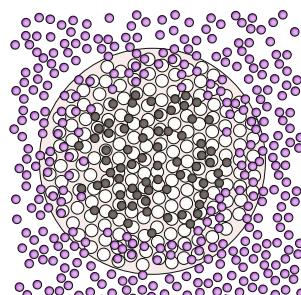
effect on polydimethylsiloxane (PDMS) hydrogels. They further demonstrated that reducing PAAm stiffness resulted in a reduction of anchor points available for cell attachment, which they did not observe on PDMS substrates of varying stiffness. This study suggests that the stiffness-dependent response of keratinocyte stemness on PAAm occurs owing to the inability of keratinocytes to robustly adhere to the substrate, rather than on substrate stiffness¹³⁷. Taken together, these findings suggest that biomechanical regulation mediated by substrate stiffness is specific to individual stem cell lineages and that the choice of hydrogels for studies investigating substrate stiffness-mediated stem cell responses should be carefully considered. With advances being made in the field of bioengineered hydrogels, recent studies have also

started to investigate the effects of matrix degradation and relaxation on stem cell fate^{138,139}, providing deeper insight into how dynamic biomechanical cues regulate stem cell behaviour.

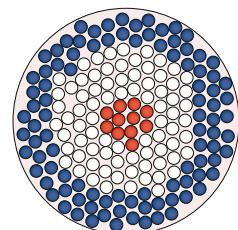
Computational models and bioreactor systems. Integrated approaches using mathematical modelling and niche engineering have proved to be powerful tools for deciphering the dynamics of stem cell systems. For example, this approach has been employed to identify the intercellular regulatory logic underlying maintenance and proliferation of HSCs^{140,141}. These studies have used mathematical models to predict the existence of coupled negative feedback loops that inhibit the proliferation of the HSC and haematopoietic progenitor

A In vitro techniques for the geometric confinement of cells**Aa Microcontact printing****Ab UV-lithography-based patterning****B Example of application: pattern formation in geometrically confined hPSC colonies**

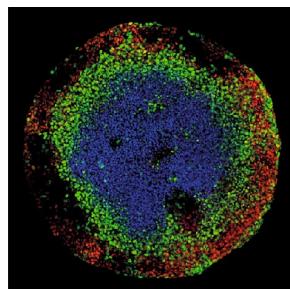
Geometrically confined hPSC colony subjected to a random morphogen distribution



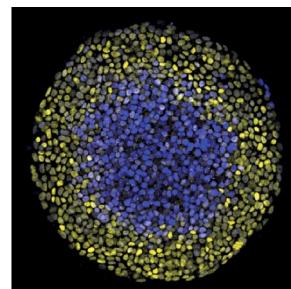
Reaction-diffusion-mediated self-organization of morphogen distribution



Morphogen concentration-dependent fate patterning

Bc**Perigastrulation-like**

■ SOX2
■ BRA
■ CDX2

Preneurulation-like

■ SOX2
■ GATA3

◀ Fig. 7 | **Niche engineering by micropatterning of cells to control colony shape and size.** Geometric confinement of cell colonies enables intercellular-communication-mediated emergence of collective behaviour. **A** | Different techniques can be employed to achieve geometric confinement of cells for in vitro studies¹⁶⁵. **Aa** | Microcontact printing is a technique in which extracellular matrix (ECM) islands are stamped on tissue culture substrates. To ensure that the cell types of interest do not adhere to nonspecific areas of the substrate, bioinert polymers such as Pluronic or polyethylene glycol (PEG) can be employed to backfill these regions, rendering them non-adhesive. **Ab** | An alternative technique of achieving geometric confinement of cells for in vitro studies uses deep-UV-mediated photo-patterning of tissue culture substrates coated with bioinert polymers such as PEG. This method results in patterned regions permitting ECM adsorption and subsequent cell attachment, whereas the rest of the substrate remains bioinert. **B** | Micropatterning has been applied to study cellular interactions based on collective behaviour such as fate patterning. **Ba** | Molecules such as morphogens, as shown for bone morphogenetic protein 4 (BMP4), can induce the expression of themselves and their inhibitors. In the case of BMP4, Noggin represents a key inhibitor. Both BMP4 and Noggin are diffusible molecules and interact in the extracellular environment. **Bb** | Micropatterning cells and presenting a random distribution of BMP4 results in self-organization of the distribution of the morphogen and subsequent spatially segregated fate patterning of the micropatterned cells. **Bc** | Representative images of human pluripotent stem cell (hPSC) colonies induced to differentiate in conditions that pattern gastrulation-associated fates (CDX2, Brachyury (BRA), SOX2) and pre-neurulation-associated fates (GATA3, SOX2). Part A adapted from REF.¹⁶¹, Company of Biologists.

compartments. Importantly, the model predictions led to the discovery of secreted factors released by mature blood cells that suppress the proliferation of HSCs^{140,141} (FIG. 6a). More recent work has started to provide deeper insight into the structure and network motifs present in the HSC regulatory network⁷⁰. With this knowledge in hand, culture technologies based on bioreactors have also been developed to considerably increase the yield of HSCs in in vitro culture¹⁴², which can provide a valuable boost to the development of cell-based therapies (FIG. 6b). Indeed, advances in bioreactor platforms have made key contributions to the development of quality stem-cell-derived cell therapy manufacturing pipelines¹⁴³. Computational modelling of HSC differentiation has also provided key insights into the clonal dynamics of long-term HSC expansion and differentiation in the in vivo environment, capturing the variability in the times to HSC differentiation after transplantation¹⁴⁴. Furthermore, the intersection of synthetic biology and cell manufacturing promises key advancement in quality assurance, potency and manufacturability of cell therapies¹⁴⁵. The advances made through these studies and avenues provide an example of how the understand–predict–control axis of EDPs can ensure rapid progress in stem-cell-based regenerative medicine strategies.

Engineering stem-cell-derived structures

Bioengineers have developed a variety of approaches, using many niche engineering technologies, such as micropatterning (FIG. 7), to generate complex spatially ordered tissues that can reveal the rules that regulate their form and function.

Tissue engineering using modular assembly approaches. One approach to engineer tissues is a bottom-up method whereby modular assembly of simpler parts gives rise to more complex structures. For example, single-cell chemical functionalization can be used to

pattern multiple layers of defined cell types into the third dimension perpendicular to the substrate, and the assembly can be released using DNase treatment to generate organoid-like microtissues¹⁴⁶. Cell-laden hydrogels with tunable chemical and mechanical properties offer another method to generate patterned cells that develop into complex tissues using a modular assembly approach. This methodology can be employed to generate tissue sheets with intricately patterned microenvironmental cues, such as growth factors and mechanical stiffness¹⁴⁷. Once engineered, these tissues can be used to study the mechanisms that regulate and maintain multicellular tissues. Furthermore, rapid advances in 3D bioprinting have made it possible to use cell-laden hydrogels to generate robust human-scale tissue constructs¹⁴⁸, which will have a direct impact on the development of tissues and organs on demand for regenerative medicine applications. Similarly, 3D engineered models enable controlled co-culture of desired cell types as well as control of oxygen gradients^{149,150}. These approaches recapitulate the 3D complexity of tissues and provide further control over the microenvironment.

Tissue and materials engineering approaches that harness self-organization. A complementary approach to the bottom-up technique for generating spatially ordered multicellular tissues is to exploit the intrinsic capability of stem cells to self-organize into developmentally and biologically relevant complex structures. Over the past decade, numerous studies have reported that stem cells can self-organize into structures that resemble mature tissues and organs in many aspects of form and function^{30,151,152}. In fact, it has recently been shown that stem cells can give rise to blastocyst-like structures, termed ‘blastoids’, in vitro through the cooperation of trophectoderm and ESCs¹⁵³. These observations have given rise to an entire field of research that focuses on employing so-called stem-cell-derived organoids to both study the emergence of form in multicellular tissues and for use in disease modelling and regenerative medicine studies. The progress made by this field has been remarkable and is detailed in some excellent reviews^{21,25,40}.

The organoid field has mainly focused on generating ordered multicellular constructs in three dimensions, observing that biomechanical support and cues are crucial for facilitating the self-organization of organoids. Conventionally, the protocols described embed stem cell clusters in ECM proteins to provide the appropriate biomechanical support. Overlaying sophisticated bioengineering techniques to further pattern these 3D tissues can improve the efficacy and fidelity of developmental models. For instance, applying biodegradable polymers to generate elongated hPSC embryoid bodies before embedding in ECM can improve the efficacy of generating human cortical plate organoids¹⁵⁴. Similarly, providing hPSC embryoid bodies with asymmetrical concentrations of ECM proteins at opposing ends can successfully recapitulate many aspects of peri-implantation human amniogenesis^{155,156}. In keeping with EDPs, bioengineers have recently employed computational modelling in conjunction with experimental observations to begin to delineate

Bioreactors

Vessels in which biological species, such as stem cells and their progeny, are grown, maintained and manipulated in a controlled environment (pH, oxygen and media change) for cell manufacturing pipelines.

Bioprinting

Utilization of printing techniques ranging from inkjet printers to 3D printers to combine cells, biomaterials, extracellular matrix, growth factors, etc. to fabricate complex tissue surrogates in vitro.

the cell–ECM interactions that underlie appropriate self-organization^{157,158}. An important general drawback of organoids for the purposes of regenerative medicine has been the use of undefined sources of ECM proteins (for example, Matrigel or Geltrex) to provide the appropriate mechanical support. Understanding the need to develop a defined, synthetic polymer to replace these unreliable sources of ECM, a recent study identified the mechanism by which Matrigel or Geltrex enables organoid formation from ISCs. They identified two different stages: one in which the ECM remained stiff and induced the nuclear translocation of the mechano-sensitive TF YAP in the ISCs and the second in which the ECM degraded owing to remodelling by the cells and thus was unable to maintain nuclear YAP in ISCs¹⁵⁹. Having identified these dynamics in nuclear YAP, and given the crucial role that YAP plays in tissue development, the authors engineered a synthetic hydrogel that mimicked this dynamic regulation and demonstrated the ability of the hydrogel to give rise to, and maintain, ISC organoids¹⁵⁹. Similarly, process engineering approaches have been applied to develop more robust organoid-generation pipelines. For example, Arora et al. have shown that automated approaches to sort developing intestinal organoids on the basis of diameter can significantly enrich for pre-organoids, thus moving closer to establishing a robust protocol for high-quality intestinal organoid generation¹⁶⁰. Recently, automated platforms for high-throughput generation of organoids have also shown promise for genetic and drug screening platforms¹⁶¹. These high-throughput platforms enable large-scale studies with stem-cell-derived structures.

Self-organized formation of spatially ordered tissues has also been demonstrated in 2D starting populations of human^{39,162} and mouse stem cell cultures^{163,164}. Using micropatterning technologies, these platforms enforce geometric confinement in the starting populations^{39,165,166}, indicating the importance of controlled tissue geometry in allowing emergent self-organization. These platforms have recapitulated aspects of the organized fate patterning that occurs during the onset of a developmental event called gastrulation and have predominantly focused on BMP4 treatment to induce these events. The BMP signal transduction pathway results in the phosphorylation and nuclear translocation of an effector protein called SMAD1, which offers an ideal readout of spatial localization of active BMP signalling and enables the mapping of BMP activity within the patterned fates. Using a combination of mathematical modelling and experimental approaches that track the localization of phosphorylated SMAD1, we and others have identified the importance of the dynamics between morphogens and their secreted inhibitors in giving rise to the self-organized spatial ordering in BMP4-treated, geometrically confined hPSC populations^{39,167} (FIG. 7B). Notably, although these proposed morphogen-based models are consistent with the experimental data from hPSCs^{39,167} or mEpiSCs¹⁶³, mechanisms that underlie the organization observed in starting populations of mESCs seem to be independent of self-organized gradients of morphogens¹⁶⁴. Instead, the organization seems to be biophysical in nature, whereby certain geometric cues

induce localization of differentiated fates within the micropatterned mESC colonies¹⁶⁴.

Genetic engineering and other approaches. There has also been exciting progress in genetically engineering emergent self-organization of stem-cell-derived tissues. A recent study employed a heterogenous genetic pulse of a TF, GATA6, in 2D hPSCs to give rise to a highly complex fetal liver bud-like tissue¹²¹. This tissue contained spatially organized stromal cells and, surprisingly, was capable of inducing fetal haematopoiesis within 14 days¹²¹.

Finally, rapid advances are being made in understanding the underlying principles that regulate the formation and maintenance of stem-cell-derived multi-component tissues. Computational models have started to investigate the roles of feedback between different population fractions in inducing morphogenesis and fate patterning^{39,168}. Technologies such as robotic swarms offer an attractive approach to test the proposed rules of intercellular interactions in non-living and computationally programmable systems^{170,171}. Not only do these technologies complement conventional computational models of underlying self-organization principles but they also pave the way to developing artificial, biomimetic self-organizing solutions for the world of automated machinery and artificial intelligence.

Interorgan and systemic interactions

At a physiological level, different organ systems are required to interact with each other via systemic signals to maintain homeostasis⁴⁶. Successful long-term stem-cell-based therapeutics will be required not only to regenerate the depleted cell numbers in a diseased tissue but also to ensure that the regenerated tissue is able to robustly interface with the dynamic *in vivo* environment. Understanding these systemic responses has been very challenging, but studies in animal models have proved incredibly valuable.

Parabiosis. A prominent example of an animal model that has provided valuable insights into systemic response is parabiosis¹⁷¹ — a surgical technique to join animals such that they share a blood circulatory system — in studying ageing. The reduction of regenerative capacity of organisms as they age is well accepted; however, the underlying reasons for this reduction and whether any systemic factors are involved in this regulation were unknown. Using heterochronic parabiosis (parabiosis between animals of different ages), a pioneering study demonstrated rejuvenation of the proliferation capacity of skeletal MuSCs and hepatocytes of aged mice when paired with young mice¹⁷². Furthermore, they demonstrated that in old mice, Notch signalling, which is reduced in ageing MuSCs, and cEBP α complexes, the levels of which increase in ageing hepatocytes, both approached levels observed in young animals, suggesting that the age-related reduction in regenerative capacity of organs is modulated by systemic factors¹⁷². This experimental paradigm can serve as a valuable model to identify underlying mechanistic regulations in ageing tissues^{173,174}. Parabiosis studies are designed to probe the impact of alternative systemic environments, such as

Fate patterning

A process during embryogenesis in which cell fates are allocated or ‘patterned’ as a function of space and time.

Morphogens

Signalling molecules, typically soluble chemicals, for which the asymmetric distribution in a developing tissue gives rise to fate patterning and morphogenesis.

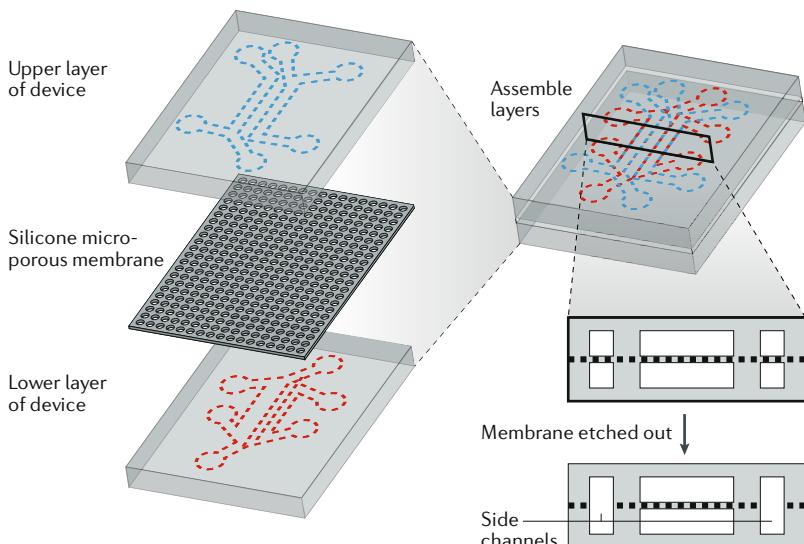
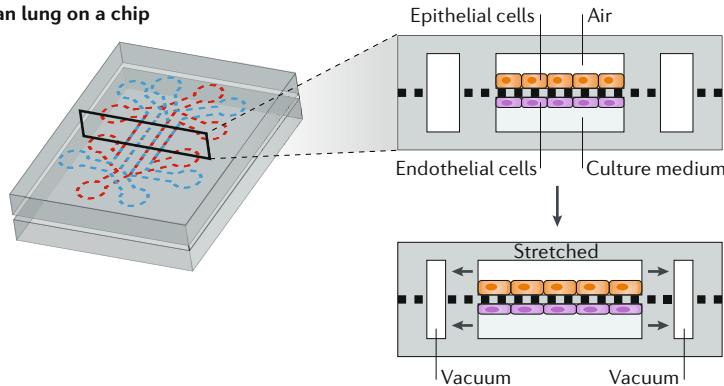
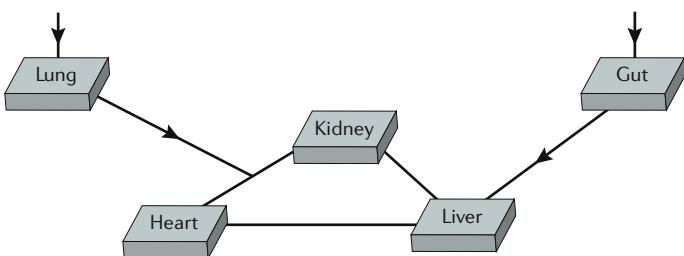
a Fabrication overview of microfluidic device to culture lung on a chip**b Human lung on a chip****c Human-on-a-chip platform: models of multiple organs interacting in a microfluidic system**

Fig. 8 | Microfluidics technologies enable organ-on-a-chip and human-on-a-chip applications. **a** | Fabrication overview of microfluidic device to culture lung on a chip as described by Huh et al. to develop a solution for organ on a chip¹⁸⁰. The layers of the microfluidic device are prepared individually with silicone rubber and assembled to generate the complete microfluidic device with a microporous membrane for culture of the lung epithelium and endothelium. The microporous membrane from the side channels is etched away. **b** | The culture of the lung epithelium and endothelium is performed such that the epithelium is in contact with air and the endothelium is in contact with the cell culture medium. Furthermore, application of vacuum through the side channels stretches the cell culture membrane, maintaining a biophysical environment reminiscent of the in vivo lung. **c** | An assembly of multiple organs on a chip interfaced with each other to provide each an in vitro organ model with a comprehensive and realistic milieu present in vivo. This human-on-a-chip model has the potential to provide valuable information that can advance drug discovery efforts. Part **a** adapted from REF.¹⁷⁷, Springer Nature Limited.

the young and old, on stem cells and stem-cell-derived cells. They represent elegant and complex solutions to understanding the niche, although the prevalence and convolution of parameters involved in the system may challenge direct and clear interpretations of results. Thus, other bioengineering approaches, discussed below, can serve to decouple the influence of individual parameters.

Human-on-a-chip systems. In addition to the exciting advances made in understanding ageing, animal models have also served as the gold standard for studying diseases and systemic effects of drugs. However, studies that employ these models are expensive and time-consuming. Furthermore, interpretation of the results of these studies in terms of the relevance to the human system has been challenging owing to species-specific differences. For instance, the mechanistic underpinnings of inflammatory diseases can vary dramatically between the same phenotype in mice and humans^{175,176}. This species-specific distinction persists in drug development studies and confounds interpretation of the findings. Indeed, as a direct consequence of this variation, many drug candidates that have shown promising data in animal studies fail in human clinical trials¹⁷⁷. Microfluidic models of organ-level function^{178–180}, on the other hand, have shown much promise for the purposes of modelling diseases (FIG. 8). Consequently, bioengineers have begun to explore avenues to develop multiorgan *in vitro* models that recapitulate human-specific pathophysiology while enabling comprehensive interactions between organ systems for drug screening studies^{176,178–180}. These ‘human-on-a-chip’ systems aim to preserve the role of intertissue interactions that occur at the systemic level while enabling drug testing in the human context (FIG. 8). Ultimately, these bioengineering platforms serve as a strategy to isolate and better understand the impact of key systemic parameters in the stem cell niche, providing more mechanistic insight and instructive control over stem cell systems.

Predictive pharmacokinetics and pharmacodynamics models. Complementing platforms that mimic systemic effects of multiorgan interactions, computational approaches such as pharmacokinetic–pharmacodynamic (PK–PD) models have also made key contributions to drug discovery and development by capturing systemic responses to input drugs¹⁸¹. These models incorporate drug-specific parameters that can often be measured *in vitro* through bioassays (such as receptor affinity and efficacy) with biological system parameters (time-dependent transduction mechanisms and homeostatic feedback mechanisms) to optimize dosing regimens and the delivery profile of drugs within clinical trials as well as for the selection of candidate drugs¹⁸¹. These models provide a basis for capturing and predicting the temporal dynamics of organism-level responses to drugs, often measured through biomarkers, and may represent the whole body as a network of individual organs that are connected through blood flow. Interestingly, multiorgan *in vitro* models such as human-on-a-chip technologies^{182,183} (FIG. 8) provide a means of measuring key parameters that are used in

PK–PD models, which have been limited to in vitro cultures of human cell lines for estimation^{176,184}. Indeed, the advancement of engineered platforms that capture multi-organ feedback will provide not only tools to experimentally probe the role of systemic interactions on tissue homeostasis, repair and development but also a basis for improving the predictive power of computational models that aim to effectively capture systemic dynamics.

Conclusions and perspectives

From an engineering perspective, stem cells are a system that can be reverse engineered and forward engineered. This paradigm challenges stem cell researchers to view cells as programmable units of life that can be predictably controlled to self-organize on the basis of simple encoded rules. Once we better understand these rules, we can reliably programme them by manipulating the cell's GRN and microenvironment, ultimately coaxing single cells to yield desired complex outputs. Here, we argue that to achieve this goal, we should use an engineering framework based on EDPs: designing the cell's programming language, editing that code and driving perturbations along a prescribed route to reach a complex outcome by manipulating single cells.

Stem cell bioengineering approaches have begun to unpack the layers of complexity that hinder our ability to reliably predict and control the behaviour of stem cell populations. As we have discussed, advances have been made along the understand–predict–control axis through the development of cellular GRN models and

niche engineering. Nevertheless, there is a continuing need to apply EDPs to understand the core rules that govern cells and their interactions with one another and their microenvironment.

Beyond uncovering the fundamental rules that govern individual cell behaviour, there is a need to better understand how the collective actions of cells, based on these underlying rules, give rise to emergent behaviour and self-organizing events that coordinate tissue and organ development such as symmetry breaking, lineage commitment or pattern formation. The outcome of this non-intuitive coordination is robust and reproducible (for example, the structural and functional features of each organism) but cannot be easily predicted from the behaviours and decisions of the individual cells. By deciphering the hierarchies of information control and uncovering the nonlinear control systems that connect each layer of complexity — within the cell, between cells and their microenvironment and in multicellular tissue and organ systems — we may ultimately be able to engineer designer tissues and organs from the bottom up and realize the central goal of regenerative medicine: the ability to produce organs on demand for clinical use. Uncovering the principles governing the emergence of collective behaviour in living and non-living systems may also have broad and powerful implications in the design of advanced machines and devices that cross the machine–human interface.

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