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Current Opinion in
Biomedical Engineering

Capturing complex epigenetic phenomena through human multicellular systems

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Abstract

Epigenetic states inherently define a wide range of complex biological phenotypes and processes in development and disease. Accurate cellular modeling would ideally capture the epigenetic complexity of these processes, as well as the accompanying molecular changes in chromatin biochemistry including in DNA and histone modifications. Here, we highlight recent works that demonstrate how multicellular systems provide a natural approach to capture complex epigenetic phenomena. They accomplish this through more closely matching the *in vivo* environment and through the intrinsic nature of multicellular systems being able to generate and model multiple distinct cellular states, all within one system. We also discuss challenges and limitations of such systems, efforts to tune and modulate epigenetics directly in multicellular systems, and how molecular interventional approaches could advance and improve the utility of these models.

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Current Opinion in Biomedical Engineering 2020, **16**:34–41

This review comes from a themed issue on **Molecular & Cellular Engineering**

Edited by **Kris Saha and Ethan Lippmann**

https://doi.org/10.1016/j.cobme.2020.05.008

Keywords

Epigenetics, Organoids, Development, Aging, 3D, Multicellular.

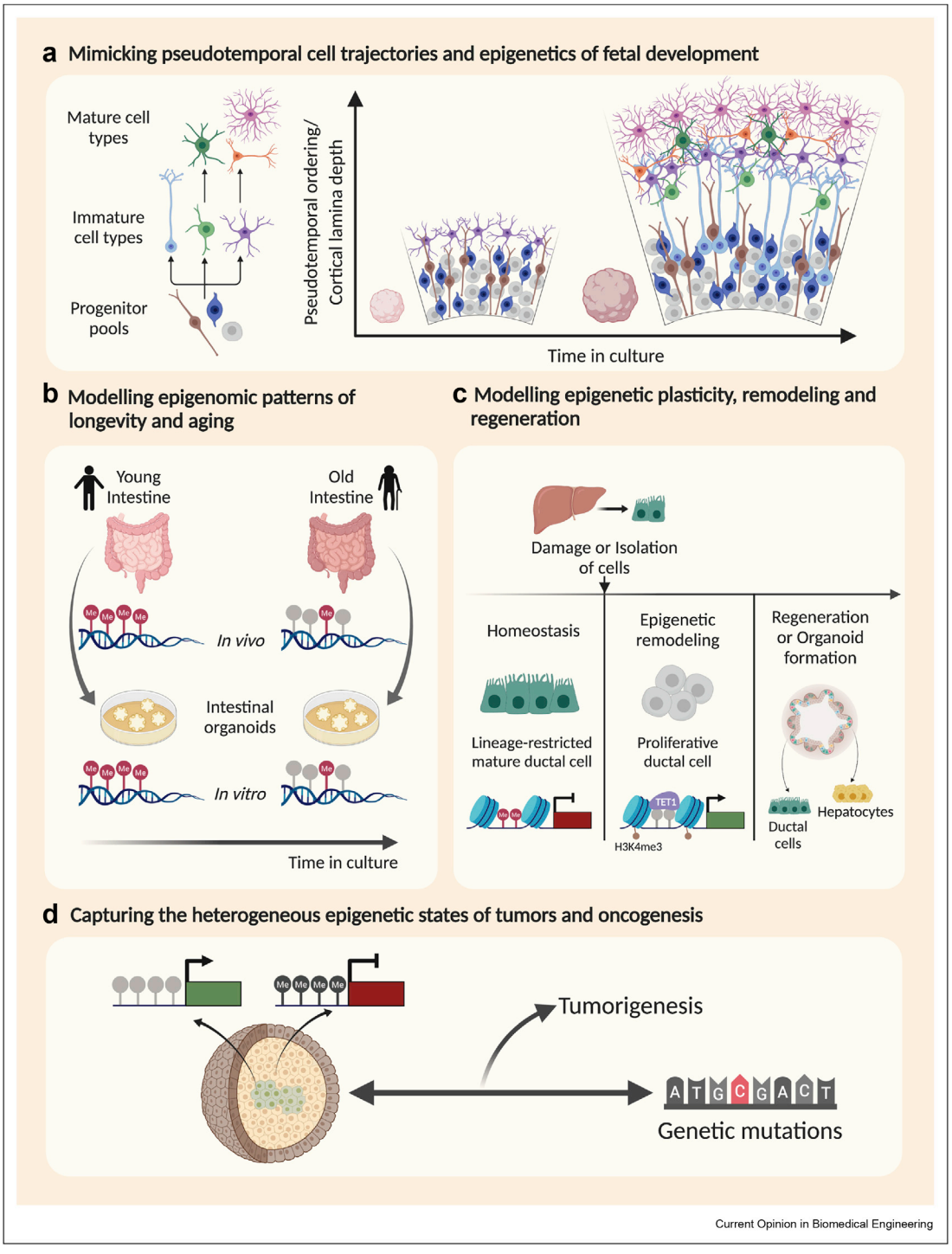
Introduction

Many questions in biology and biomedical engineering inherently involve epigenetics, defined broadly as stable features or phenotypes not conferred through changes in genetic sequence. Some are the following examples: a diverse set of cell lineages are generated during embryonic development from just one genomic sequence; specific alleles are turned on or off in specific cells within a tissue in a process called imprinting that is important in neural development; and subsets of cells within a tumor can have different pathogenic potential or resistances to drugs. These epigenetic states are often defined in the literature by distinct static compositions of mRNAs, miRNAs, proteins, and/or histone and DNA modifications; however, epigenetic states are not driven or maintained by static compositions but rather by a common dynamic mechanism, namely feedback, among the molecules. The different molecules within cells interact in complex feedback networks that reinforce and maintain their current levels and compositions, leading to epigenetic heritable stability. How the different epigenetic states that define, for example, cell types or tumorigenic potential, rely on initial transient perturbations from the environment that 'tip' cells down different developmental or oncogenic paths to eventually establish self-maintaining epigenetic states even once the initial perturbation signals are gone. Thus, to fully understand epigenetics and recapitulate epigenetic processes, it is important to consider the environmental signals provided to cells.

The multicellular nature of human tissues is a major contributing environment of signals that can establish the cascades of molecular events that establish epigenetic states within individual cells. When one considers the many examples of epigenetics at work, there is an inherent connection to multicellularity that becomes apparent: cells with the same genomic sequence but distinct epigenetic states are created often through or with interactions with other cells in a multicellular environment or through paracrine responses to factors released by other cells. There is often also a spatial correlation between cellular lineages or phenotypes that arise within a tissue. Thus, when modeling natural or disease processes, multicellular systems could inherently provide a means to induce epigenetic states and capture epigenetic phenomena. Furthermore, because multicellular systems can create microenvironments more closely resembling native tissues, cells may develop more mature or accurate cellular and molecular epigenetic states.

A large class of *in vitro* multicellular systems includes 3D systems and organoids, often derived from pluripotent stem cells. These models often recapitulate several key features of *in vivo* organ development, structure, and function [1]. For example, they have already provided useful insights into host–pathogen interactions, diseases with developmental origin, and multicellular interactions in cancer [2]. In addition, comparisons provided by single-cell RNA sequencing studies show that 3D models of the liver, kidney, and brain recapitulate the transcriptional programs of primary cell types with reasonable fidelity [3–5]. This review focuses specifically on recent examples of how these systems

Figure 1



Modeling of complex epigenetic phenomena with multicellular systems. (a) Human cerebral organoids can recapitulate the pseudotemporal cell trajectories of human fetal development. **(b)** DNA methylation analysis of human intestinal organoids derived from young and old tissues show that organoids have similar epigenetic ages to the tissues from which they were derived. Studies also show that over time in culture, fetal tissue-derived organoids show a significant change in DNA methylation and gene expression patterns toward those of tissues derived from older individuals, indicating a degree of *in vitro* maturation. **(c)** Lineage-restricted ductal cells isolated from the liver experience extensive epigenetic remodeling during *in vitro* organoid formation resembling the changes that happen *in vivo* upon extensive tissue damage. **(d)** Organoids have been shown to capture the proper physiological conditions and cellular microenvironment to model heterogeneous epigenetic states of tumors and age-associated oncogenesis.

have been able to capture epigenetic phenomena, illustrating their power to recapitulate and access features such as pseudotemporal cell trajectories of development, aging, and cancer, plasticity and regeneration, as well as molecular features of epigenetic remodeling. We also discuss the current challenges facing such systems that may result in incomplete epigenetic programming, as well as new approaches, tools, and future directions for modulating epigenetic and cellular states in multicellular systems.

Mimicking pseudotemporal cell trajectories and epigenetics of fetal development

During *in vivo* development, each cell makes fate decisions that are influenced by signals from other cells in their microenvironment in conjunction with intrinsic transcription factors and epigenetic programs. Thus, cells are not transitioning as a relatively homogeneous cohort between distinct states over time as in traditional adherent and suspension cultures; rather, cells in natural systems differentiate and proliferate relative to the other cells around it. This raises some challenging issues with analysis due to cellular heterogeneity in time and space; however, when these challenges are sufficiently addressed, multicellular systems can provide a pseudotemporal ordering that allows for the existence of, and powerful comparisons between, diverse but related cell types within the same experimental sample or system [6]. Furthermore, identification of molecular epigenomic changes, for example, in DNA methylation, can provide insights into the biology and accuracy of the models themselves.

A recent example harnessed single-cell RNA sequencing as a tool to address the challenge of heterogeneity in human cerebral organoids derived from pluripotent stem cells [4]. Comparison of single-cell transcriptomes between cerebral organoids and human fetal tissue revealed that organoids recapitulated the pseudotemporal cell trajectories of the first trimester of human fetal development. In particular, organoids captured the birth of prenatal cortical neuronal subtypes from subsets of progenitors and the gradual appearance of more mature neuronal subtypes as organoids aged. Immunohistochemistry further showed that this ordering tracked with the layered structure of the cortex with apical progenitors to pial neuronal subtypes (Figure 1a).

These systems have subsequently been used to identify molecular changes in chromatin that track with this development: a recent study showed that cytosine methylation in cytosine-guanine (CG) and non-CG nucleotides adopted patterns closely resembling the human fetal brain [7]. It is important to note that although this study demonstrates the potential of 3D systems in capturing complex developmental epigenetic remodeling, it also highlights how some of the epigenomic patterns seen in organoids are specific to *in vitro* conditions. In particular, their results show that *in vitro* neural cultures including cerebral organoids and neurospheres derived from primary cells are characterized with decreased methylation in pericentromeric differentially methylated regions. They also demonstrate that in a subset of these differentially hypomethylated regions, there is a significant loss of the histone modification H3K9me3 which could suggest potential heterochromatin decompaction. As these regions are important in genome stability, addressing these types of incomplete epigenomic remodeling may be critical for the long-term stability of organoid cultures and for therapeutic applications. Another difference identified in this study is increased CG methylation in the regulatory elements of cerebral organoids while neurospheres exhibited reduced CG methylation more similar to the fetal cortex. This difference between models may be due to an altered balance between de novo methylation and demethylation. These studies demonstrate the power of organoid systems in recapitulating pseudotemporal cell trajectories but also emphasize the importance of using genomic and epigenomic methods to understand the nature and limitations of these systems in drawing mechanistic and therapeutic insights. Screening-based studies of different culture conditions could then help identify which environmental factors contribute to these altered epigenomic patterns in organoids. In addition, models with improved spatial patterning could help control tissue heterogeneity while molecular interventions discussed in the following paragraphs could be used to 'correct' aberrant epigenomic profiles.

Epigenomic measurements can provide a potential roadmap with which the accuracy of *in vitro* multicellular systems can be to improved or tuned. This opportunity is illustrated nicely in a study of intestinal epithelial organoids derived from adult and fetal gut samples. While DNA methylation signatures of adult cell-derived organoids were stable over prolonged culture periods, DNA methylation and gene expression patterns changed significantly over time in fetal cell-derived organoids (Figure 1b) [8]. This study also showed that the majority of these epigenetic changes observed in fetal cell-derived organoids overlapped with those that differ between purified fetal and pediatric epithelial cells, thus indicating a degree of *in vitro* maturation. Together these epigenomic measurements showed that intestinal epithelial organoids not only could serve as useful models to investigate the dynamic epigenetic changes of epithelial development but also that simply growing organoids, longer or choosing age-matched cell sources, could tune their accuracy in modeling distinct developmental stages.

Modeling molecular epigenomic patterns of longevity and aging

In addition to capturing fetal cell types and developmental progressions, epigenomic patterns, specifically DNA methylation, have gained significant interest as powerful biomarkers of long-term aging where the methylation status of distinct subsets of CpG sites is strongly correlated with age on the timescales of years and decades. In fact, DNA methylation patterns have gained substantial traction as an 'epigenetic clock' to accurately predict the biological age of human subjects [9,10]. To study the epigenetics of aging, animal models have been critical for uncovering relevant pathways. However, animals are limited in their ability to capture human cell type and molecular specificities, timescales, and mechanisms associated with complex human diseases [11]. Human cell culture studies are widely used for aging studies as well but lack the necessary cell—cell and cell—matrix interactions to capture tissue-specific features of aging [12].

Recent work has shown that multicellular *in vitro* systems can capture the molecular changes from this broad age range as well. A recent study used the pan-tissue epigenetic clock by Lewis and colleagues as a biomarker to determine if human intestinal organoids derived from the small intestine and colon could recapitulate intestinal stem cell behavior during aging in the absence of an *in vivo* aging niche [13]. Their results demonstrate that intestinal organoids show global DNA methylation patterns and epigenetic ages similar to the tissues from which they were derived, and they are viable models for studying intestinal stem cell—intrinsic aging. Interestingly, the Horvath pan-tissue epigenetic clock has shown that not all tissues within the same individual age at the same rate [10]. In support of this, Lewis et al [13] also showed that organoids derived from the small intestine showed striking epigenetic age reduction relative to organoids derived from the colon. These results suggest that organoids generated from multiple tissues of the same organism could be compared to understand the relative systemic and tissue-specific effects of age-associated mechanisms and pathologies.

Modeling epigenetic plasticity, remodeling, and regeneration

Embryonic development is characterized by waves of DNA methylation loss and acquisition. These epigenetic changes are essential to direct cells toward their future lineages by serving as fundamental barriers that ensure that differentiated cells remain lineage restricted [14]. However, epigenetic phenotypes are not fundamentally irreversible, with selected cases of reversibility found to occur as natural processes. For example, the adult liver has an extensive capacity for regeneration partly due to the acquired cellular plasticity of lineage-restricted ductal cells upon extensive liver damage. These cells can regress into an undifferentiated state and regenerate both ductal cells and hepatocytes (Figure 1c) [15].

Multicellular systems can be used to study the molecular mechanisms that allow adult committed cells to exit their lineage-restricted state and initiate differentiation, proliferation, and regeneration, as recently demonstrated by Aloia et al [16]. In their study, they used a recently reported liver organoid culture system where they isolated lineage-restricted nonproliferative ductal cells capable of generating organoids [17]. They showed that these cells underwent profound rewiring of their transcriptome, DNA methylome, and hydroxymethylome during the initiation and formation of organoids. These changes resembled the molecular changes observed in *in vivo* regenerative responses to tissue damage. In this case, the very act of creating the organoid through multicellular assembly mimicked the regeneration needed upon tissue damage and provided proof of principle that liver organoid generation provides a viable model to study the epigenetic mechanisms of tissue regeneration.

Capturing the heterogeneous epigenetic states of tumors and oncogenesis

The connections between cellular malignancy and epigenetics are extensive: It is readily apparent from cancer genetics that mutations in genes encoding epigenetic machinery are collectively a leading driver of cancers [18]; the cancer stem cell hypothesis, or the more general idea that only a subset of cells in a tumor are capable of driving cancer progression, initiation, and metastasis, directly alludes to the idea of epigenetically distinct cells within a multicellular tumor; furthermore, DNA methylation abnormalities at promoter CpG islands of many genes that control stemness, differentiation, and senescence have been correlated with cancers and may interact with the susceptibility to genetic lesions (Figure 1d) [19]. Given the clear epigenetic connections, a central question arises, why do some cells become malignant and others do not? Multicellular *in vitro* systems can provide strong models to tackle this question for several reasons: They provide systems with built-in internal controls to compare normal and transformed cells with each other all within one organoid or sample; experimental conditions can be readily adjusted; interactions with cells and their environment which are known to be important in controlling cellular malignancy can be tuned [20]; and the lineage relationships between cells can be tracked.

3D multicellular models have exploited these advantages to study the origins of mutational signatures in

early tumorigenesis [21,22]. In a recent example, Tao et al [23]linked age-associated promoter CpG island methylation to cancer predisposition using mouse colon-derived organoids. Their results show that in addition to tumor suppressor *Cdkn2a*, Wnt antagonist genes such as *Sfrp1, Sfrp2, Sfrp4, Sox17, Cdx1*, and *KI* in aged mouse colon-derived organoids are spontaneously silenced due to DNA promoter hypermethylation which activates the Wingless/Integrated (Wnt) pathway and makes the older organoids more susceptible to $Braf^{V600E}$-induced transformation and tumorigenesis. This study demonstrates the utility of organoid models in capturing the proper physiological conditions of the intestinal stem cell microenvironment by mimicking the dynamics of stem cell renewal and differentiation. They simultaneously provide sufficient experimental control to track difficult parameters such as age and to make robust epigenetic measurements and perturbations. We also direct the reader to reviews that discuss the many other genes that have exhibited epigenetic alterations in malignant cells and that include discussion of how many of these genes tend to overlap developmental genes characterized by bivalent epigenomic states in stem cells [19,24].

Tuning the epigenetics of multicellular systems: challenges and progress

While *in vitro* multicellular systems provide the many advantages described earlier, their artificial nature is a double-edged sword: they provide direct control over experimental conditions, but the effects of synthetic culture conditions could have unanticipated effects that may not be physiologically relevant. For example, while human cerebral organoids can model the transcriptomic changes of early-to-mid fetal cortical development, they also show significant differences in their expression of extracellular matrix genes, potentially due to the embedding of the synthetic tissues within Matrigel droplets as part of some artificial organoid generation protocols [7]. Externally supplying tissues with extracellular matrix proteins, especially chemically variable matrices such as Matrigel, may affect patterning and maturation of tissues contributing to well-described batch-to-batch differences reported in many organoid models. Furthermore, compared with their *in vivo* counterparts, 3D multicellular models lack vascularization, limiting the distribution of nutrients and oxygen to the cells in the center which results in extensive cell death in their core regions leading to significant cellular stress [25]. Bhaduri et al [26] characterized the degree of cellular stress in human cerebral organoids and its molecular consequences. Their results showed that cerebral organoids contain a smaller number of cellular subtypes and that these cells often aberrantly coexpress marker genes from multiple lineages resulting in physiologically aberrant pan-radial glia or pan-neuronal cells.

In addition to cellular stress, multicellular models generated from pluripotent stem cells face developmental roadblocks toward long-term maturation and remain stuck in relatively immature states, limiting their scientific potential [27]. While there are some models that are able to recapitulate the more adult epigenomic signatures of the tissues from which they were derived [8,13], most stem cell—derived organoid models more closely resemble immature prenatal tissues and fail to mature regardless of duration in culture. Interestingly, some studies have transplanted organoids into various sites in mammalian hosts resulting in organoid maturation and increased tissue functionality [28—30], while others have used physical slicing or induced vascularization to promote growth and maturation [31,32]. These studies show that organoid models are capable of maturing when provided with additional cues present in the *in vivo* environment. One caveat of the transplantation approach is that it is challenging to identify the exact factors in the host environment that are responsible for organoid maturation.

Another major limitation of multicellular models is the stochastic nature of undirected differentiation and the lack of spatial patterning factors. Currently organoids do not mimic the overall shape of human organs. The lack of embryonic body axes is a likely cause of this along with the heterogeneity and variability in current organoid protocols [27]. Achieving spatial control of patterning factors and tissue organization is an important step in improving these systems. Microfluidic devices or biomaterials engineered to deliver specific patterning factors in a spatially controlled manner could be developed to address this issue.

In addition to cellular and tissue level heterogeneity, most multicellular models lack specific cell types, some of which are critical to their utility in asking specific development or disease questions [33]. Furthermore, the lack of signaling factors from these specific cell types could adversely affect the establishment of accurate epigenetic landscapes. In some cases, this challenge can be addressed through optimizing conditions for coculturing or exogenously incorporating these missing cell types. For example, a recent study cocultured epithelial tumor organoids with peripheral blood lymphocytes and demonstrated the expansion of tumor-specific T cells which has important implications in studying patient-specific anticancer properties of these cells [34]. Similarly, microglia can be incorporated into human cerebral organoids to potentially study their roles in neurodevelopment and neurodegeneration [35].

While the nonidealities of artificial multicellular culture systems clearly affect cell states, their effects on epigenetic profiles have not been extensively studied. Such studies connecting culture conditions to

epigenetic states could provide a framework for engineering improved experimental models. There is ample evidence from the more mature field of cellular reprogramming that learning from or directly modulating epigenetics may improve multicellular models as well. When somatic cells were first reprogrammed to pluripotency and then to many different cell types, the low efficiencies of these reprogramming processes were widely noted. It quickly became apparent that the somatic cell type of origin could affect reprogramming efficiency, that chemical modulation of epigenetic states could boost reprogramming efficiencies, and that epigenetic profiles of somatic cells could predict their reprogramming potential [36]. How could similar strategies targeting epigenetics be applied to improve multicellular systems? As discussed earlier, epigenetic signatures are strongly correlated with age and regional tissue identity [8,37]. Chemical or protein-based perturbations of the epigenome could potentially be used to accelerate the maturation of multicellular systems or tune and enhance the generation of specific distributions of cell types. We also direct the reader to additional reviews that discuss more differences between organoid and *in vivo* systems beyond epigenetic implications [2,27,38].

Future directions

Experimental models are important for the scientific method especially in the biomedical sciences where human systems are extremely complex and, in most cases, unethical to use in controlled studies. This is especially true given the cellular and molecular mechanistic complexities of epigenetic phenomena. Recent advances in multicellular systems have already improved the representation and reach of human biological studies by providing a powerful tool with which to access human epigenetics. Their ability to recapitulate human-specific manifestations of disease and the dynamics of tissue development and regeneration make them attractive models for therapeutic discovery and developmental biology.

However, many challenges remain. Foremost is addressing the compositional, structural, and epigenetic variability of multicellular systems. Reflecting this concern, recent research efforts have largely moved away from the development of novel 3D multicellular models toward improving the reproducibility of existing systems [39]. In addition, the maturation and fidelity of cells within these systems is important, and mapping the epigenetic landscape of these models may help address this challenge. Ultimately, continued improvements in multicellular models will require feedback between developing a deeper understanding of epigenetics in such systems and how modulating epigenetics directly or indirectly could bring them closer to their *in vivo* counterparts. The impact of this work will extend beyond fundamental knowledge but also improve the success and safety of therapeutics discovered with and tested on human multicellular models.

Improved systems would also expand the breadth of epigenetics and the types of questions researchers can ask. For example, epigenetic mechanisms form the bridge between exposures to environmental factors or toxins and changes in cellular function. Thus, models with an accurate representation of these epigenetic networks are crucial to capture accurate environmental responses. 3D multicellular models already more closely mimic the *in vivo* environment, making them promising models to capture the epigenetic responses to environmental toxins such as alcohol or drugs of abuse during development [40]. Furthermore, the ability of organoids to recapitulate the transcriptional programs and the epigenomic states of fetal tissue development [4,7] indicates that they could be used to model more complex epigenetic mechanisms such as genomic imprinting and associated diseases.

To achieve these goals, it will be important to advance the sophistication of *in vitro* culture environments by applying multidisciplinary engineering approaches. Physical approaches such as spatiotemporal control over differentiation using tunable matrices or organ-on-chip systems may allow more control over organoid development. Furthermore, molecular strategies such as genome and epigenome editing tools, developmental switches that turn on/off at specific times to control differentiation programs, and targeting enhancer regions for multigenic manipulations could all be used to control and tune cell fate determination in multicellular models with greater precision. Even with these approaches, no model will be perfect; it will be important to master the strengths and weaknesses of current and future multicellular models before using them. Fortunately, multicellular models not only provide access to epigenetic phenomena but the converse is true as well: epigenetics provides insights into the quality fidelity, and engineering of multicellular models.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by a Simons Foundation Simons Foundation Autism Research Initiative (SFARI) Explorers Grant (495112), the National Science Foundation (NSF) Emerging Frontiers in Research and Innovation program (Emerging Frontiers and Multidisciplinary Activities (EFMA)-1830910), an National Institutes of Health (NIH) Avenir Award (DP1-DA044359, -02S1), NIH R21-E0234477, NIH R21-CA240162, and a fellowship from the American Association of University Women (to DS).

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By utilizing a recently reported liver organoid system, this study uncovers that TET1-mediated hydroxymethylation activates the regenerative program as a response to tissue damage. They demonstrate that lineage-restricted ductal cells go through rewiring of the transcriptome, DNA methylome and hydroxymethylome during organoid formation which resembes embryonic reprogramming, and they generate both ductal cells and hepatocytes.This study shows the utility of organoids as a model to study the mechanistic aspects of tissue regeneration and human liver diseases characterized by prominent ductal proliferation and hepatic fibrosis.

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