Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Biotechnology

Creating artificial signaling gradients to spatially pattern engineered tissues

Sherry Li Zheng and Kyle M Loh

Artificially constructing a fully-fledged tissue — comprising multiple cell types whose identities and spatial arrangements reflect those of a native tissue — remains daunting. There has been impressive progress in generating three-dimensional cell cultures (often dubbed 'organoids') from stem cells. However, it is critical to appreciate that not all such three-dimensional cultures will intrinsically self-organize to spontaneously recreate native tissue architecture. Instead, most tissues *in vivo* are exogenously patterned by extracellular signaling gradients emanating from organizer cells located outside the tissue. Innovations to impose artificial signaling gradients — using microfluidics, optogenetics, or introducing organizer cells — could thus prove decisive to create spatially patterned tissues *in vitro*. Additionally, unified terminology to describe these tissue-like simulacra as 'aggregates', 'spheroids', or 'organoids' will be critical for the field.

Address
Department of Developmental Biology, Stanford Institute for Stem Cell Biology & Regenerative Medicine, Stanford University School of Medicine, Stanford, CA 94305, USA

Corresponding author: Kyle M Loh (kyleloh@stanford.edu)

Current Opinion in Biotechnology 2022, **78**:102810

This review comes from a themed issue on **Tissue, Cell and Pathway Engineering**

Edited by **Laura Segatori** and **Jennifer A. Maynard**

https://doi.org/10.1016/j.copbio.2022.102810

Introduction

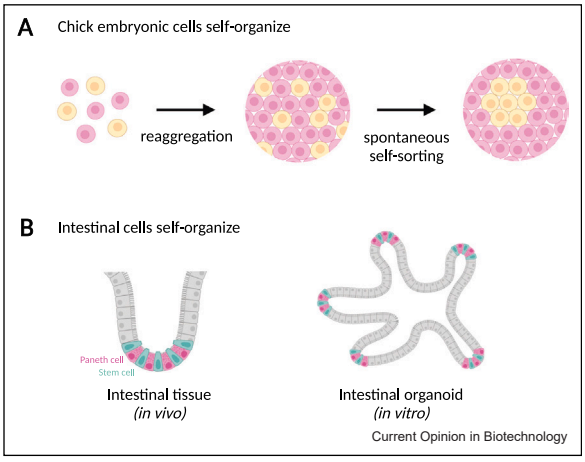
Engineering artificial tissues is one of the grandest challenges in modern biology. To even grasp the scope of this challenge requires one to first consider: what is a tissue? Consensus holds that tissues are defined by two hallmarks: 1) the coexistence of multiple cell types and 2) their spatial organization relative to one another [1,2]. This second hallmark is crucially important: spatial organization transforms cells into tissues [3]. For instance, a brain that has been dissociated and reaggregated contains all the right cell types in the correct proportions, but is clearly no longer a brain because the precise spatial pattern of neural connections has been destroyed. Spatial organization is thus preeminent in evaluating whether a tissue-like simulacra approximates a real tissue [2].

In recent years, organoids or cultured 3D tissues purporting to mimic the cell-type composition and organization of *in vivo* tissues, have garnered much attention [1,4–9]. Organoids were partially inspired by historical embryological findings from the 1960s that when particular cell types from chicken embryos were mixed together in culture, they 'self-organized' into tissue-like entities [10] (Figure 1a). This was even more dramatically borne out in the 1970s by the finding that if an entire hydra was dissociated and then reaggregated into a ball, it spontaneously recreated its original architecture within several days [11]. Certain cell types, when left to their own devices *in vitro*, can thus self-organize to a surprising degree.

These pathbreaking findings inspired the 3D culture of many different explanted tissues. For instance, adult intestinal stem cells cultured in specific conditions generate 3D intestinal organoids, which largely fulfill the two above criteria for tissues [7,12]. First, they comprise multiple intestinal cell types, including enterocytes, goblet cells, Paneth cells, and more [12]. Second, some of these cell types are spatially arranged in crypt-like domains generally reminiscent of those seen *in vivo* [7,12] (Figure 1b). This architecture remarkably forms in the absence of specific efforts to drive patterning *in vitro*, highlighting the intrinsic ability of intestinal tissues to self-organize. Other cell cultures, such as pluripotent stem-cell (PSC)-derived optic cups, also exhibit impressive self-organizing abilities, forming C-shaped structures containing both photoreceptors and retinal pigmented epithelial cells, analogous to the back of the eye [13].

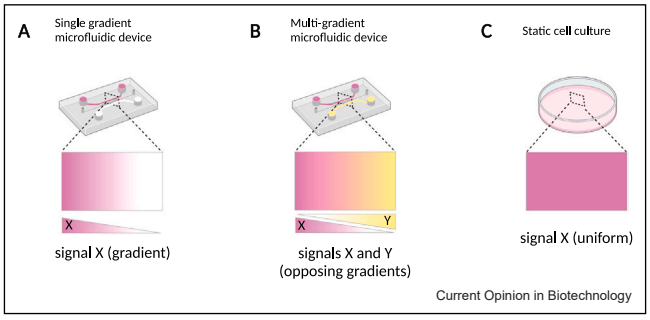
However, not all tissues can self-organize in all respects. For instance, current-generation 3D liver cultures contain the two principal liver epithelial cell types (hepatocytes and cholangiocytes), but they are seemingly interspersed randomly with one another [14]. 3D cultures of other cell types instead exhibit transient organization. This is exemplified by forebrain organoids generated from human PSCs: they initially comprise rosettes of neural progenitors ensconced in concentric rings of various cortical neurons [15–19]. However, after several weeks, these cell types mix together and their

Figure 1



Some tissues are capable of self-organization. **(a)** When mixed *in vitro*, some cell types spontaneously self-sort such that like cells cluster together [10]. **(b)** Like *in vivo* intestinal tissues (left), intestinal organoids (right) contain both stem cells (green) and Paneth cells (pink), and form crypt- and villus-like domains *in vitro* [7,12].

Figure 2



Microfluidic devices generate signaling gradients *in vitro*. **(a–c)** Microfluidic devices can generate simple unidirectional signaling gradients **(a)** or countervailing gradients of multiple signals **(b)**, in contrast to standard cell cultures wherein signals are uniformly distributed **(c)**.

original spatial organization largely fades [20]. A prominent message that emerges is that despite the recent surge of interest in 3D cell cultures, not all 3D cultures stably adopt tissue-like spatial organization. Given that the term 'organoid' denotes tissue-like architecture, the two terms — 3D cultures and organoids — should not necessarily be conflated.

To engineer complex *in vivo*-like architectures, perhaps we could be guided by nature: afterall, the embryo assembles marvelously complex tissues with near-unerring precision. In development, tissues are spatially patterned by gradients of extracellular signals (morphogens) originating from an external 'organizer' cell [21–25]. These morphogens diffuse so that cells closer to the organizer receive higher levels of signals than those farther away, causing cells in different locations to adopt different fates [21–25]. Emerging methods to create these signaling gradients *in vitro* may thus prove decisive in improving the spatial organization of engineered tissues. Here, we will briefly review four such methods: microfluidics, optogenetics, organizer cells, and self-generating gradients.

Microfluidics: creating synthetic morphogen gradients

One method to create signaling gradients *in vitro* is through the use of microfluidic devices [26]. Microfluidic devices can control the spatial distribution of signaling molecules, such that cells at different positions within the device receive differing concentrations of signals [26] (Figure 2a,b). By contrast, in standard cell cultures, cells are bathed in uniform media (Figure 2c).

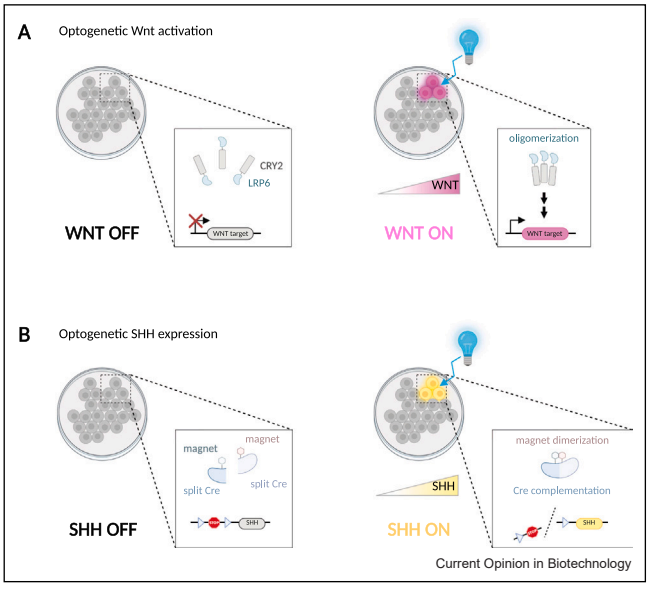
Microfluidics have been used to expose PSC-derived neural progenitors to spatial gradients of Hedgehog or Wnt signals emanating from one side of a device [27,28]. Consequently, neural cells of different identities arose at different positions in the culture [27,28]. Alternatively, PSCs exposed to a microfluidic gradient of primitive streak-inducing signals (bone morphogenetic protein (BMP), Fibroblast growth factor (FGF), Transforming growth factor β (TGFβ), and WNT) yielded primitive streak cells at one side of the device [29].

A distinctive advantage of microfluidic systems is that they theoretically afford great precision in tuning the range and slope of signaling gradients. A particularly exciting idea is to microfluidically expose cells to countervailing signaling gradients along a given axis (e.g., adding the agonist vs. antagonist for a single pathway or two different pathway agonizts at opposing poles [27] (Figure 2b)). Even more intricate would be the creation of multiple orthogonal signaling gradients along different spatial axes [27], therefore emulating anterior–posterior, dorsal–ventral, and left–right tissue patterning *in vivo*. However, microfluidic devices are usually quite small, and therefore may not easily scale to differentiate billions of cells and to create large-scale tissue simulacra. Additionally, complex microfluidics may be challenging for most biologists to adopt, although simpler devices are relatively low-cost and user-friendly [29], and can easily expand a cell-culture toolkit.

Optogenetics: activating signaling pathways using light

Through the agency of optogenetic tools, light can be controlled to activate desired signaling pathways. Therefore, by shining light on one region of a cell culture, signaling pathways can be activated in a precise place and time. For instance, Wnt signaling can

Figure 3



Optogenetic devices generate signaling gradients *in vitro*. **(a)** In the optoWnt system, blue light activates Wnt signaling by driving expression of Wnt target genes [30]. **(b)** In the split-Cre SHH system, blue light activates the Cre-driven expression of SHH ligand [31].

specifically be activated in one region of human PSC cultures using the 'optoWnt' system, thereby triggering localized primitive streak differentiation only at that location [30] (Figure 3a). OptoWnt cells express the Wnt coreceptor Low-density lipoprotein receptor-related protein 6 (LRP6) fused to the light-sensitive domain cryptochrome 2 (Cry2); light induces LRP6–Cry2 oligomerization to activate Wnt signaling [30]. Importantly, the optoWnt system is reversible: Wnt signaling ceases upon light termination [30].

In another strategy, light can be exploited to elicit expression of the signaling ligand Sonic hedgehog (SHH). In this system, light-induced dimerization of a split-Cre system leads to permanent *SHH* expression [31] (Figure 3b). This technique has been used to create spatially patterned cultures of human neural progenitors from PSCs: regions exposed to light adopted a ventral neural fate, whereas distant regions acquired a dorsal neural fate [31], consistent with how Shh gradients pattern the developing forebrain along the dorsal–ventral axis *in vivo* [32]. In this approach, light irreversibly activates *SHH* expression [31].

In summary, light can be wielded to achieve localized signaling-pathway activation, thereby affording spatial control over stem-cell differentiation. Additionally, light can be rapidly turned on or off within seconds, thus potentially providing fine temporal control over signaling. However, cells must first be genetically engineered to carry these optogenetic systems; additionally, LEDs or other light sources must be deployed to cell cultures. Light exposure, or expression of optogenetic proteins, could also potentially alter cell state. The advent of optogenetic systems to activate additional signaling pathways, including Extracellular signal-regulated kinase (ERK) and TGFβ [33,34], or to potentially multiplex several signaling pathways in parallel using different frequencies of light, will extend the remit of these light-induced patterning strategies.

Introducing artificial organizer cells to create gradients

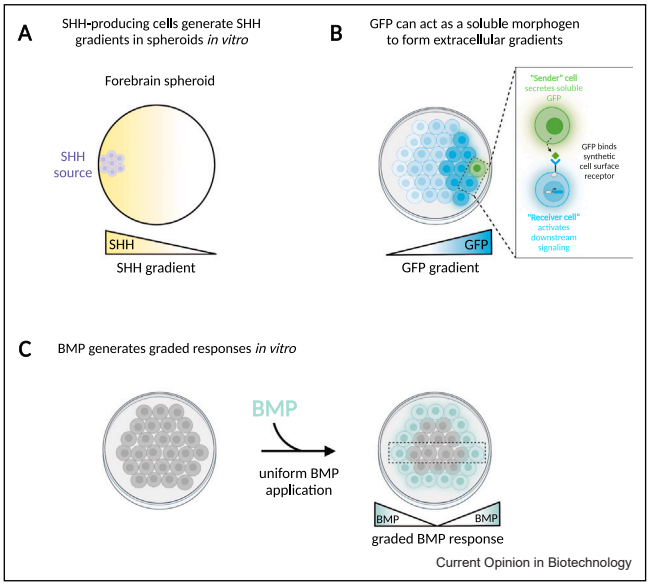
If existing cells cannot serve as organizers, artificial organizers can be introduced into cell populations instead. In developmental biology, there is a rich history of introducing cells or beads presenting a given signaling ligand to create artificial signaling gradients *in vivo* and *in vitro* [35–37]. Recent *in vitro* examples of synthetic organizers include cells engineered to express SHH signaling ligands [38,39]. By introducing these synthetic organizer cells only into one side of the culture dish, it is possible to create artificial signaling gradients whereby cells nearby the organizer receive and respond to the signal, but distal cells do not [38,39]. In one salient example, SHH-expressing PSCs were positioned at one end of PSC-derived brain organoids: this SHH signaling gradient spatially patterned the organoids, generating ventral neural cells next to the SHH source, and dorsal cells further afield [38] (Figure 4a).

Of course, natural signaling pathways come with their own limitations. For instance, almost all of them elicit expression of signaling antagonists, thereby distorting the shape of the signaling gradient over time [39]. Some have thus abandoned natural signaling pathways and replaced them with partly artificial ones. For example, inert molecules such as GFP can be used to generate extracellular gradients, serving as alternatives to classical signaling molecules to pattern tissues *in vitro*. To this end, some cells were engineered to secrete soluble GFP molecules, which formed extracellular gradients [40,41]. Other nearby cells were engineered to express artificial GFP receptors on the cell surface, which were coupled to downstream signaling cascades [40,41] (Figure 4b). Coupling these artificial GFP receptors to differentiation-inducing signaling programs (e.g., BMP) endowed GFP with the power of a developmental morphogen [40].

Gradients that essentially create themselves

While microfluidics and organizer cells can be used to generate signaling gradients, they may not always be necessary. Some signals, when added uniformly to static culture media, do not act on all cells equally, rather they exert spatially graded effects on cells. For instance,

Figure 4



Artificial organizers generate signaling gradients *in vitro*. **(a)** SHH-expressing cells create spatial gradients of SHH in forebrain spheroids [38]. **(b)** green fluorescent protein (GFP) can be employed as a diffusible signaling molecule that binds artificial receptors on nearby cells to generate graded responses within cell cultures [40,41]. **(c)** Owing to the basolateral localization of BMP receptors, uniform application of BMP in a culture will lead to a graded BMP response, where only cells on the edges of colonies will respond to BMP signaling [42,43].

when BMP4 is added to hPSC colonies, only cells along the periphery experience BMP signaling [42,43] (Figure 4c). BMP receptors are located at the basolateral surfaces of cells [43]. Consequently, cells in the center of the colony cannot receive BMP4 located in the media located above them [43].

This idiosyncratic property of BMP, whereby it predominately affects peripheral cells within a cluster, has been exploited in interesting ways. *In vivo*, the ectoderm germ layer is spatially patterned into epidermis, neural crest, and neural progenitors by decreasing BMP concentrations [44]. Remarkably, 2D or 3D clusters of hPSC-derived ectoderm progenitors exposed to BMP4 differentiated to generate concentric layers of epidermis (outermost cells), neural crest, and neural progenitors (innermost) *in vitro* [45,46].

Consequently, controlling cell colony size may be crucial, yet widely overlooked in stem-cell differentiation experiments. In essence, the size of the cell colony dictates the extent to which BMP influences the colony interior [42]. Variable colony sizes across different experiments could thus dramatically alter differentiation outcomes. Emerging methods to achieve reproducible hPSC colony sizes, coupled with signals that influence peripheral cells (e.g., BMP), can therefore create radially patterned cell cultures [42,45,46].

It is not just gradients! Other tissue-patterning mechanisms

While this brief review has primarily concerned itself with long-range signaling gradients as decisive forces in tissue patterning, there is a much wider repertoire of tissue-patterning mechanisms, including mechanical forces [47,48], cell migration [49], differential cell adhesion and cell sorting [1,10,50,51], short-range signaling [52], cytoneme-delivered signals [53], and more. Although beyond the scope of the present review, these additional mechanisms might one day be combined with engineered signaling gradients to spatially pattern cell cultures. For instance, cells engineered to express either N-cadherin or P-cadherin spatially segregate from one another in cocultures, as cells expressing the same cadherin preferentially adhere to each other [54]. Thus, differential cell adhesion can be exploited to generate multilayered cell aggregates [54].

A note on terminology: 'organoids', 'spheroids', 'aggregates', and more

Finally, 3D cell cultures have been variously referred to as organoids, spheroids, and aggregates. But what qualities distinguish an organoid, spheroid, and aggregate? Community-wide consensus is needed to define and consistently apply these terms. Not all 3D cell cultures closely resemble *in vivo* organs, and thus not all 3D cultures should be dubbed 'organoids'. 3D cell cultures that comprise only one cell type, or that contain multiple but randomly interspersed cell types, may instead qualify as 'spheroids' or 'aggregates'. The moniker 'organoid' should be reserved for 3D cultures containing multiple cell types that are spatially positioned relative to one another in a way reminiscent of *in vivo* tissues [1,2]. Other neologisms may need to be created as well, to describe spheroids with transient spatial organization such as forebrain spheroids [20].

Of course, one should not be overly didactic in nomenclature because tissue patterning exists on a spectrum: how can we quantitatively classify a cell culture as an organoid (or a spheroid, aggregate, or something else)? Indeed, even if one applies some of the patterning methods discussed above, is the resultant culture truly an organoid? Tissues are complex communities often encompassing nerves, vasculature, immune cells, mesenchyme, and other cell types [4] often lacking from most 'organoids' (with some notable exceptions [55,56]). Many open questions thus remain. For instance, if a culture displays some degree of tissue-like functionality [1], but is not spatially organized akin to a tissue, does it qualify as an organoid? How close does one need to get to the real tissue before calling it an organoid? While

single-cell RNA sequencing can quantify how transcriptionally similar cultured cells are relative to their *in vivo* counterparts, quantifying the 3D spatial organization of cell populations is not trivial [2]. New tools to quantify the spatial locations of multiple cell types relative to one another in 3D will be transformative. Such advances will help the tissue engineering and stem-cell biology communities to quantify how closely 3D cultures mimic native tissues. To that end, the ability to create artificial signaling gradients will avail us in our perennial quest to engineer increasingly realistic tissues and to emulate the perfection of native tissues.

Conflict of interest statement

Nothing declared.

Data Availability

No data were used for the research described in the article.

Acknowledgements

We thank Shuo Han, Macy Vollbrecht, Carolyn Dundes, and Alex Pollen for feedback and Alex Dunn, Leeya Engel, Kiara Cui, and Lay Teng Ang for discussions regarding microfluidics. We apologize to all researchers whose work we were unable to cite owing to brevity. S.L.Z. is supported by the Stanford Graduate Fellowship and the U.S. National Science Foundation. K.M.L. is a Packard Foundation Fellow, Pew Scholar, Human Frontiers Science Program Young Investigator, and The Anthony DiGenova Endowed Faculty Scholar. Figures were created with Biorender.com.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest.

1. Lancaster MA, Knoblich JA: **Organogenesis in a dish: modeling development and disease using organoid technologies**. *Science* 2014, **345**:1247125.

2. Fowler JL, Ang LT, Loh KM: **A critical look: challenges in differentiating human pluripotent stem cells into desired cell types and organoids**. *Wiley Interdiscip Rev Dev Biol* 2019, **113**:823-891.

3. Fletcher DA: **Bottom-up biology: harnessing engineering to understand nature**. *Dev Cell* 2016, **38**:587-589.

4. Takebe T, Wells JM: **Organoids by design**. *Science* 2019, **364**:956-959.

5. Sasai Y: **Cytosystems dynamics in self-organization of tissue architecture**. *Nature* 2013, **493**:318-326.

6. Paşca SP: **The rise of three-dimensional human brain cultures**. *Nature* 2018, **553**:437-445.

7. Sato T, Clevers H: **Growing self-organizing mini-guts from a single intestinal stem cell: mechanism and applications**. *Science* 2013, **340**:1190-1194.

8. Hofer M, Lutolf MP: **Engineering organoids**. *Nat Rev Mater* 2021, **6**:402-420.

9. Velasco S, Paulsen B, Arlotta P: **3D brain organoids: studying brain development and disease outside the embryo**. *Annu Rev Neurosci* 2020, **43**:375-389.

10. Steinberg MS: **Reconstruction of tissues by dissociated cells. Some morphogenetic tissue movements and the sorting out of embryonic cells may have a common explanation**. *Science* 1963, **141**:401-408.

11. Gierer A, Berking S, Bode H, David CN, Flick K, Hansmann G, Schaller H, Trenkner E: **Regeneration of hydra from reaggregated cells**. *Nat New Biol* 1972, **239**:98-101.

12. Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, van Es JH, Abo A, Kujala P, Peters PJ, *et al.*: **Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche**. *Nature* 2009, **459**:262-265.

13. Eiraku M, Takata N, Ishibashi H, Kawada M, Sakakura E, Okuda S, Sekiguchi K, Adachi T, Sasai Y: **Self-organizing optic-cup morphogenesis in three-dimensional culture**. *Nature* 2011, **472**:51-56.

14. Huch M, Dorrell C, Boj SF, van Es JH, Li VSW, van de Wetering M, Sato T, Hamer K, Sasaki N, Finegold MJ, *et al.*: **In vitro expansion of single Lgr5+ liver stem cells induced by Wnt-driven regeneration**. *Nature* 2013, **494**:247-250.

15. Lancaster MA, Renner M, Martin C-A, Wenzel D, Bicknell LS, Hurles ME, Homfray T, Penninger JM, Jackson AP, Knoblich JA: **Cerebral organoids model human brain development and microcephaly**. *Nature* 2013, **501**:373-379.

16. Pasca AM, Sloan SA, Clarke LE, Tian Y, Makinson CD, Huber N, Kim CH, Park J-Y, O'Rourke NA, Nguyen KD, *et al.*: **Functional cortical neurons and astrocytes from human pluripotent stem cells in 3D culture.** *Nat Methods* 2015, **12**:671-678.

17. Kadoshima T, Sakaguchi H, Nakano T, Soen M, Ando S, Eiraku M, Sasai Y: **Self-organization of axial polarity, inside-out layer pattern, and species-specific progenitor dynamics in human ES cell-derived neocortex**. *Proc Natl Acad Sci USA* 2013, **110**:20284-20289.

18. Qian X, Nguyen HN, Song MM, Hadiono C, Ogden SC, Hammack C, Yao B, Hamersky GR, Jacob F, Zhong C, *et al.*: **Brain-region-specific organoids using mini- bioreactors for modeling ZIKV exposure**. *Cell* 2016, **165**:1238-1254.

19. Mariani J, Simonini MV, Palejev D, Tomasini L, Coppola G, Szekely AM, Horvath TL, Vaccarino FM: **Modeling human cortical development in vitro using induced pluripotent stem cells**. *Proc Natl Acad Sci USA* 2012, **109**:12770-12775.

20. • Bhaduri A, Andrews MG, Mancia Leon W, Jung D, Shin D, Allen D, Jung D, Schmunk G, Haeussler M, Salma J, *et al.*: **Cell stress in cortical organoids impairs molecular subtype specification**. *Nature* 2020, **110**:20233-20284.
This manuscript found that the spatial organization of brain 'organoids' derived from human pluripotent stem cells changed over time. Initially, stereotyped neural progenitor clusters arose, encircled by differentiated neurons. However, after 2.5 months of culture, this spatial organization was lost.

21. Briscoe J, Small S: **Morphogen rules: design principles of gradient-mediated embryo patterning**. *Development* 2015, **142**:3996-4009.

22. Rogers KW, Schier AF: **Morphogen gradients: from generation to interpretation**. *Annu Rev Cell Dev Biol* 2011, **27**:377-407.

23. Sagner A, Briscoe J: **Morphogen interpretation: concentration, time, competence, and signaling dynamics**. *Wiley Interdiscip Rev Dev Biol* 2017, **6**:e271.

24. Stapornwongkul KS, Vincent JP: **Generation of extracellular morphogen gradients: the case for diffusion**. *Nat Rev Genet* 2021, **22**:393-411.

25. Lander AD: **Morpheus unbound: reimagining the morphogen gradient**. *Cell* 2007, **128**:245-256.

26. Sonnen KF, Merten CA: **Microfluidics as an emerging precision tool in developmental biology**. *Dev Cell* 2019, **48**:293-311.

27. Demers CJ, Soundararajan P, Chennampally P, Cox GA, Briscoe J, Collins SD, Smith RL: **Development-on-chip: in vitro neural tube patterning with a microfluidic device**. *Development* 2016, **143**:1884-1892.

28. • Rifes P, Isaksson M, Rathore GS, Aldrin-Kirk P, Moller OK, Barzaghi G, Lee J, Egerod KL, Rausch DM, Parmar M, *et al*.: **Modeling neural tube development by differentiation of human embryonic stem cells in a microfluidic WNT gradient**. *Nat Biotechnol* 2020, **38**:1265-1273.

This work created a sheet of human neural progenitors patterned along the anterior-posterior axis. In a microfluidic device, human pluripotent stem cells were exposed to a spatial gradient of a WNT agonist. Neural progenitors farther or closer to the WNT source respectively differentiated into anterior vs. posterior neural progenitors.

29. Cui KW, Engel L, Dundes CE, Nguyen TC, Loh KM, Dunn AR: **Spatially controlled stem cell differentiation via morphogen gradients: a comparison of static and dynamic microfluidic platforms**. *J Vac Sci Technol A* 2020, **38**:033205.

30. •• Repina NA, Bao X, Zimmermann JA, Joy DA, Kane RS, Schaffer DV: **Optogenetic control of Wnt signaling for modeling early embryogenic patterning with human pluripotent stem cells**. *bioRxiv* 2019,.

This study created an optogenetic system to turn on WNT signaling in human pluripotent stem cells, using blue light. Shining blue light onto one side of a human pluripotent stem cell culture selectively induced primitive streak cells at that location. Using optogenetics, light can thus be wielded to differentiate specific cell-types at defined times and places.

31. • De Santis R, Etoc F, Rosado-Olivieri EA, Brivanlou AH: **Self-organization of human dorsal-ventral forebrain structures by light induced SHH**. *Nat Commun* 2021, **12**:6768.

This manuscript reported an optogenetic system to turn on the expression of signaling ligand SHH in human pluripotent stem cells, using blue light. Shining blue light onto one side of a neural progenitor culture selectively generated ventral neural progenitors near that location, with more distant cells instead becoming dorsal neural progenitors.

32. Hebert JM, Fishell G: **The genetics of early telencephalon patterning: some assembly required**. *Nat Rev Neurosci* 2008, **9**:678-685.

33. Johnson HE, Goyal Y, Pannucci NL, Schupbach T, Shvartsman SY, Toettcher JE: **The spatiotemporal limits of developmental Erk signaling**. *Dev Cell* 2017, **40**:185-192.

34. Sako K, Pradhan SJ, Barone V, Ingles-Prieto A, Muller P, Ruprecht V, Capek D, Galande S, Janovjak H, Heisenberg CP: **Optogenetic control of nodal signaling reveals a temporal pattern of nodal signaling regulating cell fate specification during gastrulation**. *Cell Rep* 2016, **16**:866-877.

35. Noji S, Nohno T, Koyama E, Muto K, Ohyama K, Aoki Y, Tamura K, Ohsugi K, Ide H, Taniguchi S, *et al*.: **Retinoic acid induces polarizing activity but is unlikely to be a morphogen in the chick limb bud**. *Nature* 1991, **350**:83-86.

36. Niswander L, Tickle C, Vogel A, Booth I, Martin GR: **FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb**. *Cell* 1993, **75**:579-587.

37. Pourquie O, Fan CM, Coltey M, Hirsinger E, Watanabe Y, Breant C, Francis-West P, Brickell P, Tessier-Lavigne M, Le, Douarin NM: **Lateral and axial signals involved in avian somite patterning: a role for BMP4**. *Cell* 1996, **84**:461-471.

38. •• Cederquist GY, Asciolla JJ, Tchieu J, Walsh RM, Cornacchia D, Resh MD, Studer L: **Specification of positional identity in forebrain organoids**. *Nat Biotechnol* 2019, **37**:436-444.

This study generated 3-dimensional human brain spheroids patterned along the dorsal-ventral axis. In an innovative approach, SHH-secreting human pluripotent stem cells were positioned on one end of the spheroid, therefore creating a SHH gradient. Ventral and dorsal neural progenitors respectively arose at different locations along the SHH gradient.

39. • Li P, Markson JS, Wang S, Chen S, Vachharajani V, Elowitz MB: **Morphogen gradient reconstitution reveals Hedgehog pathway design principles**. *Science* 2018, **360**:543-548.

In this work, synthetic SHH gradients were created *in vitro* by positioning SHH-secreting fibroblasts at one end of a cell culture. Synthetic morphogen gradients provided a powerful experimental system to analyze gradient generation and response in ways hitherto difficult to achieve *in vivo*. It was found that SHH signaling leads to negative feedback that attenuates the gradient, revealing complexities with natural signaling pathways that might be circumvented using artificially-engineered signaling pathways.

40. •• Stapornwongkul KS, de Gennes M, Cocconi L, Salbreux G, Vincent JP: **Patterning and growth control in vivo by an engineered GFP gradient**. *Science* 2020, **370**:321-327.

This pathbreaking study converted extracellular GFP into a synthetic morphogen *in vivo*. Secreted GFP molecules formed spatial gradients and could bind to artificial GFP receptors expressed on the cell surface. By coupling these GFP receptors to BMP signaling, it was possible to deploy secreted GFP to create spatial gradients of BMP signaling *in vivo*.

41. •• Toda S, McKeithan WL, Hakkinen TJ, Lopez P, Klein OD, Lim WA: **Engineering synthetic morphogen systems that can program multicellular patterning**. *Science* 2020, **370**:327-331.

This innovative study converted extracellular GFP into a synthetic morphogen *in vitro*. Cultured cells were engineered to secrete extracellular GFP, or to express cell-surface GFP receptors. These synthetic GFP receptors could be coupled to user-defined outputs, therefore enabling GFP signaling gradients to drive different types of cellular behaviors.

42. Warmflash A, Sorre B, Etoc F, Siggia ED, Brivanlou AH: **A method to recapitulate early embryonic spatial patterning in human embryonic stem cells**. *Nat Methods* 2014, **11**:847-854.

43. •• Zhang Z, Zwick S, Loew E, Grimley JS, Ramanathan S: **Mouse embryo geometry drives formation of robust signaling gradients through receptor localization**. *Nat Commun* 2019, **10**:4516.

This study illuminated the mysterious phenomenon of why BMP4, when added to colonies of human pluripotent stem cells, acts on peripheral cells but not inner cells. BMP receptors are basolaterally localized within cells. Therefore, BMP4 binds its receptors on peripheral cells, but cells within a colony cannot receive BMP4 from the overlying media.

44. Patthey C, Gunhaga L: **Specification and regionalisation of the neural plate border**. *Eur J Neurosci* 2011, **34**:1516-1528.

45. •• Britton G, Heemskerk I, Hodge R, Qutub AA, Warmflash A: **A novel self-organizing embryonic stem cell system reveals signaling logic underlying the patterning of human ectoderm**. *Development* 2019, **146**:dev179093.

This study reported a remarkably elegant approach to create spatially-patterned human ectoderm cultures. 2-dimensional cultures of human pluripotent stem cells were differentiated into ectoderm. Upon treatment with BMP4—which presumably acts only on outer cells—the outermost ectoderm differentiated into skin precursors, whereas the innermost ectoderm differentiated into neural precursors.

46. •• Karzbrun E, Khankhel AH, Megale HC, Glasauer SMK, Wyle Y, Britton G, Warmflash A, Kosik KS, Siggia ED, Shraiman BI, *et al*.: **Human neural tube morphogenesis in vitro by geometric constraints**. *Nature* 2021, **599**:268-272.

This manuscript beautifully exemplifies the power of BMP signaling gradients to create spatially-organized human ectoderm structures. 3-dimensional cultures of human pluripotent stem cells were differentiated into ectoderm. Upon treatment with BMP4—which presumably acts only on outer cells—the outermost ectoderm differentiated into skin precursors, whereas the innermost ectoderm differentiated into neural precursors.

47. Goodwin K, Nelson CM: **Mechanics of development**. *Dev Cell* 2021, **56**:240-250.

48. Vining KH, Mooney DJ: **Mechanical forces direct stem cell behaviour in development and regeneration**. *Nat Rev Mol Cell Biol* 2017, **18**:728-742.

49. Scarpa E, Mayor R: **Collective cell migration in development**. *J Cell Biol* 2016, **212**:143-155.

50. Wilkinson DG: **Interplay of Eph-Ephrin signalling and Cadherin function in cell segregation and boundary formation**. *Front Cell Dev Biol* 2021, **9**:784039.

51. Takeichi M: **Self-organization of animal tissues: cadherin-mediated processes**. *Dev Cell* 2011, **21**:24-26.

52. Clevers H, Loh KM, Nusse R: **Stem cell signaling. An integral program for tissue renewal and regeneration: wnt signaling and stem cell control**. *Science* 2014, **346**:1248012.

53. Kornberg TB, Roy S: **Cytonemes as specialized signaling filopodia**. *Development* 2014, **141**:729-736.

54. Toda S, Blauch LR, Tang SKY, Morsut L, Lim WA: **Programming self-organizing multicellular structures with synthetic cell-cell signaling**. *Science* 2018, **361**:156-162.

55. Takebe T, Sekine K, Enomura M, Koike H, Kimura M, Ogaeri T, Zhang R-R, Ueno Y, Zheng Y-W, Koike N, *et al*.: **Vascularized and functional human liver from an iPSC-derived organ bud transplant**. *Nature* 2013, **499**:481-484.

56. Workman MJ, Mahe MM, Trisno S, Poling HM, Watson CL, Sundaram N, Chang CF, Schiesser J, Aubert P, Stanley EG, *et al*.: **Engineered human pluripotent-stem-cell-derived intestinal tissues with a functional enteric nervous system**. *Nat Med* 2017, **23**:49-59.