Biomaterials Science

REVIEW

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Biomaterials-based approaches to model embryogenesis

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Understanding, reproducing, and regulating the cellular and molecular processes underlying human embryogenesis is critical to improve our ability to recapitulate tissues with proper architecture and function, and to address the dysregulation of embryonic programs that underlies birth defects and cancer. The rapid emergence of stem cell technologies is enabling enormous progress in understanding embryogenesis using simple, powerful, and accessible *in vitro* models. Biomaterials are playing a central role in providing the spatiotemporal organisation of biophysical and biochemical signalling necessary to mimic, regulate and dissect the evolving embryonic niche *in vitro*. This contribution is rapidly improving our understanding of the mechanisms underlying embryonic patterning, in turn enabling the development of more effective clinical interventions for regenerative medicine and oncology. Here we highlight how key biomaterial approaches contribute to organise signalling in human embryogenesis models, and we summarise the biological insights gained from these contributions. Importantly, we highlight how nanotechnology approaches have remained largely untapped in this space, and we identify their key potential contributions.

Introduction

The largely unexplored molecular and cellular mechanisms accompanying implantation are often referred to as the

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Valeria Caprettini

'black box' of human development.[1] Insights into post-implantation events are crucial to comprehend the cellular differentiation of embryonic stem cells (ESCs) accompanied by morphogenesis and growth of the embryo. In fact, a better understanding of embryogenesis (Box 1), more specifically related to the events occurring during gastrulation, can improve our ability to direct cell fate, thereby enabling the engineering of complex cellular architectures. This improved understanding is critical to dissect the complex signalling networks regulating development and to advance biomedical research for tissue regeneration and diseases of developmental derangement such as birth defects and cancer.[2] Recent decades have seen rapid and significant improvements in establishing approaches to model and dissect embryonic development. The rise of sophisticated lineage tracing animal models has played a crucial role in shedding light over cell fate, migration and arrangement through morphogenesis and identifying the key driving events that regulate gastrulation. Yet, investigating the early processes of human embryogenesis *in vivo* is challenging because of the difficulties in accessing and thus visualizing individual cells.[3] Furthermore, despite some conserved morphological landmarks between mammalian blastocysts, timing and molecular expression differences exist between species.[4] Therefore, the degree of relevance that knowledge obtained from animal models holds for humans remains uncertain.[5] For example, contrary to the mechanisms involved in mouse embryogenesis, fibroblast growth factor (FGF) signalling might not be required for hypoblast formation in human, thus suggesting that mechanisms occurring in mice do not directly translate to other mammals.[6–8]

Box 1 Embryogenesis

Embryogenesis is the process regulating the formation and development of the embryo. During embryonic development, the first differentiation event leads to the formation of the inner cell mass (ICM) surrounded by a layer of trophectoderm cells. Successively, the ICM shift towards one pole of the blastocysts and just before implantation it segregates in a 'salt and pepper' arrangement forming the embryonic ectoderm (epiblast) and the primitive endoderm (PE, hypoblast).[9] The resulting epiblast and hypoblast cell types are spatially arranged in two distinct tissues which undergo gastrulation to establish the three embryonic cell layers and sets the basic axes (anterior–posterior, dorsal–ventral and left–right) of the body. In mouse, the epiblast cells express Nodal signalling that acts on the adjacent visceral endoderm which in turn acts as a source of Wnt and Nodal antagonists, migrates towards the anterior domain and form the anterior visceral endoderm (AVE). As the AVE continues to secrete antagonists, an anterior–posterior gradient of Wnt and Nodal signals is established leading to the onset of gastrulation. A groove forms in the cranial end of the epiblast that elongates as epiblast cells proliferate and migrate to form the primitive streak. The epiblast cells experience epithelial to mesenchymal transition to ingress through the primitive streak forming the mesoderm and definitive endoderm.[10] The suppression of Nodal and Wnt signalling pathways by their antagonist in the anterior epiblast has proven to be essential for neural induction.[11] For a deeper and more exhaustive summary of the current understanding of the molecular and cellular mechanisms occurring during early human development we refer the reader to excellent recent reviews of the subject.[12,13]

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Ciro Chiappini

Glossary
Embryo – The early stages of growth, development and differentiation of a multicellular organism.
Blastocyst – A hollow spherical structure consisting of:
Inner cell mass – a pluripotent cellular mass within one side of the blastocyst's interior forming the embryo.
Trophoblast – the outer layer of the blastocyst giving rise to extraembryonic tissues.
Epiblast – One of the two layers forming the inner cell mass deriving the three germ layers.
Hypoblast – The other layer forming the inner cell mass which gives rise to the yolk sac.
Embryoid body – A spherical aggregate of stem cells that can differentiate towards multiple cell lineages.
Primitive streak – A temporary elongated depression marking the gastrulation site.

Germ layers – The three layers, namely endoderm, mesoderm and ectoderm, that commit to all adult tissues and organs formation as the embryo develops.

Gastruloids – *In vitro* 3D aggregates displaying key embryo-like features similar to those occurring after implantation.

Gastrulation – An early embryonic developmental process whereby the three germ layers are established.

Extracellular matrix – A 3D structural scaffold consisting of glycoproteins and growth factors among other macromolecules, providing external biochemical and physical support.

Autocrine signalling – A form of diffusible signalling secreted from one cell that binds to its own receptors or act on a neighbouring targeted cell of the same type.

Paracrine signalling – A form of diffusible signalling secreted from one cell type to a neighbouring targeted cell type.

Approaches using embryonic tissue explants improve access, visualisation and manipulation of single cells within certain limits, but either cannot address inter-species developmental variations or pose significant ethical issues when using human tissues. Culturing the human embryo *in vitro* in the absence of maternal tissues established important insights into the early post-implantation phases of human development. These studies displayed a series of key morphogenetic rearrangements which provided a new understanding of the self-organizing capacity of the human blastocysts.[14,15] However, the ethical guidelines of the Warnock report limit these experiments to pre-gastrulation stages.[16,17]

The rapid progress of organotypic embryonic models from human pluripotent stem cells (hPSCs) has contributed to otherwise inaccessible human-relevant insight into implantation and gastrulation events. The establishment of human ESC derived from blastocysts[18] has provided an alternative 'bottom-up' approach to studying embryogenesis. Additionally, the development of induced pluripotent stem cells (iPSCs) through the reprogramming of adult human fibroblasts, has served as a powerful tool to dissect the fate of pluripotent cells while significantly mitigating the ethical dilemma associated with the use of ESCs.[19,20] Both types of pluripotent stem cells can extensively proliferate, and upon manipulation of their environment, they can differentiate into the three embryonic lineages, namely the endoderm, mesoderm and ectoderm. Therefore, stem cells hold a great promise as models to recapitulate processes of early human embryology.[21] Indeed, the early use of uniformly distributed morphogens as isolated stimuli to direct embryogenesis in uniform hPSC cultures has provided some valuable insights into key regulatory mechanisms directing embryonic differentiation, but has often proved insufficient to dissect the orchestrated signalling associated with embryonic patterning.[22]

Improved design of the embryonic niche combining spatiotemporally regulated biochemical and biomechanical stimuli are emerging to shed light over the processes regulating axis determination. In fact, the self-organizing capacity of hPSCs needs to be complemented by specific physico-chemical cues to modulate cellular development in an organized, context-dependent manner. As such, self-organization and differentiation are dependent on local cues which comprise, among others, morphogen dynamics[23,24] and mechanical stresses.[25,26] Even if animal development mechanisms differ from the human ones, a number of vertebrate models suggest that some basic key signalling pathways responsible of determining cell fate and patterning are conserved, including Wnt, transforming growth factor (TGF-β) and FGF.[27] Yet, uncovering how the signalling activities are orchestrated in space and time to pattern the epiblast into the three germ layers is still an open challenge. Indeed, controlling differentiation in an organized manner towards the desired outcome is of paramount importance in hPSC research. Initial attempts investigating the morphogen gradients within the embryoid body *in vitro* consisted in the generation of ESC aggregates followed by exposure to Wnt3a morphogen. These ESC aggregates established the formation of the so-called embryoid bodies (EBs) with a spontaneous self-organizing gradients driving cells to an anteroposterior polarity and primitive streak characteristics.[28] These findings suggest that although external signals are required for activation, the ensuing processes are self-reinforcing after initiation. Small aggregates of mouse ESC and more recently human ESC stimulated by CHIR99021 (CHIR), a Wnt agonist, resulted in aggregate elongation and expression of markers resembling the embryonic tail bud. Furthermore, the transcriptional programmes of these gastruloids recapitulated the embryo-like spatiotemporal patterns of gene expression, hence mimicking the processes occurring during symmetry breaking and axial organization.[29–31] Undoubtedly, these 3D models are promising substitutes of embryos that will significantly contribute to expand our knowledge of early developmental processes. Nevertheless, fundamental differences persist between these advanced gastruloid models and early embryos. The lack of effective spatiotemporal regulation of stimuli that drive developmental signalling pathways plays a crucial role in the observed differences. Indeed, biomaterial sciences are providing key contributions to recapitulate the developmental niche by supplying these signals. Apart from providing the appropriate biochemical and biomechanical cues, biomaterials can mimic their spatial and temporal organization to recapitulate the stem cell microenvironment and induce the desired differentiation fate. Nanomaterials, thanks to their emergent properties yielding intrinsic multifunctionality, have the yet unfulfilled potential to provide unique contributions to control embryonic development through high resolution, spatiotemporally controlled and environmentally aware delivery of stimuli that regulate biogenesis. In this perspective, we summarise the advances that biomaterial-based approaches offer to the study of human embryonic patterning and highlight how inputs from nanotechnology can further evolve the field (Fig. 1).
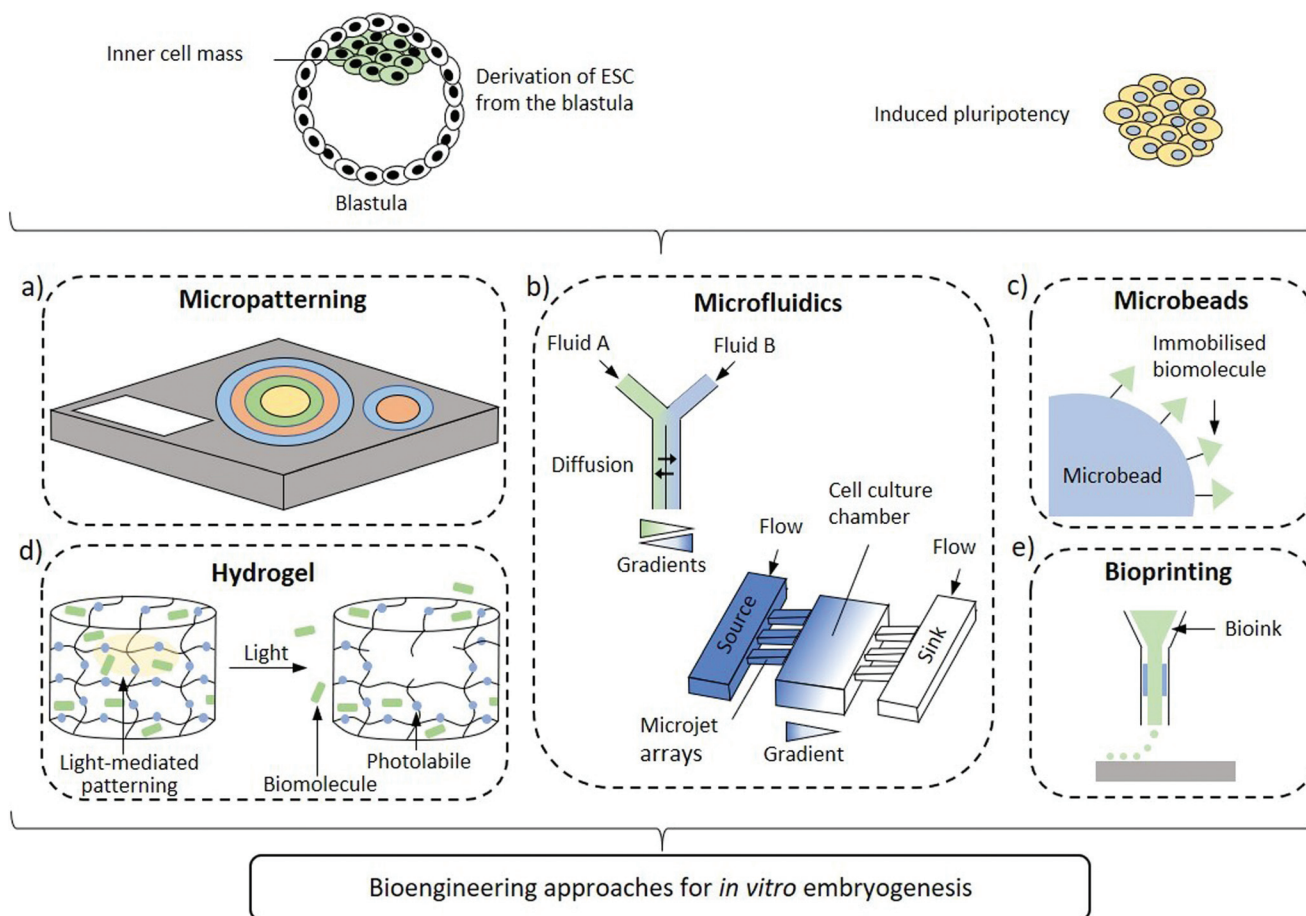
Fig. 1 Schematic representation of biomaterial approaches to organize signalling in models of embryogenesis. The cellular material employed in these models is typically embryonic stem cells derived from the inner cell mass of the blastocyst or induced pluripotent stem cells. (a) Manipulating and controlling the size and shape of the stem cell colony through a defined micropattern regulates the spatial arrangement of paracrine signalling to induce germ-layer specification (the different colours indicate the variation in the differentiation fate of the cells). (b) A Y-shaped (upper image) and a source-sink (lower image) microfluidic device can provide spatiotemporally organized gradient of soluble morphogens to direct the organisation of stem cell colonies. (c) Biomolecules can be immobilized on the surface of the microbeads providing immobilized cues to cells in contact with the beads, inducing differential responses. (d) Hydrogel systems with photolabile elements can 3D pattern biophysical and biochemical cues to mimic the spatial organization of the extracellular matrix and induce axial orientation. (e) Bioprinting allows assembling quasi-arbitrary arrangements of cells, biophysical and biochemical cues by additive manufacturing with bio-inks, potentially creating arbitrary 3-D models of developmental niches.

2D Micropatterning

Two-dimensional micropatterning can provide effective spatio-temporal control over topographic, biochemical and biophysical cues enabling orchestrating organisation in stem cell colonies. Confining hESCs *in vitro* within micropatterns of a defined shape and size (Fig. 1a) induces spontaneous, spatially ordered organisation of the colony and patterned differentiation which mimics aspects of embryonic development. Micropatterning can be achieved by topographic modification of the substrate using micro/nanostructures that confine and direct the arrangement and adhesion of cells, or by surface chemical modifications to locally enhance or prevent cell adhesion (Fig. 2a).[32,33] These approaches typically comprise photolithography, direct writing, microcontact printing, possibly alongside more exotic microtechnology approaches.[34,35]

Mouse epiblast-like cells cultured on micropatterned substrates supplemented with appropriate morphogen signalling (Wnt, BMP, ACTIVIN, FGF), induce differentiation events and a spatial arrangement of cell fate comparable to those occurring when establishing the mouse gastrula *in vivo*.[36] Geometrical confinement of pre-streak-like stem cells can control patterning of Brachyury (Bra)/T expression to guide the position of the primitive streak and attain asymmetric patterning of pluripotent stem cells. Indeed, growing cells on an ellipse micropattern instead of circular ones preferentially induces localised T-expressing cells at the tips of the ellipse rather than on the entire circumference (Fig. 2b). The simplicity, robustness, stringent control over key geometrical features and accessibility to molecular readouts of this approach highlights its transformative potential for investigating how geometric asymmetries – such as edges or elongations – in homogenous stem cells colo-
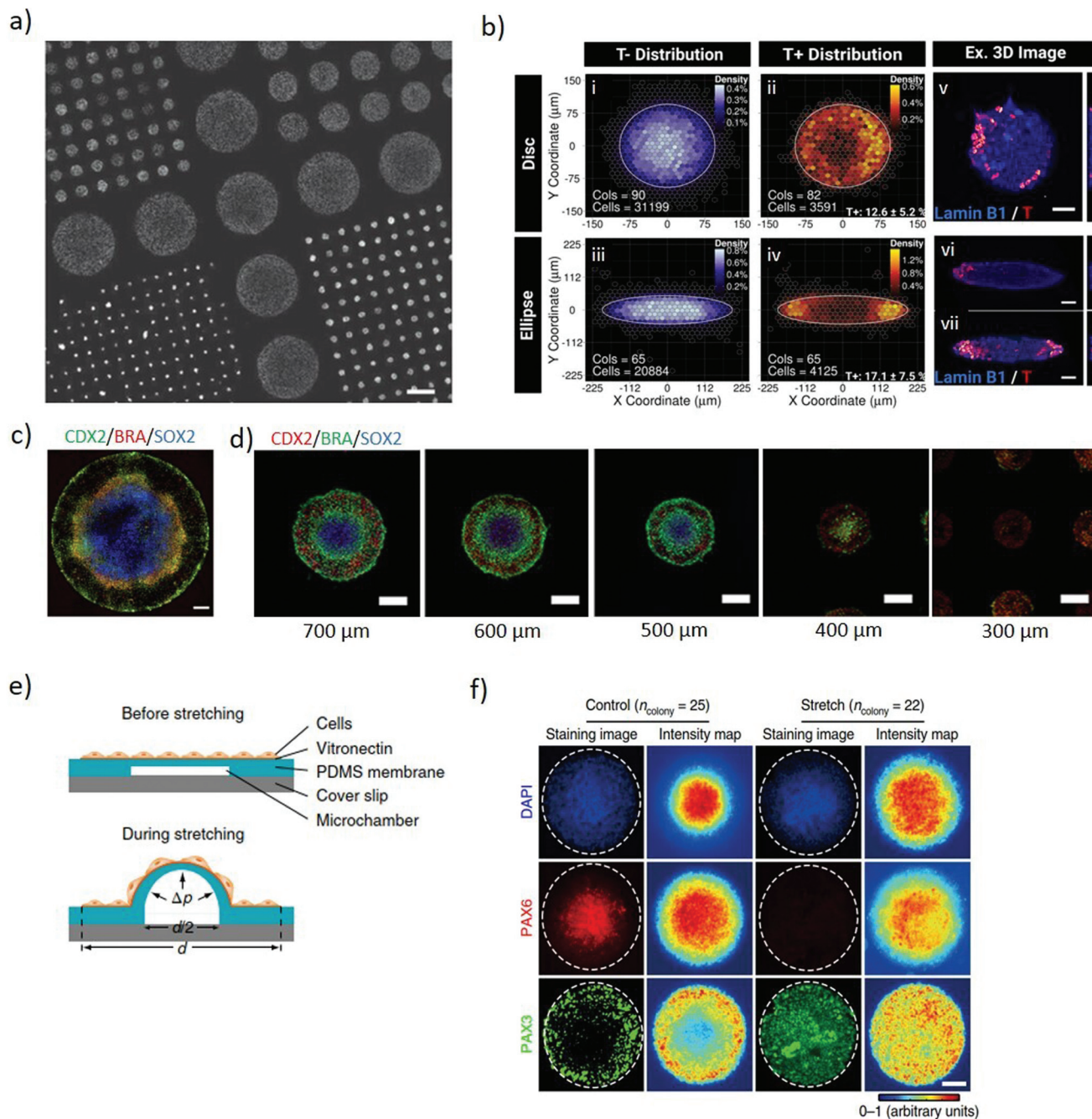
Fig. 2 Micropatterning stem cell colonies induces differential germ layer specification. (a) Glass coverslip coated with poly-D-lysine micropatterns of different diameters for selective hESCs adhesion to the functionalised regions. Adapted with permission.[39] Copyright 2014, Nature Publishing Group. (b) The geometry of the micropatterned colonies influences the pattern of Brachyury (T) expression. T expression identifies the position a pre-streak like population. Binned density maps (BDMs) images (i–iv) and confocal images (v–vii) show that brachyury positive cells (T+, red) are positioned at the tips of the ellipse micropattern whilst in the disc micropatterns, T+ cells are located on the entire circumference. Cols = number of colonies analysed; cells = number of cells analysed. Scale bars = 50 μm. Adapted with permission.[41] Copyright 2018, The Company of Biologists Ltd. (c) Immunofluorescence of micropatterned hESC treated with BMP4, displaying radial organization of the trophectoderm (CDX2) and germ layers markers: mesoderm (BRA) and ectoderm (SOX2). Scale bars = 100 μm. Adapted with permission.[39] Copyright 2014, Nature Publishing Group. (d) Immunofluorescence images showing pattern-size dependence of germ-layer domains. As size is reduced SOX2 expression declines and eventually disappears. Scale bars = 200 μm. Adapted with permission.[32] Copyright 2017, The Company of Biologists Ltd. (e) Schematic of the micropatterned cell colonies on locally-stretchable PDMS membrane. The central region of the patterned colony stretches upon expansion of the underlying microfluidic channel. (f) Immunofluorescence images and average intensity maps of unstretched (control) and mechanically stretched (stretched) colonies at the central portion at day 8. Staining the colonies with Pax6 (neuroepithelial cell marker) and Pax3 (neural plate border marker), suggest that mechanical stimulation plays a role in regulating neural plate border differentiation. White dashed lines mark the colony periphery. Scale bar = 100 μm. (e and f) Adapted with permission.[42] Copyright 2018, Nature Publishing Group.

nies influence the colony's response to morphogens and contributes to introduce heterogeneity in cell fate specification.

The size of 2D micropatterned hPSCs is a major determinant in the maintenance of pluripotency within a colony. When exposed to identical stimuli, circular larger colonies maintain pluripotency whereas smaller ones spontaneously differentiate over time. Possibly, the size of hESC colony influences the ratio of pSMAD1 agonist and antagonist expression, important for mediating self-renewal and differentiation. The higher local cell density present in large colonies correlates with higher concentrations of autocrine/paracrine morphogen signals, such as the BMP antagonist GDF3, and induces repression of SMAD1 leading to retaining expression of the pluripotency marker, Oct4. In contrast, small colonies express excess pSMAD1 agonists favouring differentiation.[37] When stimulated with BMP2 and activin A, large colonies tend to differentiate towards the mesoderm whilst small colonies differentiate towards the endoderm lineage.[38] Uniform soluble BMP4 stimulus applied to patterned circular hPSCs colonies induces concentric differentiation towards the germ layers. The inner portion of the pattern acquires endodermal specification, surrounded by a mesodermal ring, itself encircled by ectoderm whilst the outer layer of the colony acquires a trophectoderm-like phenotype colony (Fig. 2c).[39] Size plays an important role for these micropatterns, with the outer ectodermal and mesodermal regions largely independent of the overall colony size, while the inner endodermal specification occupying the remaining of the pattern. Indeed, small colonies of less than 250 μm diameter do not exhibit significant mesodermal or endodermal specification, which progressively arises as the size of the pattern expands (Fig. 2d).[32,39] This approach enables investigating the interplay between BMP4 and its inhibitor Noggin and the localization of the BMP4 receptors along a radial symmetry. The sensitivity of BMP4 is reduced from the edge to the centre of the colony, both due to the radial changes in localization of the BMP receptors from the apical to the basolateral surface of the cells and because of differential Noggin activation and diffusion.[40] These systems indicate that the size and shape of micropatterns influence the response of cells to uniform diffusible molecules thus establishing local gradients of biochemical cues which induce controlled pattering within stem cell colonies. Identifying that asymmetric micropatterns lead to breaking the radial symmetry suggests a means to further study and secure the positional robustness of the streak.[41]

Two dimensional micropatterning also enables investigating neuroectoderm patterning. Neural induction of circular micropatterned hPSC colonies induces concentric arrangement of the neural plate border and the neural crest, in accordance with their arrangement during mouse embryo development. Isolated stretching of the central portion of the pattern highlights a local activation of BMP-SMAD signalling in response to mechanical stimulation (Fig. 2e), suggesting its role in the regulation of neural plate border differentiation (Fig. 2f). Overall, this system enables studying the mechanisms inducing neuroectodermal specification with a potential to advance our understanding of neural development and its associated disorders.[42] Furthermore, the spatiotemporal regulation of 2D patterns shows promise as a screening platform for teratogenicity. Mesoendoderm generated from micropatterned hPSC treated with known teratogens displays disrupted patterning in a dose-dependent manner, highlighting the role of the drugs in inducing developmental disorders.[43]

Micropatterning is a reductionist approach that enables isolating and controlling the role of geometrical parameters to study their impact on signalling networks responsible for differentiation and morphogenesis during development. This highly controllable and simple two-dimensional approach shows promising potential for recapitulating key aspects of the early stages of embryogenesis and gastrulation but does not provide a simple avenue to model the temporal regulation of morphogenic cues that happen *in vivo*. In fact, it is typically challenging and unreliable to modify micropatterns over time after the colony has been established, or to trigger a precise gene expression or inhibition in a time-resolved fashion. This has currently limited micropatterning approaches to probe individual signalling pathways during one key developmental event, hampering more comprehensive investigations of large signalling networks across the developmental timeline.

Microfluidic devices

Soft lithography of silicones by replica moulding – among which polydimethylsiloxane (PDMS) is the most explored – is a largely established approach for rapid prototyping of microfluidic devices, thanks to its low-cost, reliability and ease of processing.[44] Microfluidics can combine gradients of soluble factors, providing fine control over the spatial and temporal arrangement of morphogens,[45] with technical limits on the number of simultaneous gradients available and their individual spatiotemporal complexity (Fig. 1b). Furthermore, regulating fluid flows allows improved control of nutrients exchange, culture conditions and shear stimuli. This increased complexity in the regulations of cues requires elaborate experimental setups, but can provide the improved versatility necessary to dissect interconnected signalling and events.[46]

Continuous flows can wash autocrine and paracrine factors away from cells in a regulated fashion, hence enabling quantitative investigation of the secreted factors involved in differentiation.[47,48] This approach allows investigating the role of FGF4 for neuroectodermal specification in mouse ESCs. Microfluidic delivery of FGF4-supplemented differentiation medium to mESCs is insufficient to induce neuroectoderm when autocrine and paracrine cell-secreted factors are washed away from the system. Instead, the presence of cell-secreted factors in addition to FGF4 recovers neuroectodermal differentiation, highlighting that FGF4 is necessary but not sufficient to establish neuroectodermal fate, thereby providing insight in manipulating diffusible signalling.[48] Approaches based on T or Y-shaped laminar flow systems can introduce solutions with different concentrations of desired biochemicals in each side

branch, establishing a gradient in the main branch by lateral diffusion. However, as perfusion depletes the autocrine and paracrine factors essential for cell growth, survival or differentiation, continuous flow restricts the study of self-organizing systems. Besides, the continuous laminar flow generates shear stresses that can affect cell behaviour in unpredictable ways.[49] Thus, diffusion-based devices are preferable as they avoid active perfusion of the culture. These systems rely on Fickian diffusion to establish gradients over a central culturing chamber interconnected with reservoirs containing desired concentrations of stimulants.[50] Such systems can establish stable, opposing linear concentration gradients of multiple signalling molecules, such as sonic hedgehog (SHH) and FGF/BMP, generated using side channels under laminar flows as reservoirs in order to regulate neuronal differentiation.[51,52] Exposing neural progenitors to these gradients stimulates a patterned neuronal differentiation with analogies to the dorsoventral specification of the neural tube. A four-channel setup of this system (Fig. 3a) can further introduce opposing retinoic acid and possibly FGF gradients to mimic the distribution of key anteroposterior specification morphogens alongside the dorsoventral SHH/BMP gradients (Fig. 3b).[52] Indeed, these systems can induce dorsoventral and anteroposterior patterning as a response to the opposing morphogenic gradients.[53]

Microfluidics is a key element of a platform that recapitulates the dorsal–ventral patterning of hPSCs epiblast-like cysts (Fig. 3c), showing landmarks of the human epiblast development and recapitulating amniotic ectoderm differentiation with the specification of primordial germ cells. The loaded ESCs reside into gel pockets within a microfluidic device, where they grow to establish a lumen, developing into epiblast-like cysts (Fig. 3d). In the presence of BMP4, the pole of the cysts exposed to BMP4 differentiates into amniotic ectoderm-like cells (AMLC) with an epiblast-like epithelium at the opposite pole not exposed to BMP4 (Fig. 3e), hence resembling a human embryonic sac before its gastrulation onset. Provided that Nanog concentration is low, the cysts acquire a prospective posterior end of the epiblast (PrePS-EPI) phenotype and thus the asymmetric sacs are referred to as posteriorized embryonic-like sacs (P-ELS). Instead, in the presence of Noggin and IWP2 (Wnt-inhibitor) stimuli the cysts retain AMLC patterning on one pole but develop a more organised epiblast-like pole, and become anteriorized embryonic-like sacs (A-ELS) (Fig. 3f).[54] To investigate the anterior–posterior symmetry events, microfluidic systems can expose 2D colonies of human pluripotent stem cells to gradients of BMP4 (Fig. 3g) and investigate the influence of cell density and morphogen concentration on the patterning outcome. A low initial cell density combined with the BMP4 gradient can induce an axial arrangement of the germ layers, while a high cell concentration combined with BMP4 and opposing Noggin gradient result in characteristic markers of the trophectoderm (CDX2), mesoderm (MIXL1, T) and endoderm (SOX17) towards the BMP4 source, and ectoderm (SOX2) present throughout the colony except near the source (Fig. 3h).[55]

Microfluidics also enables shuttling and trapping multiple EBs at precise locations and controlling their interaction, while exposing them differentially to desired combinations of morphogens. With such an approach it is possible to first trap BMP4-free EBs and then direct BMP4-treated EBs within gelatin microparticles towards the trapped EBs in order to induce their fusion. The fused EBs express spatially controlled mesoderm differentiation markers, providing a mean to manipulate and enhance precision in patterning EBs.[56]

Overall, microfluidics is a powerful tool to control the exposure to morphogens in space and time, which can effectively regulate spatial patterning of cells in 2D and 3D systems alike. These devices can develop organ-on-a-chip and organoid models that emulate key multicellular structures and physiology across key early developmental events. Despite these crucial features, handling 3D systems within microfluidic devices remains challenging, due to diffusion barriers in flow leading to uneven distribution across the tissues. Furthermore, the efficiency of long-term stem cells culturing remains highly variable and there is limited space for tissue development, whilst increasing the channel dimensions and flow rates obliviate the key microfluidic advantage of laminar flow.[46,55] Moving forward, platforms integrating microfluidics and hydrogels promise comprehensive control over the biophysical and biochemical niche enabling more accurate modelling of early developmental events in 3D cultures to model embryogenesis. Hydrogels demonstrate a strong potential in tailoring the physicochemical properties of the extracellular matrix in space and time in order to enable *in vitro* modelling, while microfluidics provides sophisticated control over soluble cues. A hydrogel precursor injected into a PDMS mould followed by gelation generates a hydrogel chip with microchannels. The surface topography of the hydrogel where cells are deposited is fabricated for flat cell cultures or spheroid-based cultures. Exposing mouse EBs to retinoic acid (RA) gradients under spatial and temporal control within this systems results in their neural differentiation, with those EB close to the RA source growing significantly larger.[57] Embedding mouse embryoid bodies in a collagen matrix and subjecting the EBs to combined gradients of RA and smoothened agonist (SAG), an SHH activator, creates a range of different microenvironment conditions within the scaffold. The combinatorial effect of the morphogens induces a graded response of motor neurons differentiation in regions of high retinoic acid concentrations similar to *in vivo* situations.[58]

Microbeads or microspheres

Immobilizing factors to solid substrates (Fig. 1c) enables investigating the role of signalling that originates from the extracellular matrix as well as induce spatial organization to both matrix and soluble-like cues. The organisation of signalling enables investigating heterogeneity in differentiation and unravelling asymmetric division within the embryoid body. Local presentation of leukaemia inhibitory factor (LIF) to ESCs
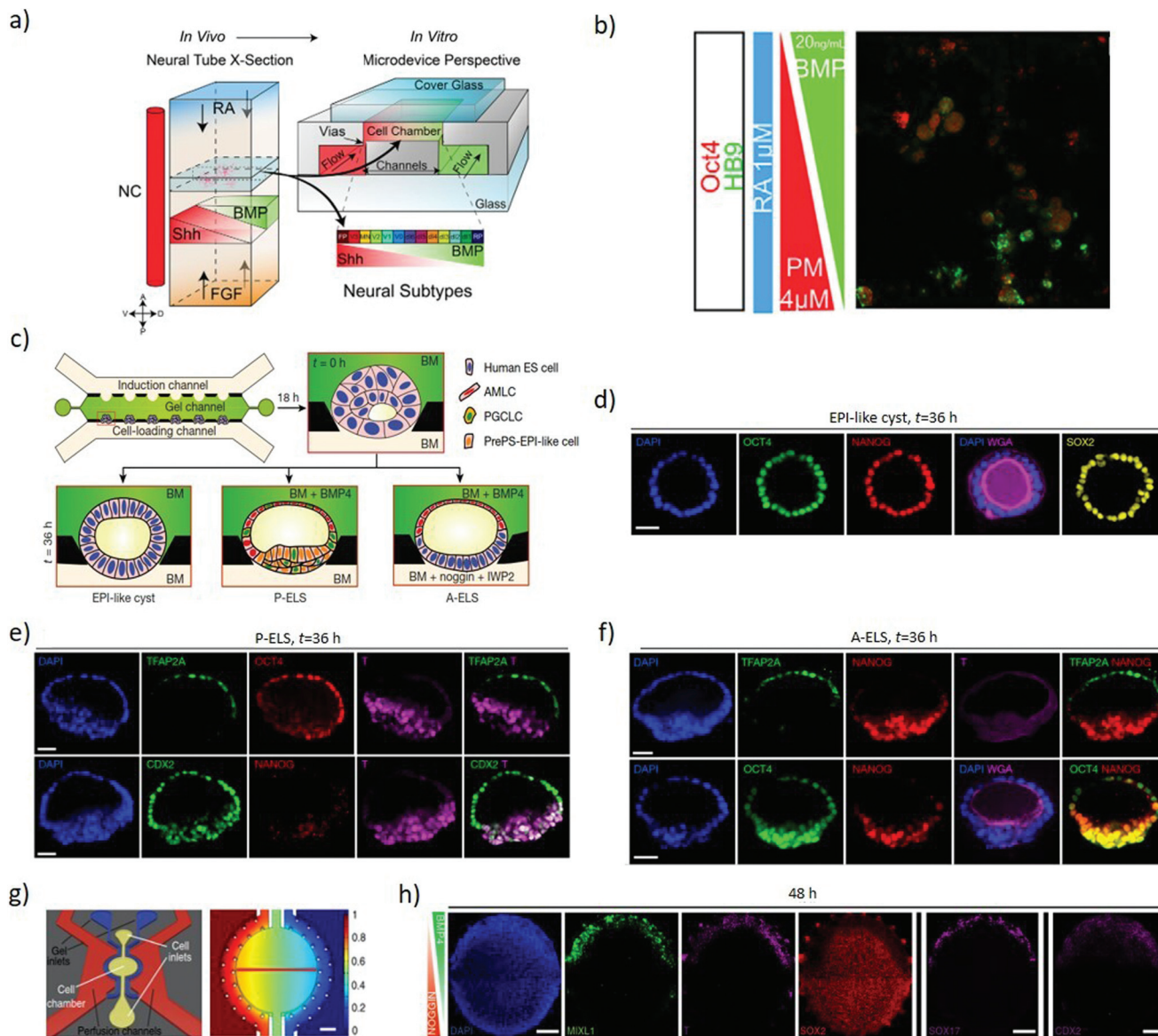
Fig. 3 Microfluidics devices establish gradients of morphogens to induce axial specification. (a) Schematic representation of the four primary signals (RA, FGF, SHH and BMP) directing neural tube patterning *in vivo* and the four-channel setup developed to recapitulate the gradients of these signals *in vitro* through a source and sink approach. (b) ESCs with HB9-GFP (a gene marker for post-mitotic motor neurons) exposed to opposing BMP4 and PM (SHH analogue) gradients, in the presence of RA. The combined gradients induced dorsoventral patterning as evidenced by localized OCT4 expression (dorsal marker) at the BMP side and HB9 expression (neuronal specification marker) at the PM side. (a and b) Adapted with permission.[52] Copyright 2016, The Company of Biologists. (c) A schematic representation of multi-channel microfluidic device with the middle channel preloaded with Geltrex forming concave pockets to host cells and provide an asymmetric environment. The lower channel is used to load cells and flow base medium while the upper channel is used to flow desired concentrations and temporal patterns of chemical inducers. (d) Confocal images showing the lumen developed within the Oct4, Nanog and Sox2 stained epiblast-like cysts. (e) Confocal images showing TFAP2A (a putative amniotic ectoderm marker) detected exclusively at the pole exposed to BMP4. The micrographs represent P-ELS formation as the epiblast-like cells at the opposite pole show the presence of CDX2 and T but a reduction in Nanog. (f) The micrographs represent A-ELS formation with more organized Nanog and Oct4 staining and diminished T staining. (c–f) Adapted with permission.[54] Copyright 2019, Nature Publishing Group. (g) Left to right: Schematic of the individual unit within the microfluidic device; and computational simulation of the gradient generated within the active chamber after 48 hours of perfusion. (h) Immunofluorescence analysis of high density hESC colonies exposed for 48 hours to opposing BMP4 (50 ng mL^{-1}) and Noggin (200 ng mL^{-1}) gradients. The colony shows markers of the trophectoderm (CDX2), mesoderm (MIXL1, T) and endoderm (SOX17) at the BMP4 source side, and ectoderm (SOX2) throughout the colony except near the BMP source side. Two rightmost panels display different replicates. Scale bars = 200 μm. (g and h) Adapted with permission.[55] Copyright 2019, Nature Publishing Group.

as an immobilized ligand maintains ESC pluripotency for at least 2 weeks, which is not achievable with soluble LIF. This approach can apply to a range of factors maintaining pluripo-

tency or directing differentiation.[59] Wnt coated microbeads can present asymmetric signalling to individual mouse ESC. Cell division in the presence of such asymmetric signalling
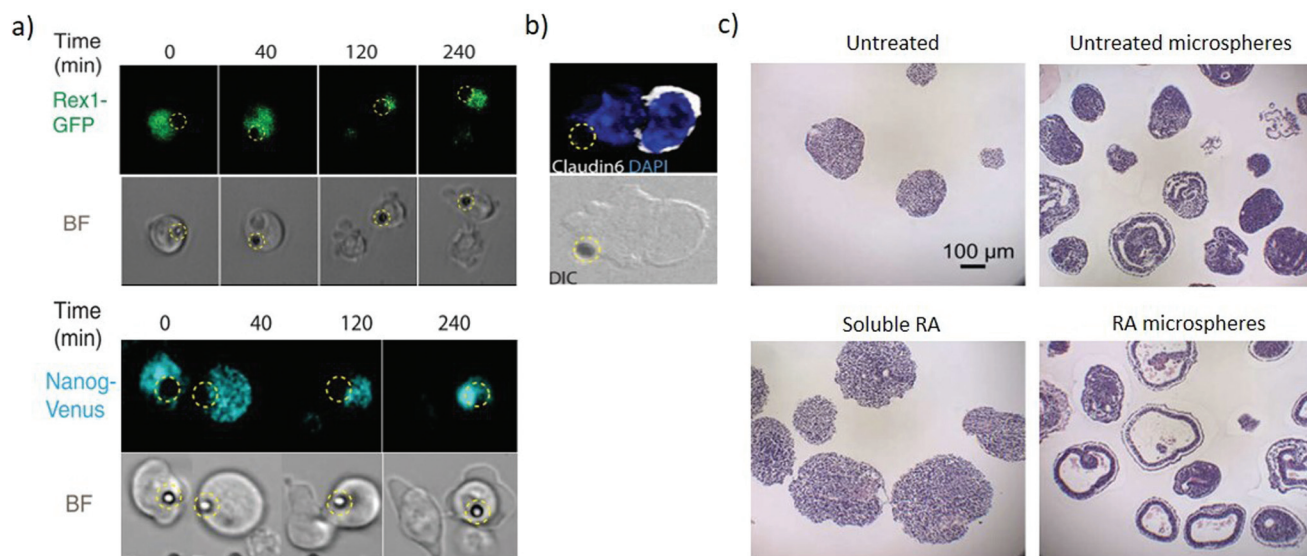
Fig. 4 Microbeads provide spatiotemporally regulated display of morphogens. (a) Pluripotency genes expression of Rex1-GFP and Nanog-Venus of selected frame from time-lapse imaging during ES cell division in the presence of Wnt3a beads (represented by the dashed yellow circle). The pluripotency markers are asymmetrically expressed with high expression in the proximal daughter cell than in the distal one. (b) The distal daughter cells expressed Claudin6 as an epiblast stem cell marker suggesting that the cell is differentiating. (a and b) Adapted with permission.[60] Copyright 2013, American Association for the Advancement of Science (AAAS). (c) Comparison analysis of the cystic organization of embryoid bodies after 10 days of differentiation under the influence of soluble or microsphere-released RA. Untreated and RA-free microspheres as controls. RA-loaded microspheres induce large cysts within EBs not present when EBs are exposed to soluble RA. Adapted with permission.[63] Copyright 2009, Elsevier Ltd.

yields pluripotent daughter cells at the Wnt microbead (Fig. 4a) and differentiated cells away from the microbead (Fig. 4b), enabling investigating the mechanism of asymmetric cell division in ESCs.[60,61]

Biodegradable microspheres enable spatiotemporal control of biomolecule release contributing to recapitulate their regulation during early development.[62,63] Poly(lactic-co-glycolic acid) (PLGA) microspheres loaded with RA can be incorporated within embryoid bodies. As the microspheres degrade, the RA induces highly organized cystic regions in a dose-dependent manner, since microspheres with low RA levels (0.3 μg mg^{-1} PLGA) display fewer cystic EBs than microspheres loaded with high RA levels (30 μg mg^{-1} PLGA). Interestingly, no cysts are observed across several concentrations of soluble RA (Fig. 4c), hence demonstrating the need for the spatially controlled presentation of the morphogen. The cystic spheroids phenotypically resemble the early streak mouse embryos (E6.75) and could be a useful model to investigate the role of secreted morphogenic factors during early differentiation events.[63] Microfluidics approaches can also provide sustained release and gradients of soluble factors, however, currently they require significantly higher complexity and can only generate concentration gradients from outside the cell colony. Contrastingly microbeads enable inside-out gradients with spherical symmetry in a simple package. The material composition of the bead itself also influences cell fate, with equally sized microbeads of agarose, PLGA and gelatin influencing the differentiation proportions in PSCs 3D aggregates.[64] These findings suggest that tailoring the physical properties of a

bead can influence signalling to the stem cell aggregate and hence direct differentiation.[65]

Hydrogel and patterning

Bi-dimensional cultures can only mimic a part of the complex and highly three-dimensional scenario that gives rise to the development of a human embryo. Preliminary reports indicate that human ESCs grown on patterned substrates can originate asymmetrical 'gastrulation-like' nodes, a model that could dissect the influence of tension and confinement on regulating early development *in vitro*.[66] Importantly, human ESC cultured in disc-shaped colonies within modified polyacrylamide hydrogels exhibit stiffness-dependent response to Wnt stimulation while retaining stiffness-independent self-renewal potential. Taken together these results demonstrate that the mechanical properties of the microenvironment alter the cellular response towards biomolecules influencing the differentiation potential, hence, such an approach can be useful in identifying mechanosensitive molecules and mechanisms regulating embryonic development.[67] Extracellular interactions have a substantial influence on morphogenesis, differentiation and proliferation of cells as is well observed in 3D somatic cell reprogramming towards iPSCs.[68] Therefore, recapitulating the spatiotemporal milieu of cell–cell interactions and extracellular matrix *in vitro* are key components needed to properly regulate stem cell fate.[69] Three-dimensional microarrays enable high-throughput screening of matrix-initiated cues by combi-

naturally testing hydrogels parameters (composition, stiffness, bioactivity) to identify their role in stem cell regulation.[70] In particular, mechanical forces within the microenvironment are known to regulate embryonic organization, and controlling hydrogel stiffness is a simple approach to regulate the mechano-environment of *in vitro* models of embryonic development. Indeed, controlling stiffness in fibrin gels regulates the establishment of concentric, organized formation of endoderm, mesoderm and ectoderm in 3D colonies, highlighting that colony-matrix interactions, as demonstrated by the variations in the stiffness, have a significant impact on the spatial patterning of the germ layers.[71] These findings indicate that mechanical and biochemical cues act synergistically to recapitulate embryogenic-like processes.

Dissecting the chemical and the physical cues guiding mammalian embryogenesis provides a lead to model early developmental events. Indeed, emerging hydrogel approaches enable independent regulation of stiffness, degradation and biochemical cues density allowing improved mimicking of *in vivo* niches.[72] Besides controlling the biophysical environment, hydrogels play a crucial role in presenting matrix-bound biochemical cues, which complement the role of soluble factors. Matrigel can provide support for the co-culture of 3D aggregates of mouse embryonic and extraembryonic trophoblast stem cells enabling their mutual interaction (Fig. 5a). This leads to their fusion and co-development of a common lumen directed through Nodal signalling secreted by the embryonic stem cell compartment (Fig. 5b). Analysis of the symmetry breaking events in the embryo suggests that, unlike embryonic stem cells alone, the presence of the trophoblast stem cell compartment induces regionalized cells of mesoderm lineage which also express mesenchymal markers. Furthermore, Wnt signalling is essential for the expression of mesoderm markers and BMP signalling induces primordial germ-like cells at the boundary between the two compartments.[73] The Matrigel provides cues that partially compensate for the visceral endoderm, which is necessary for the mouse ESC to progress towards lumenogenesis and epiblast polarization.[74] Further development leads to the asymmetric induction of mesoderm markers similarly positioned to that of the natural embryo.[73] Neural tube induction in 3D systems is possible by culturing single mouse ESCs suspensions in Matrigel under neural induction conditions where the cells differentiate along the neural lineage forming apical-basal neuroepithelial cysts with a single lumen. Remarkably, the addition of high concentrations of RA achieves neural tube dorsoventral patterning. PEG hydrogels, which ablate matrix biochemical cues yield similar results although to a lesser extent, indicating that matrix composition is not an essential requirement for such patterning.[75] Further investigations on the conditions driving neural tube patterning focused on the combinatorial dissection of cues in the cell microenvironment, allowing elucidating

Fig. 5 Hydrogel-based approaches to induce asymmetric embryonic microenvironments. (a) Schematic representation of the set-up used to mimic the epiblast architecture. Small clumps of trophoblast stem cells and single embryonic stem cells are suspended in Matrigel in order to establish a dialogue between them through their mutual interaction in a 3D extracellular matrix scaffold. The Matrigel is allowed to solidify followed by culturing in a suitable ETS-embryo medium for the stem cells types to fuse and codevelop into a common lumen. (b) Mouse embryonic stem cells and extra-embryonic trophoblast stem cells co-embedded in Matrigel according to (a) interact and codevelop into a common lumen (top panel) resembling the blastocyst stage of mouse (bottom panel), as represented by the ETS-embryo images (upper panel). Staining includes Oct4 as embryonic marker, EOMES as extraembryonic marker and DAPI nuclear counterstaining. Dashed line outlines the cavity. Scale bars = 30 μm. (a and b) Adapted with permission.[73] Copyright 2017, American Association for the Advancement of Science (AAAS). (c) Schematic representation of light-mediated patterning for spatially-resolved regulation of mechanical and biochemical signals. Photolabile crosslinkers cleave upon light interaction to locally soften the gel matrix. (d) Bioligands bound to the hydrogel with the active group masked by a photo-sensitive caging group are activated upon exposure to light, leading for patterned tethering of biomolecules. (c and d) Adapted with permission.[82] Copyright 2014, The Company of Biologists.

the role of stiffness and extracellular matrix (ECM) composition on neuroepithelial cyst patterning.[76] Therefore, this tractable approach enables deciphering some of the complexity that exists in the developmental processes.

A 3D human ESCs model can achieve anteroposterior symmetry breaking in the absence of extra-embryonic tissues. A single-cell suspension-cultured into polymeric hydrogels supplemented with Matrigel and a precisely controlled uniform dose of soluble BMP4 induces regionalized expression of molecular signatures of the germ layers. In this system, low BMP4 concentrations (0.1 ng mL^{-1}) yield SOX2+ expressing cells whilst high concentrations (5 or 10 ng mL^{-1}) result in morphological and molecular properties changes of the cells, fitting the description of trophectoderm-like cells. Intermediate levels (1 ng mL^{-1}) cause half of the colonies to generate a spatially separated expression of SOX2+ and BRA+ cells, serving as a human model for studying the mechanism leading towards anteroposterior symmetry breaking.[77] Apart from modelling post-gastrulation processes, seeding the human pluripotent stem cells at the interface between a Geltrex ECM solution and soft gel bed enables modelling aspects of peri-implantation amniogenesis, a pre-gastrulation event. The physical cues from the soft gel are sufficient to induce squamous cysts, since reducing the thickness of the soft gel bed increases its rigidity and inhibits cysts development. However, in the absence of maternal and extraembryonic biochemical cues, only the combination of soft gel bed and Geltrex ECM induces a combination of transcription factors that resemble differentiation towards amnion-like tissue.[78] Concurrently, a small fraction of cysts show asymmetric differentiation with amniotic ectoderm on one side and an opposing epiblast-like pole, resembling the post-implantation amniotic sac. In addition, post-implantation amniotic sac embryoids proceed to develop molecular hallmarks of the posterior primitive streak.[79]

Light-mediated patterning of hydrogels improves control over the spatiotemporal regulation of cues. Light patterning interacts with photosensitive chemical groups within hydrogels and can induce reversible or irreversible mechanical or chemical changes based on the light pattern (Fig. 1d).[80–82] Patterning in the presence of photoinitiators can mediate increase in hydrogel stiffness by triggering additional local crosslinking within the gel.[83] In contrast, hydrogels with embedded photolabile chemical groups are cleaved upon light irradiation, resulting in local softening of the gel (Fig. 5c).[84,85] Additionally, biochemically patterned hydrogels provide fine 3D control over the distribution and availability of the signals to the cells through light-mediated approaches[86] whereby the molecules are presented or removed through photocaging and photocleaving respectively. In photocaging, the bioligand is bound to the hydrogel with its active site masked by a photodegradable moiety and becomes activated upon light irradiation (Fig. 5d).[87] Orthogonal photopatterning enables generating photoreversible hydrogels where bioactive proteins are first conjugated to the hydrogel by photopatterning and subsequently removed through the same mechanism, enabling sophisticated spatiotemporal control of extracellular matrix

cues.[88] These approaches could improve the localization of extracellular cues for differentiation which can promote symmetry-breaking events with spatial control. Nonetheless, many challenges remain to be addressed including identifying hydrogels that reversibly respond to light and improving the efficiency of visible-light photo-reversible and photolabile chemical groups to ensure integrity of the biomolecules and cytocompatibility.[87]

Although bioconjugation allows effective functionalization of biomolecules, it often reduces their biological activity. Non-covalent binding can provide effective biofunctionalisation with improved biomolecular activity. Several growth factors have a high affinity for heparin and micropatterned dual-cross-linked alginate/heparin hydrogels provide a means to sequester those growth factors in designated spatial locations. The immobilized growth factors can direct migration and differentiation of human mesenchymal stem cells (MSC) encapsulated within the micropatterns.[89] However heparin-based systems are subjected to batch-to-batch variability which can hamper their application.[90]

Hydrogel-based approaches are a versatile tool to study complex and dynamic biological processes and can be applied to investigate phenomena occurring during early embryogenic development. While offering support to the culture, hydrogels can accurately orchestrate the extracellular chemical and physical cues in space and time, mimicking those *in vivo*. Thanks to the flexibility and ease of use of these approaches it is possible to combinatorially explore the matrix microenvironment during development to identify its effect on embryogenic events and processes.

Bioprinting

Bioprinting is a bottom-up approach to assembling tissues by precisely depositing bioinks, composed of living cells and biomaterials, onto a substrate thus crafting an artificial 3D architecture to mimic physiological organization (Fig. 1e).[91] Crucially, bioprinting is a complex process with low throughput and variable cytotoxicity that allows for extremely precise spatial manipulation of cell arrangement.[92] Broadly available bioprinting strategies include inkjet and microextrusion bioprinting, typically applicable for materials with low and high viscosities respectively. A less common technique is laser-assisted bioprinting which overcomes the issues of nozzle clogging and the limitations due to material viscosity.[93,94] Bioinks can either print biomolecules to mimic the extracellular matrix, cells to provide their desired spatial arrangement or a combination thereof, integrating organisation of cells and matrix. Alternatively, modified microcarriers with bioactive molecules can be included in the bioink to promote lineage commitment.[95]

Importantly, bioprinting has demonstrated its ability in handling stem cells and promoting EBs formation. For instance, a composite bioink composed of alginate, agarose and carboxymethyl-chitosan (CMC) forms a porous 3D scaffold
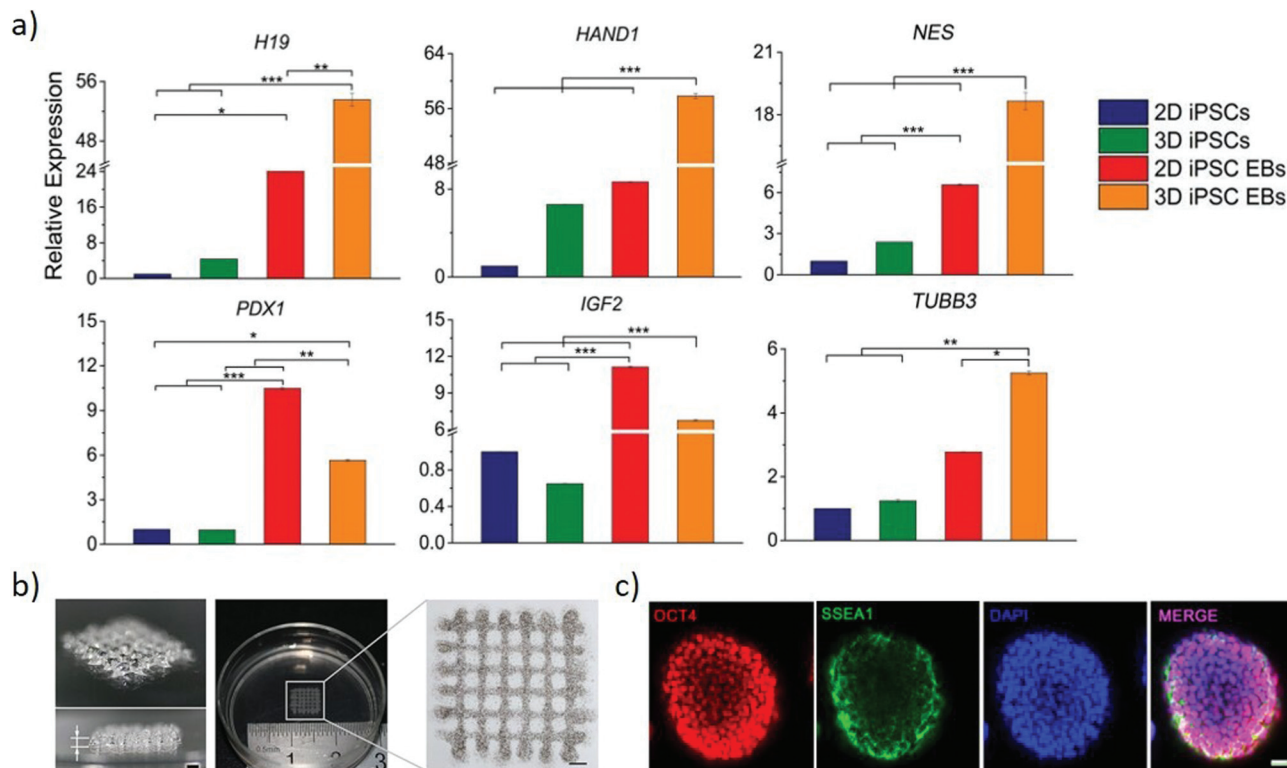
Fig. 6 Bioprinting can generate arbitrary 3D stem cell arrangements. (a) Relative genes expression of endodermal markers (H19 and PDX1), meso-dermal markers (HAND1 and IGF2) and ectodermal markers (NES and TUBB3) to compare conventional 2D cultured iPSCs and EBs with 3D printed gel-encapsulated iPSCs and EBs after 10 days iPSCs differentiation (15 days post-printing). Increased expression of all the markers emphasizes the potential for the bioprinted cells to differentiate. *P < 0.05; **P < 0.01; ***P < 0.001. Adapted with permission.[97] Copyright 2017, Wiley-VCH. (b) Images of 3-D bioprinted grid-like cell-laden hydrogel construct to generate high throughput EBs. (c) Immunofluorescence staining of EBs grown in the 3-D bioprinted cell-laden hydrogel by proliferation (right panel). Oct4 and SSEA1 suggest their pluripotency is maintained at day 7 post-printing. (b and c) Adapted with permission.[103] Copyright 2015, IOP publishing group.

supporting human neural stem cells differentiation to functional neurons and neuroglia.[96] Similarly, this bioink can support hiPSCs differentiation. The stem cells maintain their proliferation within the printed construct after gelation and self-assemble to form large pluripotent spheroids. Culturing iPSCs in medium free of basic fibroblast growth factor and without any specific supplements causes the cells to differentiate towards the three germ lineages (Fig. 6a). Whilst culturing in neural induction medium causes EBs to differentiate into functionalised homogenous neural tissues, indicating that cells retain differentiation ability post-printing.[97] Provided that there are few instances of iPSCs bioprinting, such a study lays out the initial steps in modelling cells of different lineages. Moreover, the variety of possible combination of cells, soluble factors, inks and spatial organisation, pave the way to high-throughput combinatorial investigations of their role in embryonic development.[98] More specifically, valve-based bioprinting of hESCs and hiPSCs maintains their viability and pluripotency comparable to non-printed cells.[99,100] Exposing the printed stem cells to hepatic differentiation conditions yields markers characteristic of hepatocyte-like cells.[100] Valve-based bioprinting approaches offer very fine control over the number of cell and volumes of biomolecules printed with high

spatial precision. Such level of manipulation can be leveraged to craft a variety of systems to investigate embryogenesis and apply these findings for meaningful advancement in tissue engineering.

The size of EBs/colonies is a critical parameter that determines gene expression, thus influencing the differentiation of the cells. Regulating the shape and size of EB by culturing mouse ESC in concave microwell arrays of different widths yields large EBs preferentially differentiating towards neuronal and cardiomyocyte differentiation than small EBs.[101] However, such an approach provides limited control in manipulating the microenvironment of the EBs within the microwells. Direct writing by laser bioprinting enables controlling both the printing density and colony size, allowing exploring these parameters independently. The final diameter of mouse EBs is dependent on the local cell density of the printed colony diameter, highlighting the influence of cell density in predicting and controlling EB size to obtain greater reproducibility during *in vitro* differentiation.[102] A versatile approach generates high throughput EBs of uniform and timely controlled sizes by bioprinting a 3D cell-laden hydrogel system (Fig. 6b). Mouse ESCs are embedded uniformly within hydrogels and cultured for multiple days to obtain pluripotent EBs (Fig. 6c),

that are formed through proliferation rather than aggregation. These approaches present a strong potential in generating a high precision tool to engineer EB of the desired size for its later application in differentiation studies and drug screening studies.[102,103]

Nanomaterials

Nanotechnology is prominently establishing approaches to transform drug delivery, biosensing and stem cell technologies, but its contribution to developing advanced models for dissecting embryonic development remains very limited. Yet, much of the functionality developed for disruptive nanomedicine approaches can translate into key advances for developmental models. Due to their size, nanomaterials display emergent properties with respect to their bulk counterpart, owing to their behaviour as quantum objects. Their dimensions are comparable to biomolecules and subcellular structures enabling direct interaction with the basic constituents of life. Their large specific surface area provides for extreme sensitivity to changes in their surroundings, enabling the design of devices that respond rapidly and efficiently to environmental changes. Overall the combination of these properties allows designing multifunctional nanomaterials with low impact on cell phenotype and capable of effective cellular delivery of multiple biological payloads simultaneously or in precise sequences, while controlling their spatiotemporal localisation either in response to external stimuli or in response to changes in the environment. Such multifunctionality enables delivering combinations of nucleic acids and other impermeable payloads to a precisely controlled pattern of cells with a desired temporal arrangement governed by either external factors or changes to the cellular environment.

Using active targeting approaches against stem/differentiating cells markers it is possible to selectively deliver drugs/biologicals to specific cell subtypes, thus controlling their behaviour. The physicochemical properties of RNA nanoparticles can be finely tuned and engineered to favour therapeutic delivery of biomolecules, and a 3 way-junction (3WJ) motifs of RNA packaging has been developed to have a predictable and stable folding.[104] Stable 3WJ motifs can assemble into 4 stranded RNA nanoparticles that can selectively bind markers of stemness such as CD133, and mediate RNA interference for gene silencing in a cell-specific fashion.[105] Using similar approaches, potent regulators of pathways controlling embryogenesis, including notch, sonic hedgehog and TGF-β, can be specifically directed towards cells based on their surface markers.[106,107] In addition, nanoparticles enable co-delivery of biological and non-biological agents which synergise to enhance the effectiveness of delivery and overall treatment.[108] Effective co-delivery enables designing complex temporal arrangements of combinations of drugs and biologicals capable of improving the effectiveness of treatments and transferrable to replicate the complex temporal variations of stimuli occurring during embryogenesis.[109]

Nanomaterials can be designed to couple effectively with physical stimuli such as magnetic, electrical and optical fields to locally induce mechanical forces, increase in heat, and generation of free radicals that can induce biological responses in a spatiotemporally regulated fashion.[110] Such interaction enables perturbing both biophysical and biochemical signalling pathways with fine spatiotemporal resolution. Magnetoplasmonic nanoparticles (MPNs) can target mechanoreceptors on the cell surface and be specifically pulled using a magnetic field to interrogate mechanoresponse with subcellular resolution. These mechanical stimuli can regulate the activation of developmentally-relevant pathways such as NOTCH signalling, regulate the spatial distribution of key effector proteins and trigger gene expression with a desired kinetic.[111] Similar approaches have the potential to orchestrate changes in the mechanical environment around stem cells with very high spatial resolution.

Inorganic Janus nanoparticles can load both hydrophobic and hydrophilic drugs (docetaxel and doxorubicin hydrochloride) and release them in response to local environmental changes. Since the drugs can be release based on the local pH or by imposing an external near-infrared stimulus (Fig. 7a), these nanomaterials can provide both spatial and temporal control based on the localisation of the endogenous or exogenous stimulus.[112] Enzyme-controlled drug delivery systems can be properly tuned to spatiotemporally regulate delivery by releasing morphogens to the target cells on demand. This approach enables releasing morphogens such as BMP2 in response to the proteases secreted by migrating hMSCs, resulting in locally-enhanced osteogenic differentiation.[113] This approach also lends itself to combinatorial delivery of stimuli, as evidenced with the spatially-controlled delivery of BMP2 and VEGF stimuli to differentially induce osteogenesis and vasculogenesis towards the development of in vitro vascularised bone tissue. These combined abilities enable nanoparticles to control the delivery of biophysical and biochemical stimuli in space and time by dynamically responding to the changes occurring to cells, their environment or by means of externally applied stimuli. Such extensive dynamic control of stimuli indicates the strong versatility of nanoparticles for the engineering of microenvironments within models of embryogenesis.

Alongside nanoparticles, extended nanostructures such as thin nanostructured films, nanotopographies and bidimensional arrays serve as effective interfaces to manipulate both the extracellular and intracellular environment. Nanotextured surfaces provide a spatiotemporal control over biophysical stimuli that can regulate stem cell fate, induce signalling clustering and control several phenotypical features including motility, proliferation, morphology and cytoskeletal arrangement. Small changes in the patterns of nanostructured surfaces can provide accurate control over the geometry and reprogramming of stem cells colonies. Slightly disordered nanotopographies induce osteogenic differentiation of hMSc which is not observed in ordered nanotopographies (Fig. 7b).[114] Vertical silicon nanostructures counter-
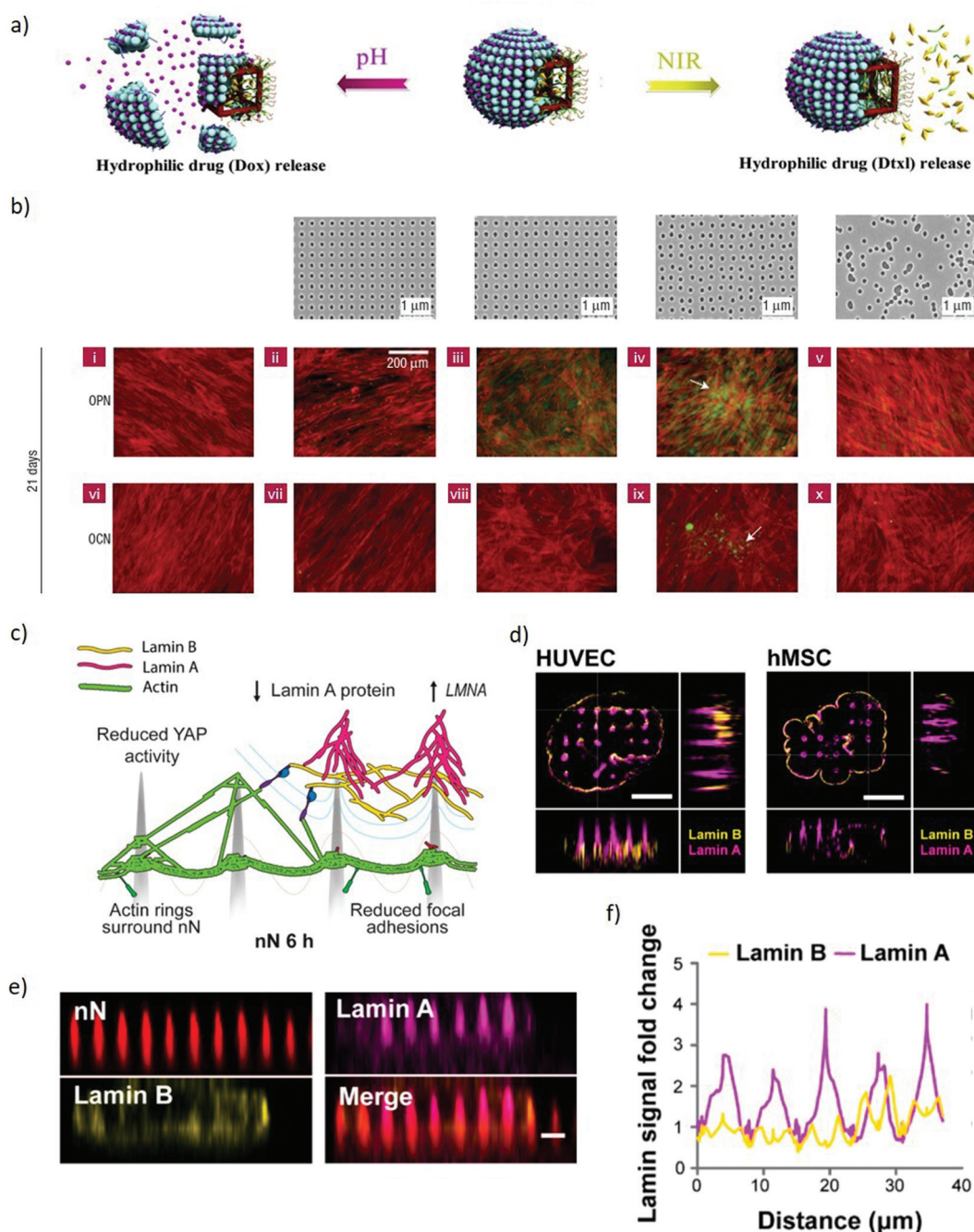
Fig. 7 Nanomaterials spatiotemporally regulate biophysical and biochemical signalling demonstrating potential to manipulate models of embryogenesis. (a) A schematic representation of the co-encapsulation of docetaxel and doxorubicin hydrochloride drugs within Janus nanoparticles. The drugs are released independently based either on pH or NIR stimuli. Adapted with permission.[112] Copyright 2018, Elsevier. (b) Upper panel, left to right: Flat control, nanotopographies with pits in a square array, pits in an array with random displacement by up to 20 nm from a square pattern (DSQ20, $\pm$20 nm from true center), pits in a displaced square 50 array (DSQ50, $\pm$50 nm from true center), and randomly arranged pits. Lower two panels: After 21 days in culture, only MSCs cultured on DSQ50 (iv and ix) exhibited both osteopontin/osteocalcin (OPN/OCN) positive regions (green) and nodule formation (indicated by the arrows), indicative of osteogenic differentiation. Red = actin, green OPN/OCN as per legend. Adapted with permission.[114] Copyright 2007, Nature Publishing Group. (c) Schematic representation of a cell interacting with nanoneedles. The nuclear envelope wraps around the nanoneedles providing mechanical stimulation to multiple intracellular elements. Lamin A reorganises at the nuclear envelope. (d) SIM imaging of human umbilical vein endothelial cells (HUVEC) and human mesenchymal stem cells (hMSC) shows lamin A (magenta) accumulating preferentially at nanoneedles whereas lamin B (yellow) distributes uniformly across the cell membrane. Scale bars = 5 μm. (e) x–z plane from confocal z-stack images showing lamin A and lamin B organization on nanoneedles, scale bar = 2 μm. (f) Quantification of lamin A and B signal intensity distribution along the basal nuclear envelope. Lamin A increases at nanoneedle sites whereas lamin B remains constant. (c–f) Adapted with permission.[139] Copyright 2019, American Chemical Society.

intuitively promote the formation of mouse iPSCs spheroids, by encouraging cell–cell interaction in contrast with cell–substrate interaction.[115] Carbon nanotubes networks promote human dermal fibroblasts reprogramming, demonstrating the ability of nanostructures to induce signs of pluripotency in somatic cells.[116] In addition, depositing nanomaterials in a nanopattern on a substrate and attaching specific biomolecules to such nanomaterial results in patterned signalling biomolecules. Using such an approach identified that, by varying the nanospacing between the cell-adhesive peptide arginine-glycine-aspartic acid (RGD) resulted for MSC to exhibit a distinction in the differentiation extents.[117]

Electrically-active nanostructures, integrated into large scale arrays, can induce highly local electroporation in order to deliver payloads to desired combination of specific cells in a temporally-controlled fashion. Nanowires,[118] nanostraws,[119] nanoantennas[120] and nanoholes[121] are particularly well suited to this approach, thanks to the tight interfacing with cells resulting in effective and local poration with relatively small and short electrical pulses, that allow reliable delivery to individual cells while minimising cell toxicity. These systems can integrate reservoirs to enable sustained and repeated delivery of the desired payloads, and/or be integrated within microfluidics systems in order to control nutrient exchange and exposure to payload. This approach delivers nucleic acids, proteins and peptides intracellularly with the potential to generate complex spatiotemporal patterns of autocrine and paracrine signalling, which could orchestrate signals in stem cells.[119,120,122–124] Furthermore, nanostraws and other nanoconduits[125] can also rapidly sample intracellular fluid with single cell resolution, potentially enabling molecular mapping of complex models of embryogenesis. Alternatively, to electroporation, nanomaterials interacting with light can also induce local permeabilization of the cell membrane, thus enabling spatiotemporally patterned delivery. Nanomaterials interacting with focussed light can locally heat up the surrounding medium inducing local reversible permeabilization of the cell membrane that can be used to gain access to the intracellular environment.[126] Plasmonic and meta-plasmonic materials can locally enhance optical fields, inducing ejection of electrons and mechano-acoustic waves in the close surroundings of the so called "hot spot" which also induce local membrane poration enabling intracellular delivery and recording of intracellular electrical activity.[127–129] How electroporation and laser-mediated poration physically disrupt the cell membrane and perturb the cytosol around has been investigated,[130] confirming the temporary and local modification of the cell membrane making the above approaches suitable to perturb stem cells system to study how different signalling pathways are influencing organogenesis. Alternatively, arrays of complex micromachines can be fabricated to allow controlled-release of multiple payloads on demand, by electrochemical dissolution of thin membranes that otherwise seal microreservoirs containing the desired molecules.[131] Such elaborate devices can be applied to stem cells cultures to regulate the triggering of chosen path-

ways, for example to study how the presence of single signalling molecules affect the development of embryos in time.

When cultured on high aspect ratio nanostructured substrates, the cell membrane wraps around such nanostructures establishing a tight sealing.[132–137,149] This physical stimulus results in a local curvature which may trigger the activation of mechanosensing pathways.[138,139] Indeed, nanopillars influence the reorganization of intracellular actin leading to a reduction in the cytoskeletal tension[140] hence, causing significant morphological alterations. Some nanopillars can also induce deformations in the nuclear envelope which modulates the expression of key mechanosensory regulators (Fig. 7c–f).[139,141] In addition to mechanical stimuli, these high aspect ratio nanostructures also enable localised delivery of payloads, potentially highlighting an approach to control the spatial organisation of signalling in embryonic models.[142,150] The mechanism of internalization is dominated by endocytic pathways stimulated by the interaction with the nanostructures, whilst physical spontaneous rupture of the cell membrane occurs when tension forces are coupled with high local curvatures of the nanostructures.[137,143–147] This permeabilisation of the cell membrane allows the direct intracellular delivery of biomolecules, thus bypassing the cells' physical barriers.[142,151] Interestingly enough, these interaction with nanostructured substrates does not greatly affect the viability of the cells.[148,149] This efficient and localised delivery capability can orchestrate signalling by patterned transfection of regulators of gene expression for key morphogens, enabling complex manipulations of stem cell colonies.

Outlook

Controlling symmetry-breaking by gaining precise spatio-temporal control over the physical and biochemical cues provided to the stem cells niche is a yet-unmet key component to accurately model *in vitro* early stages of embryogenesis and gastrulation. The biomaterials approaches highlighted here have contributed significant progress towards this ambitious goal, yet, there remain significant gaps as currently available technologies only achieve partial control over the organisation of embryoid models. Two-dimensional micropatterning can impact local biochemical signalling gradients, which induce a controlled stem cell differentiation pattern. This pattern can be partly regulated by controlling the size and shape of micropatterns. However, such control is limited, the model is two-dimensional and lacks temporal control over signalling cues, restricting the range of embryogenesis events that can be studied with this approach. Microfluidic devices can establish simple spatiotemporal gradients of soluble cues for 2D and 3D systems and they can modularly integrate with other approaches, such as micropatterning and hydrogels. Despite microfluidics efficacy at establishing patterning, the systems are complex to design and handle, can only achieve a subset of the desired gradients, and encounter challenges to handling tissue growth and long term maintenance. Hydrogels can

control both mechanical and biochemical cues in 3D cell culture systems that are essential to collectively coordinate the early developmental organisation. Patterning hydrogels enables controlling the spatial organisation of extracellular mechanical and biochemical cues, providing a high degree of local control to cell patterning. Yet, controlling soluble cues through hydrogels is complex, and temporal regulation of cues is limited to either pre-programmed design through hydrogel degradation or impractical approaches such as reversible photo-patterning. Microbeads are a simple and effective approach to present cells with asymmetrical soluble stimuli or to generate symmetrical gradients by allowing biodegradable spheres to release molecules in time. Microbeads elegantly address key challenges of establishing asymmetries by displaying signals to an individual cell or layer and outwards decaying gradients which are complex to achieve otherwise. However, they have very limited scope due to the fixed geometry which only allows displaying cues to cells on a spherical shell or establishing spherical gradients, which alone are insufficient to finely tune the chemical stimuli needed to recapitulate early stages of development. Bioprinting, although at its infant stages, is revealing an extremely powerful tool to shape tissue and embryoid bodies with high spatial and temporal control over the features of the system. Admittedly, bioprinted iPSCs retain their differentiation ability, however, in many instances cell survival, their precise placement and the low throughput of the approaches remain key barriers requiring refinement in order to recreate the microenvironmental conditions directing the stem cells to differentiate to the germ lineages in an organized manner.

We highlighted how untapped nanotechnology approaches could address some of the challenges arising from current biomaterials approach to designing niches that break symmetry in models of organogenesis. Nanomaterials can very effectively control the spatial and temporal distribution of soluble cues, using strategies developed with nano delivery vectors for cancer therapy. The topography and composition of nanomaterials can also be tightly spatially controlled and in some instances regulated in time, and their effect as biophysical cues to regulate stem cells differentiation have been established in multiple non-embryonic settings. Indeed, nanomaterials have been extensively effective at delivering biological payloads, including morphogens, with high spatial and temporal control, and they can be programmed to target specific cells, actively respond to environmental cues or be directed by external stimuli. These combined features can enable highly sophisticated design of spatiotemporal arrangement of cues, with unprecedented nanoscale resolution. This improved control and higher resolution over the patterning of cues achievable with nanomaterials, in turn can translate into regulation of stem cell fate approaching single cell resolution. Such sophisticated stem cell control would enable establishing highly localised phenotypes and investigating their role in organising patterning. Arguably, incorporating nanomaterials more effectively within the toolbox available to study embryogenesis can significantly advance our understanding embryonic development.

Conclusion

Understanding and directing the biophysical and biochemical processes regulating human embryogenesis is a very ambitious goal but it is essential to develop tools for designing tissue architecture and functions to be deployed for regenerative medicine. Only with an improved picture of the complex signalling network regulating tissue development and homeostasis, we can develop sufficiently effective bioengineered organs and personalised treatments for chronic and degenerative pathologies. However, ethical restrictions limit experimentation beyond the pre-gastrulation stages and as a result, animal models have been used as a substitute to gain insight on morphogenesis. Yet, differences between animal and human models persist, potentially leading to inaccurate or ineffective hypotheses. The rapidly developing *in vitro* stem cells technologies have provided a powerful means to decipher key pathways in early human development. Biomaterials have contributed to address challenges arising with instructing stem cell organization to mimic human morphogenesis leading to the development of improved models that can accurately mimic complex *in vivo* behaviours. Indeed, the broad array of tools available across micropatterning, microfluidics, hydrogels, microbeads and bioprinting approaches provides great flexibility to address the complex and multifactorial issue of designing accurate developmental niches for stem cell colonies. Certainly, integrating multiple approaches is beneficial to enhance the functionality, robustness and versatility of the models. Nevertheless, the available technologies still cannot provide a complete and comprehensive understanding of human early development stages and requiring key advances in our ability to control the spatiotemporal regulation of biophysical and biochemical soluble and immobilised cues which can be provided by combining both emerging technologies to manipulate individual cells and by lateral translation of mature technologies developed in other areas of bioengineering. Indeed, multiple approaches highlight a way towards the application of nanomaterials as rationally designed platforms to locally perturb the microenvironment in order to regulate stem cells organisation, differentiation and migration during the early stages of embryogenesis. The unsurpassed capability of nanomaterials for simultaneous regulation of multiple biochemical and biophysical cues with high spatiotemporal resolution indicates their potential to address outstanding challenges in orchestrating signalling for models of embryogenesis where multifactorial signalling networks can be artificially simulated in order to mimic physiological and pathological development.

Conflicts of interest

There are no conflicts to declare.

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