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Leading Opinion

Tissue assembly and organization: Developmental mechanisms in microfabricated tissues☆

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ABSTRACT

In vitro-generated tissues hold significant promise in modern biology since they can potentially mimic physiological and pathological tissues. However, these are currently structurally and functionally of limited complexity and necessitate self-organization and recapitulation of tissue development mechanisms *in vitro*. Tools derived from nano- and microfabrications along with bottom-up strategies are emerging to allow the fabrication of primitive tissues structures that can remodel overtime. Subsequently, clues are accumulating to show that, beyond genetic material, both intrinsic tissue architectures and microenvironmental cues can lead to morphogenesis related mechanisms *in vitro*. The question arises, however, as how we may design and assemble structures prone to adequate tissue remodeling, predict and manipulate those developmental mechanisms *in vitro*? Systems integrating architectural, physical and molecular cues will allow more systematic investigation of basic principles of tissue morphogenesis, differentiation or maintenance and will feedback to reproduce the dynamic of tissue development *in vitro* and form more complex tissues.

1. Introduction

In the past decades, advances in biology provided increasing knowledge of molecular components and interactions. These interactions are context-dependent, dynamically orchestrated on a microscale and promote the assembly of tissue components in spatial arrangements leading to tissue architectures and functions. To mimic this context, there is currently a need for experimental set ups of intermediate complexity between simple 2D biology and complex living organisms. It would help, for example, to understand the emergence of structures and architectures in tissues. Those models necessitate the minimum level of complexity necessary for tissue structures to arise and tools to systematically perturb, manipulate and observe them. Clearly, in spite of our knowledge of the actors and of their context-dependent behaviors, we are still missing the theatre to reproduce the scene *in vitro*.

Interestingly, clues are accumulating to show that tissue forms and architectures regulate tissue development, maintenance or function. This suggests a dynamic reciprocity of form and function [1–4] and pinpoint the importance of shaping adequate multicellular geometries to promote proper remodeling. During tissue development, most mechanisms of pattern formation are based on spatio-temporal heterogeneity inducing the formation of local environment (i.e. local gradients of soluble or insoluble factors, local streams of physical forces). Developing tools to create and manipulate the microenvironment including, for example, the location and shape of biological and physicals gradients are thus essential.

Tissues are often a combination of small repeating units assembled over several scales (Figs. 1–3). Cortical bone and skeletal muscle, for instance, are characterized by fascicles of repeating longitudinal units, respectively osteons and muscle fibers (100–500 μm diameter). Such units are "decision making modules that operate on multiple lengths scale from the molecular and subcellular level through to the cell and tissue level"[5]. Subsequently, from an engineering point of view, it was proposed to build tissues by assembling blocks mimicking those units in a *bottom-up* or *modular* approach [6–8]. This approach brings versatility and scalability to the fabrication of *in vitro* tissue models or implants.

The fabrication of those tissue models necessitates tools to create an initial architecture and to systematically manipulate their microenvironments in space and time [9]. Manipulating the remodeling of

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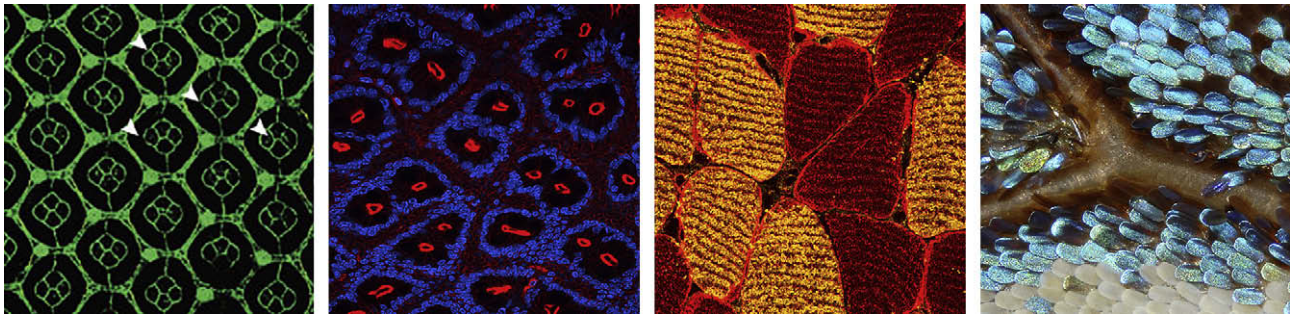

Fig. 1. Tissue architectures through different scales. Tissue architectures regulate the context of cellular signaling and gene expression by promoting the formation and maintenance of gradients of mechanical forces and biological factors. From left to right, tissue architectures at different scales: the fly retina epithelium (R.W. Carthew), the mouse large intestine (P. Appleton), skeletal muscle (I. Fischer) and butterfly wing scale (L. Gledhill).

those tissue models could help to investigate mechanisms of tissue morphogenesis, differentiation and maintenance (i.e. cancer biology) and to new applications in tissue engineering and developmental biology.

In this text we will focus on (i) technologies to assemble cells into defined *metastable* tissue constructs and (ii) strategies to create and manipulate local microenvironments promoting differentiation and morphogenesis related mechanisms.

2. Tissue assembly

2.1. Microfabrication tools

Recapitulating tissue development in microtissues is not new to biologists: growing aggregates of cells in a drop hanging from a surface is a classical method used since 1907 [10]. It has been used to grow tumor models [11] or embryoid bodies [12] and to study tissue development of liver [13], cartilage [14], retina [15] or pancreas [16]. However, more powerful technologies are emerging enabling more reproducible and precise arrangement of pools of cells. Those technologies help to form simple primitive architectures prone to remodeling. The cell printing approach is attractive and powerful and several groups are currently working on methods that are similar to traditional inkjet-printing (please see reviews) [17,18]. Laser technology [19], cell spraying through a mask [20], microfluidic [21], ultrasonic forces [22] or electro and photopatterning [23] are some of the recently developed tools to pattern cells (please see reviews for a more detailed outlook on those technologies) [24,25]. These methods allow high resolution but currently present difficulties for scaling up, generally necessitate a hydrogel support and lack the possibility to create empty space in architectures. A second method favors fabrication of Microscale Building Blocks (MBBs) that can be assembled into larger constructs. MBBs are gel encapsulated cells [8] or spontaneously aggregated cells [6]. It gained versatility and precision with microfabricated templates using photolithography [26], micromachining [27], soft lithography [28], membrane technology [29], centrifugal casting [30] or the combination of multiple processes [31]. Templates are rather cheap and allow rapid production of big quantities of MBBs of defined shape and size. Their assembly into ordered constructs requires further development (Figs. 2 and 3).

2.2. Bottom-up/modular approach

Tissue microfabrication has gained, through those technologies, the possibility to work in a bottom-up approach using either printed, molded or aggregated MBBs [8,32–35]. This bottom-up approach facilitates the fabrication of architectures using complementary shapes, the scaling up and an automated production. It uses cellular aggregates [6] or micromolded gel encapsulated cells [8]. Architectures can be achieved by complementary shapes and spatial arrangement [7,8,34,36,37] and self assembly of MBBs using microfluidic chip [38] or liquid–liquid interface [39]. Complementary shapes, forces and appropriate levels of plasticity are essential to self assembly strategies [40]. The self assembly of microscale units necessitate development to further achieve reproducible ordered objects in biologically relevant systems. Blocks can fuse [6], be cross-linked [39] and develop coordinated functionalities [6]. Examples of constructs resulting from a *bottom-up* approach include neural tubes obtained by accumulation of microtissues into an agarose template [6], MBBs encapsulating hepatocytes and covered by endothelial cells [8] or beating cardiac sheets generated by stacking of cell-sheets for patches [41,42].

3. How to orchestrate developmental mechanisms *in vitro*?

These approaches produce a metastable multicellular construct that will remodel over time according to biological and physical principles (i.e. migration of the cells, shrinkage of the hydrogel). Shapes and patterns are not inevitably translated to the final tissue. Designs must thus focus on promoting proper remodeling into the final architecture. Clearly, understanding and promoting tissue self-organization would tremendously improve tissue microfabrication (Figs. 2 and 3).

The study of the emergence of forms and functions in *in vitro* tissue models is still in its infancy. It is likely that those strategies of auto assembly can only reach a limited complexity and should be followed by more complex manipulations of the microenvironment. The rapid development of complex microfluidic systems, microbioreactors and detecting tools allows the long term culture of microscale tissues in precisely defined microenvironment. Microbioreactors permit the long term culture in controllable and continuous environment using minute amounts of biological factors [43] and to include parameters like shear stress, interstitial flow [44,45] or gradients of soluble factors [46,47]. They present great possibilities to culture microtissues in controlled, heterogeneous environments (latest development are described in this review [48,49]).

Mechanisms underlying tissue organization and the development and maintenance of tissues architecture and function are highly conserved through organisms and are better understood now than two decades ago [50]. It appears clearer that, beyond genetic regulation, the tissue architecture and microenvironment feeds back to promote its development, maintain its integrity and function [51,52]. Thus, to promote *in vitro* tissue developments, of special interest are (i) the creation of multicellular architectures prone to remodeling and (ii) strategies and tools to manipulate the microenvironment and promote *in vitro* organization. Here we

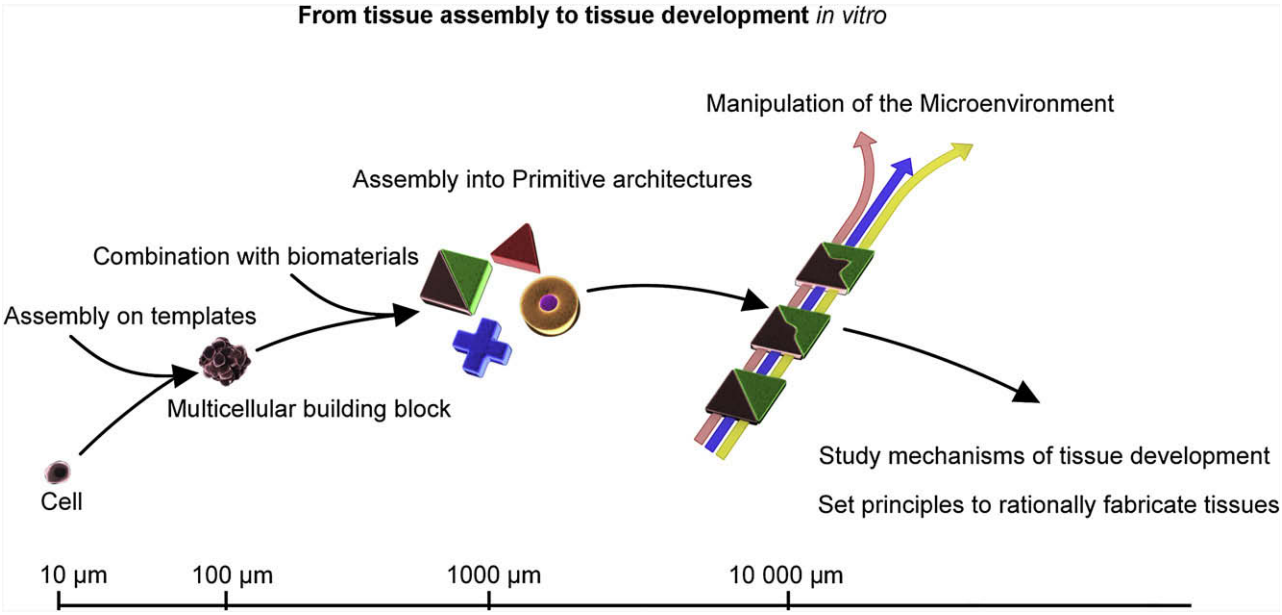

Fig. 2. From tissue assembly to tissue development *in vitro*. Tissue fabrication methods allow the assembly of cells into primitive tissue compartments, which are prone to remodeling. The tissue geometry along with the manipulation of the environment at a microscale further promote the self-organization of cells into more complex tissues *in vitro*.

describe current attempts to understand the importance of forms/geometries in the initial multicellular architecture and describe current possibilities to manipulate the microenvironment and promote developmental mechanisms in *in vitro*-generated tissues.

During tissue development, patterns of cellular states and behavior are formed through different cellular mechanisms including apoptosis [53,54], adherence [55,56] or mobility [57,58]. Those behaviors are under genetic control with feedbacks from the microenvironment. Local gradients of soluble factors [59], local production/remodeling of ECM, mechanical stress induced by a flow [60,61] or by cell–ECM contraction [62,63] induce dynamic changes of those patterns of behaviors leading to tissue morphogenesis, differentiation and maintenance. Those dynamic changes and feedback mechanisms influence coordinated local behaviors such as oriented migration, differential growth [64,65] or coordinated apoptosis [66,67] and thus promotes the emergence of organization and forms. Tissue architecture and function may primarily result from genetic material but is orchestrated by those dynamic microscale mechanisms [3,4]. Here we first describe attempts to understand the influence of microscale geometry on regulating coordinated cellular behavior *in vitro*.

3.1. Designing the initial geometry

During embryonic and post-natal development, tissues and organs go through different stages of organization. Those intermediate compartments (i.e. germ layers) promote crosstalk and further remodeling. Interestingly, geometric forms are intrinsically mediating some morphogenesis processes: they can promote the formation of local microenvironment including gradients of soluble factors or local mechanical stress thus inducing local behaviors (i.e. migration or proliferation). The creation of compartments of appropriate geometry is thus important to favor subsequent remodeling as proven experimentally in different models. In a 3D model using mouse mammary epithelial tubes, Nelson defined geometric patterns of pools of cells in a type I collagen gel using a micropatterning approach [68]. The form in itself dictated different Epithelial Growth Factor-induced branching sites. Interestingly, those branching sites matched with computer simulated profiles of secretion of autocrine inhibitory factors including TGFβ. This strongly suggests that tissue geometry conditions the formation of local microenvironments (local gradients of soluble factors) and subsequent morphogenesis processes [69]. Similarly, Nelson patterned pools of endothelial cells on different mesoscale geometric forms and showed different local patterns of proliferation. The geometry (squares, circles or rectangles) directly influenced the formation of local region of mechanical stresses subsequently inducing patterns of growth according to predicted mechanical stress [62]. In another attempt to depict the importance of initial geometrical arrangements of cells on biological function, Bhatia and her co-workers showed that the size and distribution of clusters of chondrocytes in a hydrogel directly influences the production of cartilage-related ECM [70] or its function [71]. Finally, in a study using shaped micromolded gels encapsulated mesenchymal stem cells, local patterns of physical forces induced spatial differentiation into either adipogenic or osteogenic lineage [72]. Those results pinpoint the importance of the architecture (geometry and size) of the initial cellular construct in creating local microenvironment (local gradients of factors or local mechanical stresses) inducing specific biological function or morphogenesis related processes. Those studies opened opportunities to understand how the form of multicellular systems can feedback to regulate patterns of cellular behaviors and, subsequently, how to design initial cellular constructs that promote adequate remodeling.

Beyond those initial states, strategies and technologies are necessary to engineer the microenvironment, promote and maintain further development.

3.2. Integration of signals

Creating microenvironments and integrating molecular and physical cues at microscale will promote tissue organization and help switching rationally from one pattern of cellular behavior to another. Only a few studies have focused on self-organization and developmental processes leading to patterns formation and tissue architectures. In this regard, basic work in the area of cancer or developmental biology should be carefully translated for each tissue type. The modulations of the cell–ECM and cell–cell interface

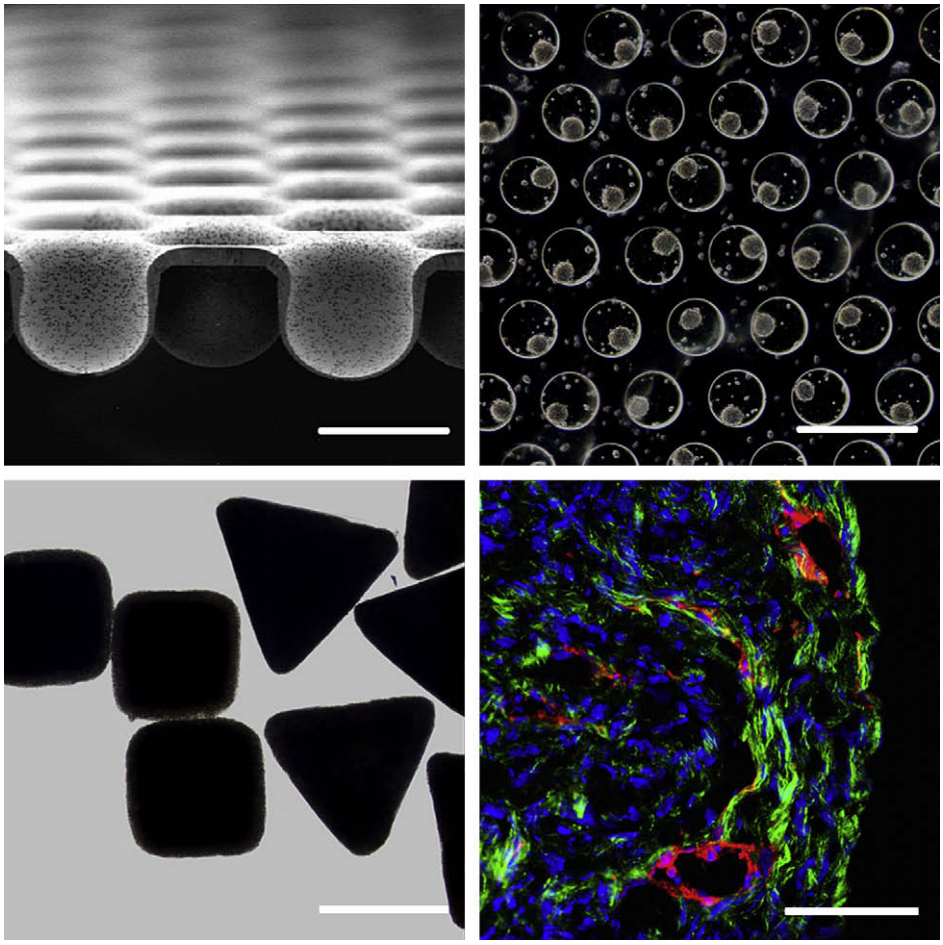

Fig. 3. Sequential events of bottom-up tissue engineering as performed in our group. Multicellular building blocks are assembled using templates including thermo-formed PLLA sheets (top left, scale bar 300 μm) or agarose chips casted on PDMS stamps (top right, scale bar 500 μm). Building blocks assemble and fuse into larger primitive compartments (bottom left, scale bar 1 mm). Complex tissues are formed by the self-organization of multiple cell types into tissue structures. Huvec and HMSC microtissues with dapi, cd31 and phalloidin markers (bottom right, scale bar 100 μm).

are of prime importance and have been under intense investigation. Other strategies, including the use of co-culture systems, the manipulation of local mechanical forces or the use of morphogens as natural patterning agents proved efficient to induce morphogenesis or differentiation related mechanisms.

3.2.1. Manipulating cell adhesion

The dynamic patterns and states of adhesive molecules influence the physical assembly of cells [73,74] and play an important role in regulating intercellular signaling partly through connections to the cytoskeleton [75]. Adhesive molecules represent a powerful link through scales for the organization of tissues. Here we focus on *in vitro* studies demonstrating the effect of modulating cadherins and integrins affinity or expression on tissue organization using genetic or pharmacological manipulation. Cadherins play an essential role during the first stage of self aggregation of multicellular systems, when the cell-secreted ECM is not yet present. *N*-Cadherin mediates embryonic compaction of mesenchymal cells and chondrogenesis [76] or the spatial arrangement of retina epithelial cells through *E*- and *N*- cadherins [77]. *In vitro*, their regulation allows the segregation of populations of cells and the formation of simple compartments [56]. Intercellular adhesion and cytoskeleton changes play an essential role in the movement of cells or sheets of cells [78] and cadherins were shown to modulate the migration of cells in a tissue model [79–81]. Similarly, the modulation of *E*-cadherins activity in pancreatic β-cells using blocking antibodies modifies both the architecture of spheroids and their functionality including the down-regulation of secretion of insulin [82]. Integrins play an essential role in cell–ECM adhesion. In a 3D model of human breast cancer, the modulation of beta1-integrin receptor and Epithelial growth factor signaling using beta1-integrin antibody and MAPK kinase inhibition led to a reversion of the malignant phenotype of 3D tissue-like acini. This change in architecture correlated with recalcitrance to growth factor stimulation [83]. Finally, cell adhesion modulates the sensitivity to some soluble factors through, for instance modification of transduction signals [84]. For example, the differentiation and maintenance of myofibroblasts phenotype by transforming growth factor beta1 is partly regulated by focal adhesion kinases and their interaction with the ECM can be modulated by pharmacologic or genetic inhibition [85]. Similarly, during vascular development, the expression of integrins is regulating cells response to angiogenic factors [84,86]. Thus, modifying the structure of binding proteins, their number or their spatial distribution can regulate the *in vitro* organization of multicellular systems [7,87,88].

3.2.2. Manipulating ECM properties

ECM is crucial for long-range communication: it transfers mechanical forces and immobilize biological factors heterogeneously in space [89–92]. The physical and adhesive properties of the ECM influence the mechanical effect of cells contracting and remodeling the surrounding matrix and feeds back to influence cellular signaling

[93,94], motility [95,96] and morphogenesis [7,97]. *In vitro*, modulating mechanical and biochemical properties can promote specific morphology either reminiscent of normal or pathologic tissue [7,97,98]. *N*-Cadherin transfected Chinese Hamster Ovary cells printed in a hydrogel, can aggregate and self-assemble in defined geometrical shape according to cell–cell and cell–matrix interactions as predicted by a mathematical model [7]. In another example, a fine tuning of concentration and molecular weight of hyaluronic acid was shown to influence the branching of ureteric bud, in kidney culture [99]. Beside this, the creation of spatial heterogeneity in architecture, mechanical properties, chemical composition and a spatio-temporal control of soluble factors are essential. Efforts in creating structural heterogeneity [100–103] or to control the distribution of soluble factors in space and time on a large scale [104,105] should prove useful in creating coordinated cell behavior. For instance, PEG hydrogel co-polymerized with fibrinogen show a correlation between structural variation in molecular architecture, degradation properties and migration behavior of pools of smooth muscle cells [101].

3.2.3. Multicellular crosstalk

Heterotypic cooperation is an essential process in the differentiation of developing tissues including kidney [106], lung [107] or heart [108]. The interaction between multiple cell types is therefore relevant to promote *in vitro* tissue development. Maturation and maintenance of blood capillaries are driven by the cooperation between Endothelial cells (EC) and mural cells [109,110]. For example, 10T1/2 mouse mesenchymal precursor cells or human mesenchymal stem cells are essential to stabilize the formation of blood capillaries of Human Umbilical Vein Endothelial cells in collagen type I [111,112] where they play the role of perivascular cells. Human Mesenchymal stem cells support the initial phase of vascular development in a co-culture with Huvec [113] and enhances the production of specific structural protein (glycoaminoglycan) in co-culture with primary articular chondrocytes (hPAC) [114]. Supportive cell type 3T3-J2 fibroblasts also maintain the biological function of hepatocytes in a microscale culture including morphology, albumin secretion or urea synthesis [71]. Generally speaking, stromal cells play an important role, in those models, in promoting or maintaining biological function or morphogenesis. This depicts the importance of heterotypic interactions in *in vitro* tissue development and the possibility to include cell types as temporary enhancers of tissue organization or maintenance.

3.2.4. Manipulating local mechanical forces

Tissues are self-deforming systems with local streams of mechanical forces [115]. During embryonic development, mechanical forces can regulate the expression of genes [116,117] and genes regulate mechanical forces [118]. These forces modulate protein expression [119], proliferation [62], cell movement [120], cell polarity [121], cell differentiation or apoptosis [122] and thus are critical regulators of morphogenesis as shown in developing tissues [123,124]. Along with the use of proper ECM, the modulation of patterns of internal forces can be achieved by direct manipulation of cell tension: direct biochemical manipulation of the cytoskeleton forces using inhibitors of ROCK (Rho associated kinase) or Rho activators can respectively inhibit or accelerate lung branching and capillary formation in embryonic mouse lung [63]. Similarly, the inhibition of the Rho and ROCK pathways block the contraction of collagen gels by epithelial cells and disrupt tubulogenesis *in vitro* [125]. A proper regulation of those pathways, partly mediated by the matrix stiffness, is necessary to the formation of proper 3D structures.

3.2.5. Morphogens

Morphogens (Hedgehog, Wnt, Bone Morphogenic Protein or Transforming Growth Factor families) are natural inducers of tissue patterning and organization. Morphogen gradients have a wide range of form generating effects by providing positional information, cell-lineage decision [126,127] or by making cells competent to respond to other factors [128]. Aggregates of mouse and human Embryonic Stem Cells (mESC, hESC) isolated from the inner mass and termed embryoid bodies can differentiate *in vitro* and self-organize using morphogens. For example, the combination of Sonic Hedgehog and retinoic acid induces the spatial organization (caudalization and ventralisation) of mESC and their differentiation into neurons that become functional after implantation [129]. Endoderm progenitors derived from mESC partially differentiated into a hepatic population by induction of BMP4, bFGF and Activin-A. Although immature, this hepatic population showed functionality including albumin secretion, glycogen storage and some integration after implantation [130]. In a keynote paper, Ten Berg show that modulation of Wnt signaling can promote the self-organization of mouse embryonic stem cells with the establishment of anteroposterior polarity and the formation of a primitive streak-like region in the embryoid body [131]. Although not completely understood yet, the use of morphogens signaling should play an important role in promoting *in vitro* tissue development.

3.2.6. Integrating native features: vascular and/or nervous network

Mimics of vascularized and innervated tissues must include such networks: They not only promote tissue survival and integration after implantation but play a far more complex role in tissue homeostasis and development [132–134]. For example, the vascular system, besides its classical role in mass transport of gas, liquid, molecules and cells, takes part in organ communication and interaction [135], in tissue patterning, tissue differentiation and tissue development [133,134]. A capillary network can grow and become mature *in vitro* [136,137]. To become stable and functional, *in vitro*-generated endothelial tubes need to produce a basement membrane and to be stabilized by mural cells/pericytes. This was achieved *in vitro* using a tri-culture system with mouse embryonic fibroblast as pericyte-precursors [138]. *In vitro* vascularized tissues have the potential to rapidly anastamose to the host environment and improve the implant survival and integration [113,138,139]. Neural network also plays a role in tissue development and organization and is essential for limb regeneration in Urodeles [134]. In skin, peripheral nerves determine the patterning of arterial branching by stimulating localized secretion of VEGF [140,141] thus, playing a role as intrinsic regulators of tissue development. Those systems should help to reveal the basic mechanisms of formation of tissue-specific capillary architectures or the interaction of the endothelium with its microenvironment and his impact on tissue development.

4. Conclusion

Converging technologies and emerging strategies allows for the assembly of multicellular systems in primitive architectures along with simple manipulations of their microenvironments. On the other hand, clues accumulate to show that microfabricated primitive compartments can follow a succession of temporary equilibrium states that can be manipulated through microenvironmental control. Of special importance are the role of geometries of multicellular systems in regulating the formation of local environment [62,69] and the possibility to create and perturb local environments using technological or biological tools. This will be useful to recapitulate and investigate *in vitro*, mechanisms of tissue organization including morphogenesis, differentiation and maintenance of physiological and pathological tissues. This knowledge could feedback to rationally design tissue architectures prone to adequate remodeling; reproducing and predicting, *in vitro*, natural patterns of developmental dynamic.

The integration of those *in vitro*-generated tissues in the chain of models of increasing complexity, from basic 2D culture to *in vivo* models, will provide new possibilities to understand the development of tissues and to rationally built and maintain *in vitro*, functionalized tissues with both therapeutic and ethical impact.

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