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Vascularized microfluidic organ-chips for drug screening, disease models and tissue engineering

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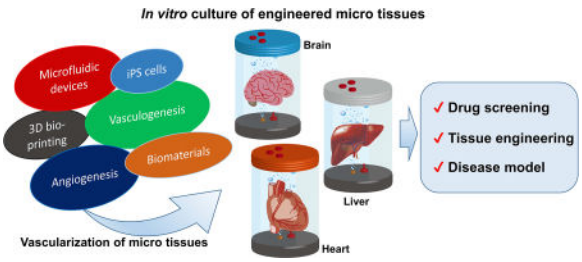
Abstract

Vascularization of micro-tissues *in vitro* has enabled formation of tissues larger than those limited by diffusion with appropriate nutrient/gas exchange as well as waste elimination. Furthermore, angiocrine signaling from the vasculature may be essential in mimicking organ-level functions in these micro-tissues. In drug screening applications, the presence of an appropriate blood-organ barrier in the form of a vasculature and its supporting cells (pericytes, appropriate stromal cells) may be essential to reproducing organ-scale drug delivery pharmacokinetics. Cutting-edge techniques including 3D bioprinting and in vitro angiogenesis and vasculogenesis could be applied to vascularize a range of tissues and organoids. Herein, we describe the latest developments in vascularization and prevascularization of micro-tissues and provide an outlook on potential future strategies.

Graphical abstract



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Introduction

In vitro micro-technologies to engineer microscale versions of various organs and tissues have attracted significant attention recently. These micro-tissues have been developed for transplantations of cornea [1], retina [2], bladder [3], or heart [4] [5], to recapitulate organ level function for drug screening [6] [7], drug discovery, personalized medicine and disease models, as well as for basic scientific understanding of *in vivo* like cell-cell interactions. In spite of growing demand for these systems, however, current ones are mostly limited to thin or avascular tissues, and for these there have been successful clinical trials [8].

Most major tissue engineered solid organs, though, lack perfusable microvascular networks. Vascularization of micro-tissues is necessary for a variety of reasons: 1) replicate angiocrine signaling [9] for the formation of micro-tissues mimicking developmental processes, 2) construct tissues of macroscopic scale, larger than can be maintained by diffusion alone with appropriate nutrient delivery and waste elimination, 3) mimic blood tissue or organ barrier such as the blood-brain barrier. The latter is especially relevant in immune cell interactions as well as tumor cell transmigration which are significantly influenced the organ specific barrier. We focus this paper on state-of-the-art developments in the vascularization of these micro-tissues, the limitations of current systems and potential future strategies to overcome them.

In vitro vascularization

There are several techniques that could be used to vascularize tissues *in vitro*. For instance, Miller et al. used 3D printing to mold dissolvable vascular channels around which any engineered hydrogel based tissue could be grown. Subsequently, the molds were dissolved and endothelialized. This technique allows building large scale vascularized tissues with tunable vascular geometries (Fig. 2A) [10]••. These robust perfusable vascular networks could be cultured with other parenchymal cells and micro-tissues. However, with the current 3D printing technology, engineering capillary structures is limited to those larger than ~100 μm in diameter. However, one can engineer sub-100 μm capillary structures (even down to 10 μm) using the other major strategies of vascularization through angiogenesis [11] or vasculogenesis [12] [13] [14] [15] (Fig. 2B) which draws upon the emergent behavior of endothelial cells. Using angiogenesis or vasculogenesis techniques, it is possible to achieve sub-100 μm capillary structures even down to 10 μm. In particular, Nashimoto et al. recently induced angiogenesis *in vitro* producing vascularized and perfusable tissues in a microfluidic device containing prevascularized spheroids (Fig. 2C) [16]••. This work demonstrated a prevascularized spheroid with lung fibroblasts and endothelial cells that was placed in the microfluidic device in a collagen gel. Then, other endothelial cells that were adhered to the wall of the gel migrate and sprout towards the spheroid, resulting in anastomoses between the vascular networks. Lastly, Sekine et al. demonstrated long-term culture of prevascularized skin-like micro-tissues by using an *ex vivo* vascular bed (Fig. 2D) [17]•. They used a rat vascular bed that was extracted from a rat's femoral muscle tissue along with the branches of arteries and veins. Stacked cardiac sheets were placed onto this vascular bed in order to form a connection between the prevascularized stacked cardiac sheets and the rat vascular bed. This group also demonstrated that the vascularization

improves the survival of tissues in vitro, after transplantation. Even though these techniques are becoming established, the development of *in vitro* vascularized organs using them is a field in its infancy. It is a nascent field in which the majority of vascularized engineered tissues rely on vascularization following implantation. The host response tends to induce vascularization of constructs containing a mixture of target tissue cells and endothelial cells, leading to a connected, perfusable tissue [18] [19]. In the vascularization technique described in [18], a millimeter scale tissue is implanted in an animal with surgery. This has the drawback of a having to perform a surgery for implantation. Likewise, the work in [19] requires 2 surgeries to produce a vascularized tissue, one for implantation of the target tissue that needs to be vascularized, and a second one to recover the vascular graft tissue. Both these techniques are cumbersome, and may not be as scalable and reproducible as other techniques that may involve purely *in vitro* vascularization. In this brief review, we will discuss the current state-of-the-art in the development of various vascularized micro-tissues for solid organs (skeletal muscle, heart, brain, liver, bone, lung, tumors) as well as potential steps for improvement.

Brain

Neurospheroids, derived from neural stem cells or neural progenitor cells, are versatile cell aggregates that model forebrain and cerebellar development [20][21]. This model could be utilized to study several brain pathologies including synaptopathy and epilepsies. A significant advance in *in vitro* brain models occurred several years ago with the first reports of cerebral organoids [22]. These organoids which are typically produced by the emergent behavior of human stem cells such as ES cells and induced pluripotent stem (iPS) cells in a bioreactor represent a complex human brain model that may be useful for drug screening. Recently, fused forebrain-like organoids were formed by placing two spheroids next to each other in order to more closely mimic the forebrain physiology [23] (Fig. 1a(i)).

Although neurospheorids and cerebral organoids represent the future of human *in vitro* brain models there remain several important challenges to advancing these models. For example, current cerebral organoids fail to recapitulate many late brain development processes such as gliagenesis and vasculogenesis mainly due to the longer time needed [24] to obtain functional brain organoids. Furthermore, vascularization of these cerebral organoids is essential for long-term maintenance [25]. To overcome these barriers to progress, other vascularized brain models are needed, for example, Phan et al.'s co-culture model of astrocytes and pericytes with a perfusable vasculature in a microfluidic device [26]•• (Fig. 1a(ii)).

Opinion

At this time, no vascularized brain model exists, even including prevascularized models. However, the co-culture of neurosphere or organoid with endothelial cells using either molding [10]•• or vascularization techniques [16]•• could be used to develop perfusable and functional brain models *in vitro*.

Lung

Gas exchange in the body takes place in the alveoli where inspired air comes into intimate contact with the circulating blood. Huh et al.'s alveolar-chip incorporating alveolar epithelial and endothelial cells on an engineered, mechanically stretched substrate represents one of the most advanced alveolar models to date [27] [28]•• (Fig. 1b).

Opinion

Inflammatory responses and toxicology studies have been conducted on this chip and compared qualitatively to responses in *ex vivo* mouse lungs with generally impressive results. Two of the primary limitations of this system, however, are the morphology of both the endothelial channel and the airways neither of which recapitulate that of their *in vivo* counterparts. Capillaries *in vivo* are approximately 10 μm in diameter, while the endothelial lumens in the alveolar-chip are an order of magnitude larger. This may influence the adhesion of cells such as neutrophils, tumor cells etc. due to differences in the shear stress, flow velocity, or interactions with the endothelium, which could in turn affect the dynamics of extravasation of these cells. Likewise, the hierarchical structure of the in vivo airway leading to the alveoli is not recapitulated in this device, perhaps leading to exposure of the epithelial cells to a different profile of aerosol particle sizes. This is in contrast to *in vivo* systems in which only the smallest particles ever reach the alveoli. Future versions of this system could benefit from having a bronchial epithelial chip upstream to the alveolar chip with a hierarchical structure in between, thus correctly representing the aerosol/nanoparticle distribution in the respective compartments.

Bone

Bone is a highly vascularized tissue containing vessels categorized into two major systems, central or "Haversian" canals, and perforating or "Volkmann's" canals [29]. Bone consists largely of a rigid, honeycomb-like matrix formed from a composite material incorporating the inorganic mineral calcium phosphate, which gives the bone its rigidity. Therefore, incorporating an extracellular matrix with calcium particles in *in vitro* bone models is important in order to recapitulate the mechanical strength of *in vivo* bone.

To vascularize bone tissues for Haversian's canals, 3D bioprinting technology has been widely used to create a relatively large vessel. For instance, 3D bioprinting can precisely control the location of cells and extracellular matrix (ECM) for engineering complicated micro structures [30]. Cui et al. used a novel technique to engineer a vascularized bone biphasic construct using dual 3D bioprinting PLA and gelatin methacrylate hydrogel [31] (Fig. 1c). This sequential printing allows fabrication of a hierarchical bone structure with a vasculature. To create Volkmann's canal-like capillary networks in bone tissue, vasculogenesis using an encapsulation of endothelial cells and mesenchymal stem cells or osteoblasts has proved an effective strategy [32]. Correia et al. demonstrated bone grafts with micro-vascular networks by co-culturing human umbilical vein endothelial cells (HUVEC) and mesenchymal stem cells (MSC) in a decellularized bone scaffold [33]. They found that sequential application of growth factors for endothelial cells and osteoblasts helps to form microvascular networks followed by osteogenic differentiation. *In vitro* bone models

have also been used to study diseases such as cancer. For instance, Jeon et al. developed a tumor extravasation model in a microfluidic vascularized bonelike tissue [34].

Opinion

In the future, the combination of 3D bioprinting techniques and vasculogenesis of endothelial cells with a stiffer scaffold could prove a sueful approach to model highly rigid bone with both types of vascular structures.

Tumors

The primary goal of making vascularized 3D tumor models is to represent the tumor microenvironment more accurately. Given the intimate proximity of tumors to the vasculature, it is likely that interaction between the two could influence expression of target proteins in the tumor, affect drug delivery and drug response. The vasculature is known to play a key role in cancer metastasis (both intravasation and extravasation). To address these concerns, Sobrino et al. developed a microfluidic tumor model with a range of tumor cells suspended in a 3D ECM, surrounded by a perfusable human cell based vasculature [15]• (Fig. 1d). They demonstrated that IC50 values for the effect of Oxaliplatin (a chemotherapeutic drug) on HCT116 cells is an order of magnitude larger for cells growing in the microfluidic vascularized micro-tumors, compared to 2D cultures.

Opinion

For more widespread adoption of such models, a more detailed characterization of this platform is required that may characterize the relative roles of the 3D architecture and associated paracrine effects, the direct effects of signaling with the vascular cells, and the importance of drug delivery through the vasculature versus through the tumor cells in 3D or 2D. The use of patient derived iPS cells in this platform would further personalize it for therapeutic screening. The use of organ-specific stromal/supporting cells as well as a full complement of immune cells may further improve the physiological relevance of such platforms in the modeling of specific tumors.

Heart

Heart tissue is highly vascularized in order to meet its high energetic demand. Yet the most widely used heart-on-a-chip micro-tissues are not vascularized. They primarily focus on contractility measurements with either 3D cardiomyocyte based tissues grown with flexible pillar anchors [35] or 2D cardiomyocyte sheets [36] on thin flexible cantilevers. Such systems have been used in disease models [37] such as mitochondrial cardiomyopathy in Barth's syndrome. A fully-realistic model of heart micro-tissues, however, will require the addition of a functional vasculature that replicates *in vivo* like endothelial-cardiomyocyte crosstalk. In particular, nitric oxide released by endothelial cells may have a crucial cardio-protective role without which drug cardiotoxicity studies may not be representative of *in vivo* physiology. Attempts at solving this problem include an emergent self-assembled cardiac tissue containing endothelial networks by seeding defined tricultures of cardiac myocytes (primary human cardiac cells, human embryonic stem (hES) cell-derived, or iPS

cell-derived cardiac myocytes), fibroblasts (embryonic or iPS cell-derived cardiac fibroblasts) and endothelial cells (hES cell-derived, or human coronary artery endothelial cells) either on a biodegradable polymeric scaffold [38] , or in aggregating hanging drop cultures [39]. However, neither these models contain a perfusable vasculature through cardiac tissue in a way that *in vivo* tissues do, despite the demonstration of structures with lumens [38]. In Chiu et al., an engineering approach is used to plate cardiac-myocytes on top of a perfusable capillary bed [40]. In this approach, appropriate endothelial-cardiac interactions should occur. Nevertheless, this system lacks the capability to measure force as in Boudou et al. [35] or Agarwal-Parker et al. [36], which is essential to assess cardiac function. Vollert et al. developed one of the most realistic cardiac models to-date with an engineered heart tissue, both with anchors that could be used as force transducers as well as with a perfusable endothelialized channel through the tissue [41] •• (Fig. 1e).

Opinion

The main drawbacks of the Vollert model include the need for further validation in the context of several cardiac diseases, such as ischemia, as well as improved ease of fabrication and use. In the future, techniques such as 3D bioprinting could be used to further standardize such models making it more accessible, as well as more useful by combining the most attractive features of all the above platforms to produce an *in vivo* like heart-chip with the appropriate heterotypic cell-cell interactions and in-built instrumentation to test tissue function.

Liver

A major challenge for studying liver function in drug screening and regenerative medicine applications is that primary hepatocytes quickly lose their advanced phenotype in 2D cultures [42]. *In vitro* liver models have been used for numerous drug metabolism studies [43]. Liver spheroid cultures and other 3D cultures of hepatocytes offer effective strategies that help extend hepatocyte function lifetime and maintain high metabolic rates [44]. However, all these models have limited lifetime under even the best conditions (primary hepatocyte monolayer: 4–5 days, spheroid: ~2 weeks and co-culture spheroid with stellate cells: ~3 weeks). Another strategy is the use of cell lines and iPS cell and ES cell derived hepatocytes [45]. To further extend the lifetime of liver advanced metabolism *in vitro* [46], vascularization of liver spheroids and perfusion into micro-tissues are still needed. Timmins et al. demonstrated vascularized multi-cellular spheroid with endothelial cells and hepatocyte cell line created by the hanging-drop method [47]. They co-cultured HepG2 and human umbilical vein endothelial cells (HUVEC), resulting in the formation of a prevascularized spheroid. HUVECs are present throughout the hepatic spheroid and formed small lumen structures. However, this spheroid is not perfusable as the vasculature inside is not easily accessible.

Sasaki et al. also showed prevascularized liver tissues using a layer-by-layer fabrication method with hepatocytes and neonatal human dermal fibroblasts precoated with fibronectin and gelatin [48]. They confirmed that such prevascularized tissues could connect to the host vasculature after transplantation into mice by measuring human albumin level from mouse

serum. Liu et al. created a high cell density liver model using an aggregation of many spheroids coated with endothelial cells. They demonstrated perfusion of culture medium into this dense hepatic tissue using an endothelialized alginate hydrogel fiber *in vitro* [49].

Takebe et al, demonstrated emergent morphogenesis of a vascularized and functional liver organoid using iPS cell-derived hepatocytes, HUVECs and MSCs [50] [51]•• (Fig. 1f). They cocultured these three types of cells in Matrigel and the cells spontaneously aggregated due to the contractile forces of MSCs and subsequently became a prevascularized liver bud. The transplantation of this prevascularized bud into mice with hepatic failure significantly improved their survival.

Opinion

Prevascularized hepatic tissues from iPS cell-derived hepatocytes are adequate for most tissue engineering applications. Many groups have shown that such prevascularized tissues rapidly connect to host vasculature and support liver function. However, systems that meet the requirements for applications in drug development, vascularization and bile-duct-genesis of hepatic spheroids and organoids to maintain advanced hepatic function and metabolism *in vitro* are still a major unmet need.

Skeletal muscle

Widely used 3D skeletal muscle models include engineered muscle tissues formed by suspending myoblasts in a 3D ECM such as collagen or fibrin, and anchored to velcro or PDMS micro-pillars to measure force output [52] [35] [53] [54] (Fig. 1h(i)). Such models have been used to elucidate disease mechanisms. For instance, Lee et al. used a bioengineered 3D skeletal muscle model using primary mouse myoblasts in a collagen/matrigel based tissue to show that skeletal muscle atrophy results from length shortening [52]. The next generation of skeletal muscle models also incorporate neuro-muscular junctions [55] [56] for instance with a motor neuron spheroid in co-culture with a 3D skeletal muscle tissue in a microfluidic compartmentalized device [56]. Nevertheless, the lack of a functional perfusable vasculature in these *in vitro* models also limits the size and density of engineered skeletal muscle tissues. Furthermore, this may lead to under-representation of key vasculature-associated signaling mechanisms as there is a high capillary density in skeletal muscle *in vivo*. This could contribute to the neonatal sarcomeric protein isoforms in *in vitro* muscle [57], and also account for the small size of each muscle cell because of key missing angiocrine signaling.

Prevascularized *in vitro* skeletal muscle tissues when implanted into mice abdomen or legs become vascularized by the host [18] [19] [58] (Fig. 1h(ii)). These could serve as potential vascularized skeletal muscle models but are quite tedious and may not have the same level of control as purely *in vitro* engineered tissues. Dacha et al. have shown the first engineered skeletal muscle with endothelial cell networks, by co-culturing human skeletal muscle progenitor cells with human umbilical vein endothelial cells. However, these networks are little more than a system of connected cells [59]; perfusability of this network was not demonstrated.

Opinion

A fully vascularized and perfusable skeletal muscle tissue has not yet been engineered in *in vitro* systems to date. The use of next generation technologies such as 3D bioprinting, molding techniques to incorporate vascular tubes within engineered muscle tissue similar to Vollert et al. [41]••, angiogenesis/vasculogenesis, vascular beds with prevascularized cell sheets have potential to solve the remaining issues for skeletal muscle models.

Future outlook

We have summarized the state-of-art in vascularized micro-tissues for many major organs. Some, such as pancreatic islets, have very few reports in spite of important applications such as insulin regulation and insulinomas. Takebe et al., demonstrated prevascularized tissues with β-cells and mesenchymal cells [60] (Fig. 1g). This method was adapted from the same method used to create a liver bud as discussed earlier. Recently, there has also been progress in bioprinting of a vascularized thyroid gland [61]. Overall, there has been rapid progress in this field, and future advances in technologies such as 3D bioprinting and novel biomaterials are likely to further accelerate the work in terms of reproducing the cellular heterogeneity and complex 3D structure of *in vivo* tissues in *in vitro* micro-fluidic platforms. The combination of fundamental technologies for vascularization [10,16] [17,62] and organoid engineering [20, 21, 60, 63] could promise to obtain functional vascularized tissues. Furthermore, cutting edge genome engineering techniques will be transformative in creating genetic disease models using these *in vitro* micro-tissue platforms. With rapid progress in iPS cell differentiation technologies and manufacturing processes, we will start to see more personalized medicine applications of such chips, particularly in areas such as drug screening for patient-specific tumor (to replace time consuming patient-derived xenograft, or PDX, models).

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• of special interest

•• of outstanding interest

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Highlights

- Several viable methods currently exist to create vascularized in vitro tissues.
- Progress has been made in developing systems that mimic specific organs or tissues.
- New technologies such as 3D printing have great potential to advance the field.
- A major challenge is to vascularize organoids grown from human cells.
- Applications include drug screening or growing human implants.

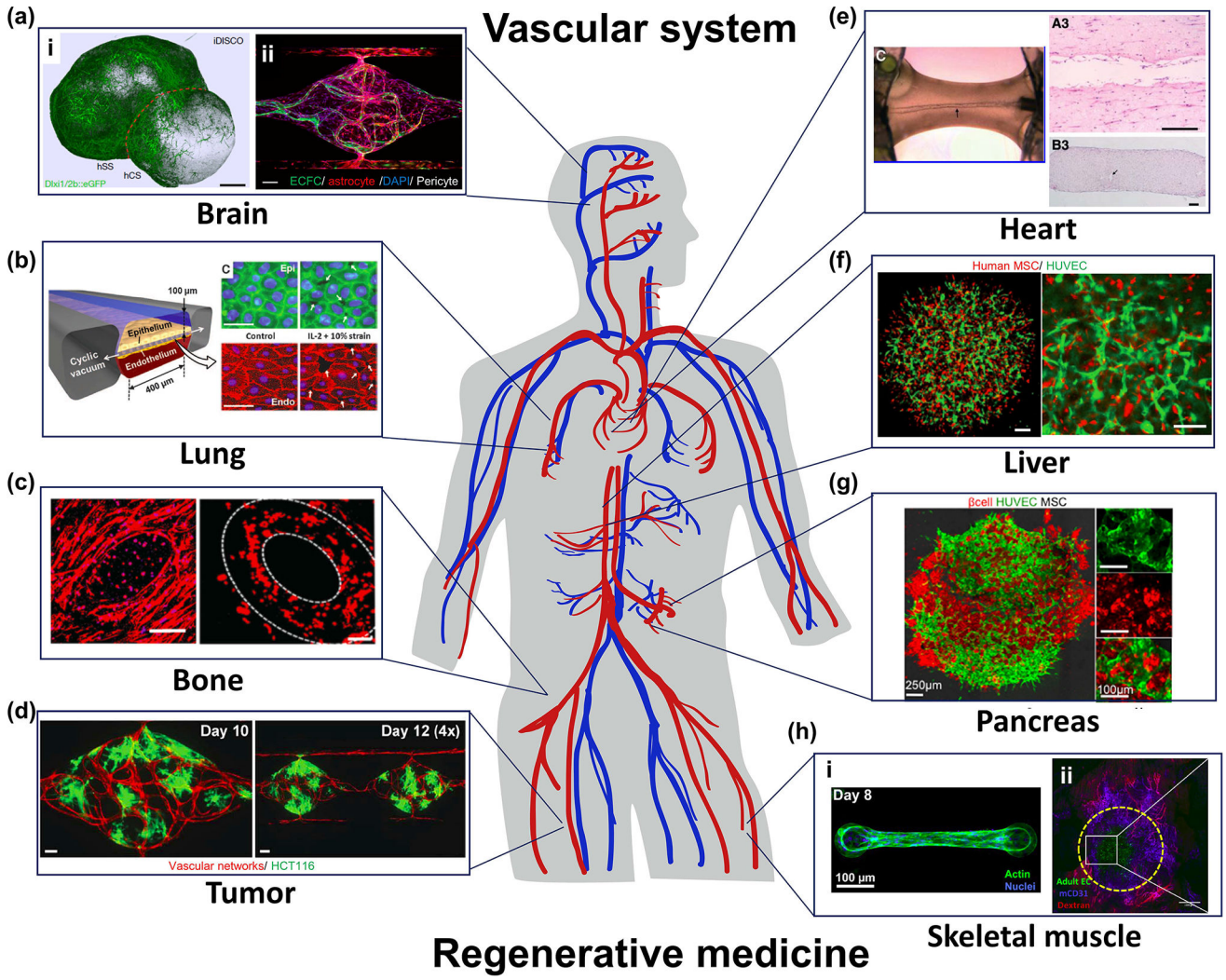

Figure 1.

State-of-the-art of prevascularization and vascularization of micro-tissues in brain (a), lung (b), bone (c), tumor (d), heart (e), liver (f), pancreas (g) and skeletal muscle(h). (a) (i) Fabrication of multi-cellular spheroid by fusing iPS cell-derived human cortical spheroids (hCS) and human subpallium spheroids (hSS) to mimic forebrain-like tissues. (ii) A microfluidic vascularized 3D BBB model developed by co-culturing colony-forming endothelial cells, astrocytes and pericytes in a perfusable microdevice. (b) A microfluidic epithelial and endothelial interface model to simulate the lung alveolar microenvironment. (c) Vascularized bone graft by using 3D bioprinting with biocompatible scaffold. (d) Vascularization of tumor tissues in a perfusable microfluidic platform. (e) Engineering endothelialized hollow-structures in a cardiac muscle fiber *in vitro.* (f) Prevascularized functional liver bud by spontaneous aggregation of human iPS cell-derived hepatocytes, human umbilical vein endothelial cells (HUVEC) and mesenchymal stem cells (MSC). (g) Prevascularized islet-like tissues with pancreatic β cells, HUVECs, and MSCs. (h) (i) Representative skeletal muscle fiber bundle attached to cantilevers in a microdevice. (ii)

Prevascularized skeletal muscle tissue aggregation and anastomosis of prevasculature with host vasculature after transplantation.

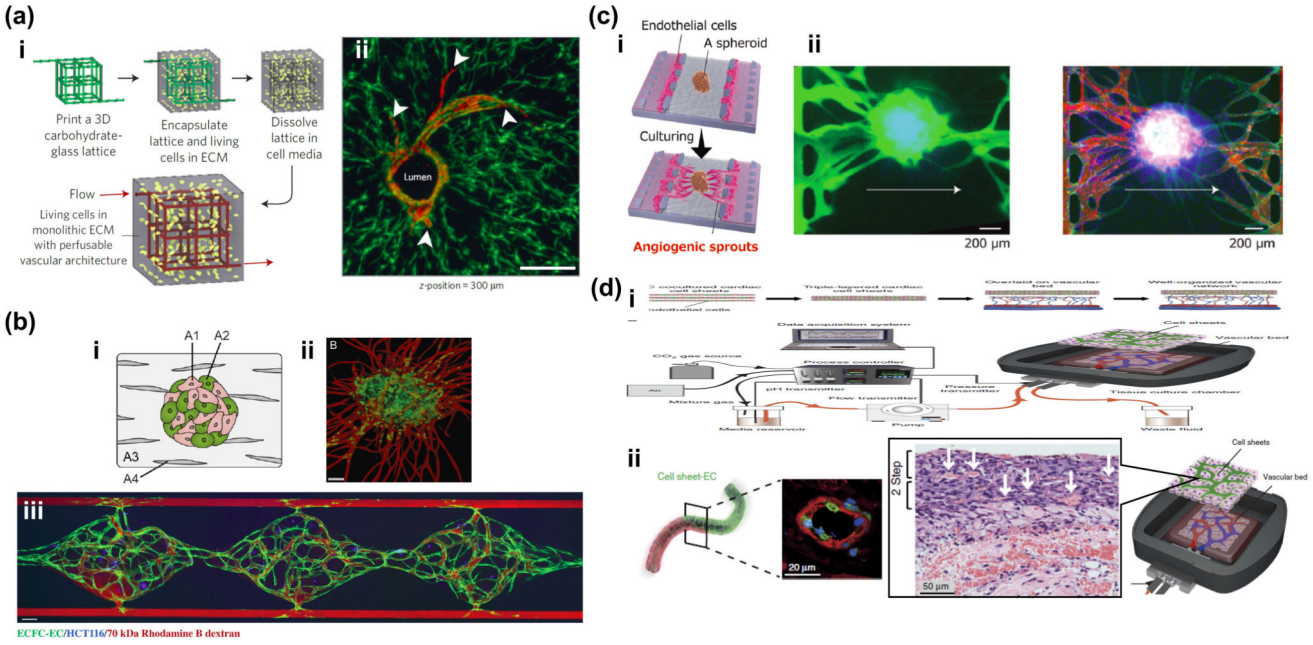

Figure 2.

Cutting-edge technologies to vascularize tissues *in vitro*. (a) Engineering of 3D vascular networks in extracellular matrix. (i) 3D casting mold was fabricated with carbohydrate glass lattice by 3D printer. After dissolving the lattice, endothelial cells were seeded into the 3D microchannels. (ii) Endothelialized microchannels and angiogenic sprouting with stromal cells. Potentially, this method could be applied to vascularization of various types of tissues, even relatively large tissues. (b) Vasculogenesis based vascularization in a microfluidic device. Flow of fluorescently tagged dextrans show the perfusability and connectivity of vascular networks to supply sufficient nutrients to HCT116 cells in a gel. This strategy is promising for high-throughput drug screening of vascularized micro-tissues (c) (i) Angiogenesis based vascularization of spheroid in a microfluidic device. (ii) Perfusion of fluorescently tagged dextrans showed that of angiogenic sprouts formed anastomoses with prevascularized networks inside the spheroid, preventing necrotic cell death in the core region. This method could be applied to vascularization of various types of spheroids and organoids for drug testing. (d) Vascularization of sheet-like tissues using an *ex vivo* vascular bed. Rat vascular bed was extracted and connected to a perfusion system *in vitro*. Stacked cell sheet with epithelial cells and endothelial cells were placed on this vascular bed. This *ex vivo* system has promise for the extension of culture time which is currently limited by the lack of vasculature (ii).