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New and Improved Tissue Engineering Techniques: Production of Exogenous Material-Free Stroma by the Self-Assembly Technique

Ingrid Saba, Weronika Jakubowska, Stéphane Chabaud and Stéphane Bolduc

Additional information is available at the end of the chapter

Abstract

Tissue engineering results from the use of cells and scaffolds to reproduce structural and spatial organization or function of a tissue. The Production of an ideal engineered tissue depends on its designed purpose. For clinical applications, the main concerns are biocompatibility and the generation of a tissue able to mimic most of its original biological functions. Moreover, the viability of an implanted tissue is associated with its stability to support vascular networks. This chapter summarizes the theory of the self-assembly approach for tissue engineering. Adjustments and modifications in stromal thickness and extracellular matrix composition for various self-assembled tissues are discussed. Methods developed to generate tissue closely mimicking the native morphology and structure, to incorporate capillary-like networks, and to reduce production time and costs are also reviewed. The self-assembly technique leads to the production of a stroma free of exogenous material and can be adapted to generate fastest, inexpensive, and near-to-native tissue bioengineering for medical and fundamental research applications.

Keywords: Tissue engineering, Self-assembly, Stroma, Epithelial cells, Endothelial cells

Abbreviations

AGE	Advanced glycation end-products
Arg	Arginine

ASC	Adipose tissue-derived stem cells
EC	Endothelial cells
ECM	Extracellular matrix
HA	Hyaluronic acid
HMVEC	Human microvascular endothelial cell
HUVEC	Human umbilical vein endothelial cell
L–Arg	L–arginine
LOX	Lysyl oxidase enzymes
LOXL	Lysyl oxidase homologues
LPA	Lysophosphatidic acid
MMP	Matrix metalloproteinases
MSC	Mesenchymal stem/stromal cells
NG2	Neuron glial-2
PLGA	Poly-lactic-co-glycolic acid
SVF	Adipose stromal vascular fraction
TGF-β	Transforming growth factor-beta
TIMP	Tissue inhibitors of metalloproteinases
VEGF	Vascular endothelial growth factor
2D	Two-dimensional
3D	Three-dimensional

1. Introduction

The extracellular matrix (ECM) is present within all tissues and organs. It constitutes the noncellular microenvironment around the cells that plays an important role in modulating their behavior and functions [1]. This elaborated milieu is very dynamic and extremely adaptable [2–4]. ECM is composed of several components that include proteoglycans, as well as collagen proteins and noncollagenous glycoproteins. Each component has several subcategories of molecules that influence the ECM physical and biochemical properties [5].

1.1. ECM deposition and assembly

The homeostasis of epithelial tissues depends on a dynamic interaction of the stroma components, such as fibroblasts, adipocytes, and nonactivated immune players [6]. In fact, fibroblasts were reported to secrete and organize type I and type III collagens, elastin, fibronectin, tenascin, and a repertoire of proteoglycans (hyaluronic acid (HA) and decorin), that maintains interstitial ECM integrity [7]. The ECM is constantly remodeled to allow the healthy tissue to

resist to a wide range of tensile pressures [8, 9]. This remodeling occurs through the synthesis of elastin, which originates secreted tropoelastin, the precursor of elastin, that assembles into fibers and becomes cross-linked on lysine residues by members of the lysyl oxidase (LOX) enzymes and lysyl oxidase homologues (LOXL) molecules [10]. LOX and LOXL catalyze the first step in the formation of collagens and elastins, a very conserved process that plays an important role in cell growth, chemotaxis, or sprouting of new blood vessels [11, 12]. On the one hand, coordinated secretion of matrix metalloproteinases (MMPs) by fibroblasts mediates ECM remodeling [13]. On the other hand, the mesh networks are counterbalanced by tissue inhibitors of metalloproteinases (TIMPs) [14] or by other enzymes such as LOX molecules and transglutaminases that stiffen the ECM [10].

2. Cellular players and ECM production

2.1. Mesenchymal stem/stromal cells

Adult mesenchymal stem/stromal cells (MSCs) are found in all postnatal organs and tissues, and they play important functions in tissue injury repair and general homeostasis [15]. These cells are one of the principal adult stem cells and the most promising tool for regenerative medicine because of their sustained proliferative capacity and their multipotent differentiation potential [15–17].

2.1.1. Fibroblasts

Fibroblasts are nonterminally differentiated mesenchymal cells derived from the embryonic mesoderm [18]. They are found in the connective tissue, a tissue that supports the whole body. Fibroblasts are spread in the ECM containing fibrous proteins and gel-like substances. In fact, fibroblasts produce the ECM proteins, such as fibrous collagen and elastin, as well as adhesive proteins such as laminin and fibronectin. Fibroblasts are also the major source of glycosaminoglycans (hyaluronan and glycoproteins) [19]. Interconnecting meshworks of extracellular protein fibers and connector proteins provide the architectural tissue structure. Moreover, this milieu forms the connections needed for cellular migration of fibroblasts, immune cells, and endothelial cells (ECs) during angiogenesis [19].

Most tissues are composed of a simple or multiple layers of epithelial cells that exhibit an apical–basal polarization. The basal part is in contact with the basement membrane, whereas the apical side is oriented toward the fluid-filled lumen [20]. Fibroblasts form a basement membrane, composed of a layer of basal lamina and a layer of reticular lamina. This basement membrane serves essentially as a structural scaffold that maintains the dynamics of a three-dimensional (3D) engineered tissue. It is also critical for tissue regeneration in wound healing and acts as a cell barrier. The basement membrane acts as a cell barrier by segregating epithelial cells from endothelial cells (ECs), thus preventing tumor invasion or metastasis.

2.1.2. Myofibroblasts: functions and origins

Myofibroblasts, or activated fibroblasts, are contractile, resistant to apoptosis and have an upregulated rate of matrix deposition. They also express different cytokine and chemokine receptors that enable fiber regulation and wound contraction at injury sites [21–24]. In this sense, activated fibroblasts not only favor wound healing, but can also cause injuries when their activation is uncontrolled, producing a pathological fibrotic response [25, 26]. The precursor of myofibroblast is not precisely known, but many cells can differentiate into myofibroblasts through different signaling pathways or gene regulation. Some of these precursors include epithelial cells, ECs, pericytes, multipotent monocytes, and fibroblasts.

2.2. Adipose tissue–derived stem cells

Fat is an abundant and accessible source of stem cells. Adipose tissue–derived stem cells (ASCs) include preadipocytes, and a subpopulation of stromal cells able to differentiate into multilineages, including neuronal cells, chondrocytes, and osteoblasts [27, 28]. Moreover, these MSCs are able to secrete cytokines and growth factors promoting regenerative processes because they can influence cell recruitment, proliferation rates, or inhibit apoptosis [29, 30]. ASCs extracted from liposuctions can be expanded in culture and used as building blocks for tissue engineering. Both connective and adipose tissues were engineered in vitro using ASCs [31–33]. Both allogeneic and xenogeneic ASCs can be transplanted in patients regardless of their immunocompatibility and without the need of immunosuppression therapy, making them an unlimited source for regenerative medicine applications [34].

3. Tissue engineering history and techniques

As medical treatments and expanded lifespan expectancies in both males and females have improved, the number of individuals waiting for organ transplants or blood vessel bypasses is constantly increasing but the availability of organs does not often match the demand. To circumvent this shortage in organ and tissue supplies, many efforts in cell culture methods were deployed to engineer tissues that could be used as an alternative therapeutical option.

3.1. Self-assembly technique

The self-assembly technique is based on the ability of MSCs to secrete and organize their own ECM to produce sheets. This tissue engineering method allows the production of autologous living tissues, free of exogenous biomaterials [35–37]. The self-assembly technique has exploited the inherent characteristics of MSCs to produce ECM. For instance, it was well documented that ascorbic acid, a vitamin C derivate, promotes collagen protein synthesis and deposition of sulfated glycosaminoglycans in human skin substitutes [38]. Once fibroblasts supplemented with ascorbic acid are cultured for 21–35 days, they form sheets of matrix where stromal cells are embedded within [35]. These sheets can be peeled from the culture dish and superimposed. The superimposed layers are maintained for an additional week for further

cell-matrix reorganization and layer fusion (**Figure 1A**). Holes can be made in the multilayer dermal equivalent and hair follicles can be added to mimic the presence of native skin component. An additional culture time is required before seeding keratinocytes. Thereafter, the skin equivalent is maintained for 21 days at an air–liquid interface to induce the cornification of the epidermis [36, 39].
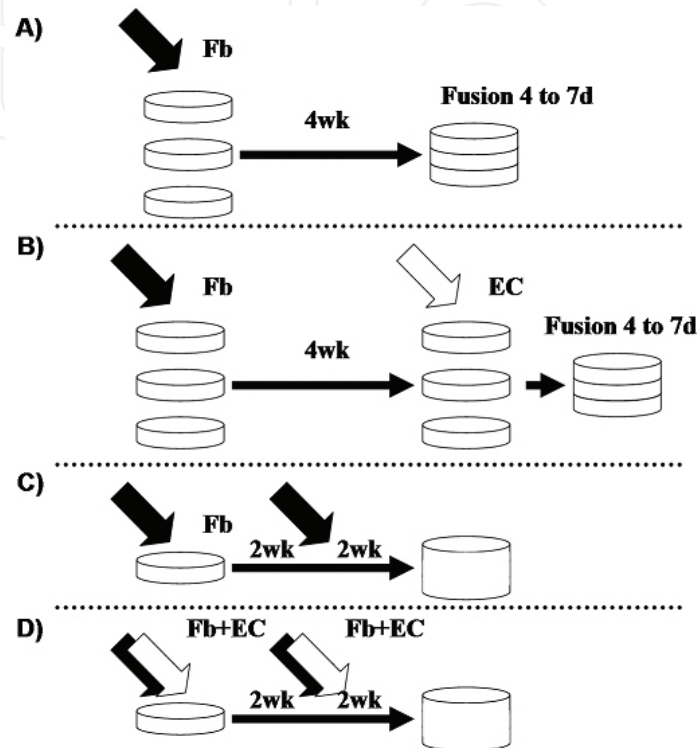

Figure 1. Schematic representation of self-assembled tissues. **A**) The classical self-assembly technique requires stacking of three fibroblast (Fb) cell sheets that are cultured for 4 weeks in the presence of ascorbic acid. Sheet fusion lasts 4 to 7 days. **B**) After 4 weeks of Fb cultures, the stroma is seeded with endothelial cells (ECs) and then a fusion set is carried on to generate endothelialized tissues. **C**) Modification to the self-assembly technique consists of an initial Fb culture for 2 weeks followed by an additional Fb reseeding. The culture is maintained for an additional 2 weeks before using the stroma without stacking. **D**) Fb and EC are co-seeded in order to generate endothelialized tissues with a 3D capillary-like network.

3.1.1. Engineered human skin substitutes

One of the great breakthroughs in medicine was achieved by engineering human skin substitutes for grafting purposes. Self-assembled skin substitutes were generated by extracting the patient's own cells, thus avoiding immunological incompatibility problems upon grafting. These skin substitutes were characterized and showed a fully differentiated epidermis, structural and morphological resemblance to native human skin. Moreover, these in vitro engineered tissues were able to deliver cytokines, chemokines, and growth factors at the grafted site, improving the wound closure [36, 40–43]. Because of all these characteristics, self-assembled human skin is clinically used for wound healing and burn treatments [44, 45]. Self-assembled skin substitutes possess a near-to-native architecture and maintain their cell growth

potential and matrix deposition. Therefore, these equivalents are free of exogenous material, cytotoxicity, and have clinically reduced morbidity in burnt patients (reviewed in Refs. [46, 47].

Over the years, self-assembled skin substitutes were also produced from extracted cells of patients having psoriasis [48]. The reconstructed tissues from psoriatic donors represent an ideal model to study one of the most common human skin diseases. In fact, this model outlines the excessive growth and aberrant differentiation of keratinocytes. It offers a reliable in vitro mean to measure the efficacy of appropriate treatments, perform tests directly on human primary cells, and avoid animal use [48–52]. Furthermore, self-assembled skin substitutes were used to extensively characterize cellular and molecular players involved in the pathogenesis of hypertrophic scars and scleroderma [53–55]. More recently, a skin substitute derived from patients diagnosed with amyotrophic lateral sclerosis (ALS) was similarly generated. As one of the early perturbations in ALS patients is skin alterations that often precede the neurological symptoms, this human skin model is designed to better identify disease-specific biomarkers and early diagnostic tools to monitor disease progression [56].

3.1.2. Cardiovascular tissues

The self-assembly technique was customized to engineer human blood vessel that displayed excellent physiological and mechanical properties without the need for any exogenous scaffold [35, 57]. Blood vessels are constituted of a functional endothelium seeded onto an internal membrane of human skin fibroblasts. In order to mimic the shape of a blood vessel, a smooth muscle cell (SMC) sheet is first rolled, followed by the fibroblast sheet around a cylindrical
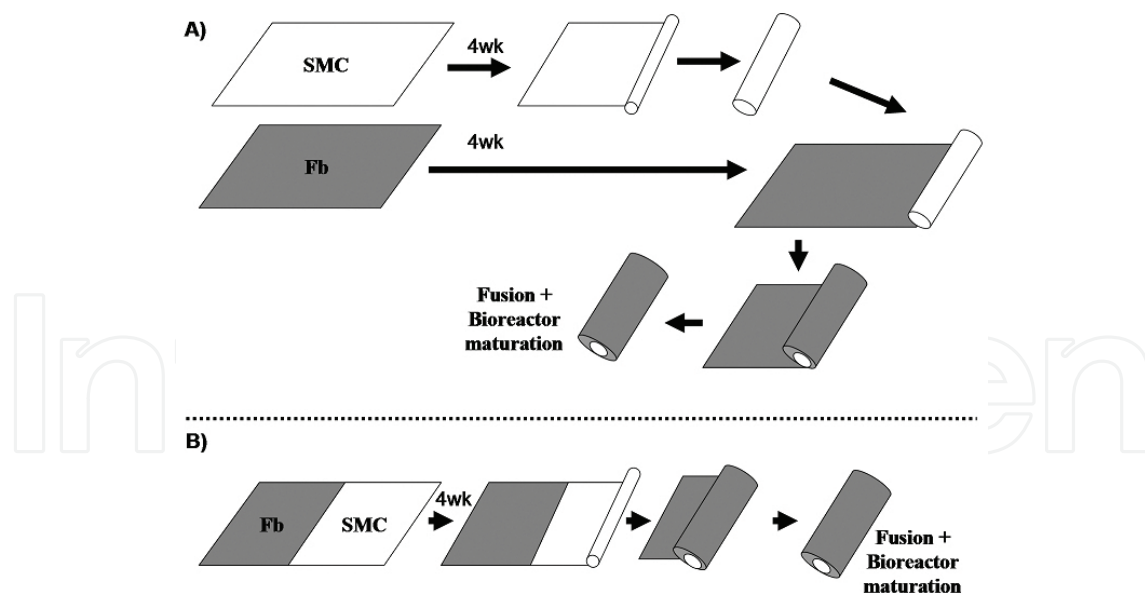

Figure 2. Vascular self-assembled tissue. **A**) A sheet of smooth muscle cells (SMC) (first) and a sheet of fibroblasts (Fb) (second) are rolled around a cylindrical support after 4 weeks of culture. The construct is allowed to fuse and mature in a bioreactor. **B**) Modification to the technique shown in (**A**) consists of co-seeding SMCc and Fb each at an extremity of the same sheet, which is rolled around a cylindrical support after 4 weeks of culture and then allowed to fuse and mature in a bioreactor.

support, and cultured until fusion (**Figure 2A**). Analyses of these in vitro engineered vessels confirmed the presence of numerous ECM proteins (collagen types I, III, IV, laminin, fibronectin, and chondroitin sulfates) and a functional endothelium [35, 58, 59].

Progress in developing self-assembled valves was reported over the years [60, 61]. Valve leaflets made of self-assembled tissue sheets can organize into a characteristic three-layer structure featuring appropriate dynamic fluidics [60]. This tissue remains to be grafted into living recipients in order to assess in vivo survival and behavior of the transplanted valve. Nevertheless, this stentless bioprosthetic offers a great alternative to artificial valves for cardiovascular surgeries [60, 61].

3.1.3. Cornea

Corneal tissue engineering was developed in an attempt to cure corneal opacity by replacing the damaged area with a clear substitute. Proulx *et al*. [62] generated a self-assembled three-layer equivalent of human cornea. These substitutes presented near-to-native stromal, endothelial, and epithelial morphology with an intact basement membrane filled with laminin V and collagen VII proteins. The differentiated epithelial layer had defined basal and wing cells that expressed Na^+/K^+ ATPase $\alpha 1$ protein, keratin 3/12, and basic keratins. This human cornea model was also used to study the pattern of MMP genes expression during corneal wound healing [63].

3.1.4. ASCs for the production of adipose tissues and other connective tissues

Adipose cell sheets can be generated in vitro using the self-assembly technique supplemented with ascorbic acid and adipogenic differentiation factors. These sheets share many adipocyte features [64]. ASCs have the ability to respond to media composition and motion allowing them to be an optimal cell type for tissue engineering. Using the self-assembly technique, fully autologous vascular tissues were also engineered from ASCs in vitro, with an organized structure and matrix components [65]. Other studies showed that ASCs could be used to bioengineer near-to-native skin [31] and bladder mucosa equivalents [66] in vitro.

3.1.5. Urogenital tissues

In vitro reconstruction of a bladder substitute using the self-assembly technique was first documented by Magnan *et al*. [67], where a single porcine biopsy was processed and allowed the generation of an endothelialized bladder equivalent. Subsequently, seeding urothelial cells on a dermal fibroblast stroma generated tubular urethral grafts for in vivo replacement. To mimic in vivo tissue architecture, the engineered construct was placed under perfusion in a bioreactor [68]. ECs were also added to the model [69]. The human tissue–engineered bladder model can be used to screen common prescribed medicine. For instance, this model was used to study ketamine, an anesthetic agent and a drug used in chronic pain management, which is excreted in the urine. The drug application on the 3D bladder model showed that ketamine directly damages the urothelium, especially the structure and the interconnections that characterize the intermediate epithelial layers, by inducing apoptosis [70].

4. Improvements of stromal thickness and mechanical properties of self-assembled tissue

The self-assembly technique has great therapeutic potential because it uses autologous cells that produce their own ECM, thus reducing allogeneic graft rejection. Although the self-assembly approach is suitable for clinical applications, the time required for tissue reconstruction and the costs are important drawbacks hindering its wider use. Hence, many strategies to reduce tissue reconstruction time and the cost associated with cell culture were investigated. Efforts to stimulate collagen deposition and matrix reorganization are detailed in this section.

4.1. Mechanical stimulation

Mechanical stimuli induce major biological modifications in the organization of the cells cytoskeleton and their ECM composition [71, 72]. For instance, the mechanical stimulation of blood flow induces the realignment of collagen fibers and strengthening of the tissue [73, 74]. At the molecular level, these changes are triggered by the activation of mechanoreceptors such as the ones containing the Arginyl-Glycyl-Aspartic acid attachment site that bind to integrins [75]. This mechanical stimulation often results in activation of extracellular signal–regulated kinase, ERK, and the c-Jun N-terminal kinase, JNK, signaling pathways that will induce cellular responses in order to adapt to new environments [75]. In some studies G-proteins seem to be also involved in the molecular signaling [76]. In response to mechanical stimuli, cells can also secrete growth factors such as transforming growth factor-beta (TGF-β) [77] that will exert paracrine or autocrine functions. Furthermore, cells can secrete and/or activate latent MMPs and other proteases [78, 79], which affect the balance between synthesis of ECM elements and their degradation. Accordingly, fibrosis was observed in mechanically overstimulated settings emphasizing that increased collagen deposition rates need to be controlled in order to remain reversible [80].

4.1.1. Improved blood vessels generation

The quality of engineered vascular tissues can be improved in a bioreactor by applying the appropriate laminar/cyclic flow. Other modification to the self-assembled blood vessel generation, such as co-seeding fibroblasts and SMCs, each at their respective half of the same sheet, before rolling around a cylindrical support [81] (**Figure 2B**) was reported. The fully autologous vascular substitutes possess high-grade mechanical strength to sustain engraftment and are readily available when needed without any immunosuppressive treatments [81–83].

4.1.2. Specific culture surface can influence fiber alignment in engineered tissues

Tissue functions can be improved using microstructured surfaces that control the interactions between cells and the ECM. With the use of a specific surface topography on an elastomeric material, it was observed that the first cell layer followed the same patterns and orientation as

the material. Subsequently, this orientation influenced the second cell layer to follow a physiologically similar alignment mimicking the structure of the native tissue. Furthermore, secreted ECM followed cell orientation in every layer, resulting in very well-structured self-assembled sheets for cornea, vascular, and dermis. A micropatterned surface on which cells are seeded have the capacity to generate multiple layers, in which cells and the ECM spontaneously organize in patterns consistent with the original tissue [84].

4.1.3. ASCs cultures in dynamic conditions

In order to reduce culture time required for tissue production, human ASCs were used to replace dermal fibroblasts in some self-assembled tissues. Self-assembled stromas generated with dermal fibroblast or ASCs can be subjected to static or dynamic conditions [85], as they can be mechanically stimulated on a 3D shaker platform. Dynamic culture conditions increased (1.5- to 2-fold) the thickness of tissues derived from ASCs compared to static conditions. Moreover, culture time could be reduced in dynamic conditions. Yet, mechanical properties of these tissues were not measured.

4.2. Enzymatic reactions and chemical stimulation

Although ascorbic acid is an essential element that contributes to collagen deposition, an increase in its concentration does not lead to enhanced collagen deposition. Ascorbic acid is an enzymatic cofactor of prolyl- and lysyl-hydroxylase [86], and its action reaches a plateau when these enzymes achieve their peak of activity. Independently of its role as a cofactor, ascorbic acid is responsible for a certain level of collagen secretion in fibroblast cultures, until it reaches its biological limits [87], albeit it can be toxic for fibroblasts if present in high dose [23].

Chemical inhibitors of MMP could also increase ECM production by restricting the extent of protease activity. Among them, galardin was used to produce self-assembled tissues and it significantly increased the thickness of treated tissues [54]. Currently, the cost associated with the use of galardin is too expensive to be a promising solution.

L-arginine (L-Arg) is converted in ornithine followed by glutamine semialdehyde and finally proline, an important amino acid that is metabolized during collagen synthesis. L-Arg supplementation to culture media was evaluated, when the stroma was produced using the self-assembly method. Although an increase in collagen synthesis and secretion (20% more collagen type-I) was observed, collagen deposition remained unchanged when compared to controls [88]. A plausible explanation would be that enzymes involved in collagen maturation were not sufficient to process the surplus of this amino acid in vitro.

4.3. Biological stimulation

Biological stimulation of ECM deposition in the field of tissue engineering is a challenge. This complexity is due to pleiotropic roles of multiple bioactive agents and their subtle effects, which could appear after a long period of time, for instance after tissue implantation. In contrary to monolayer culture studies in which experiments rarely exceed days, tissue

engineering methods can be carried out for months, especially if it involves in vivo implantation. Many proteins, peptides, and lipids can be used to stimulate collagen synthesis and deposition. Most of them are involved in fibrosis and need to be carefully handled to avoid production of pathological-like tissue.

4.3.1. Polysaccharides

Beta-glucans constitute a family of carbohydrates that stimulates fibroblasts to produce collagen [89, 90]. For instance, laminaran, a glucan from *Saccharina longicruris* seaweed, increased collagen secretion when added to dermal fibroblast culture [91]. An increase in collagen synthesis and secretion was observed in self-assembled tissues. Moreover, thicker stroma could be obtained without significant increase in cell proliferation and alpha-smooth muscle actin content, a hallmark of fibrosis [92]. The authors argued that the aggregation properties of laminaran triggered a net increase of collagen secretion without inducing a fibrotic phenotype [92, 93].

Tissue engineering often relies on glucose-rich media because glucose is the primary source of energy that allows MSCs to produce ECM compounds. Advanced glycation end-products (AGE) result from glucose metabolism and are found in elderly tissues or in diabetic patient tissues [94]. Unfortunately, the glucose concentration used during the production of most self-assembled tissues is too high. Consequently, it was reported that AGE are involved in the process of skin aging, which has an impact on mechanical and biological parameters [95]. New approaches to circumvent this issue are currently being developed and should generate promising alternatives.

4.3.2. Insulin and hypoxia

In addition to mediating glucose entry in cells, insulin also plays an active role in collagen synthesis and deposition [96]. Insulin has a long history of safety use for human therapies and microencapsulated insulin-secreting cells in hydrogels can improve collagen fiber density in diabetic mouse models [97]. Poly-lactic-co-glycolic acid (PLGA) alginate structure that releases insulin in rats was also found to increase collagen deposition and maturation [98]. In a clinical setting, wound healing is problematic for diabetic patients because their insulin metabolism is altered. Also, their tissues are less irrigated because of microvascular network changes caused by the loss of ECs. When capillary networks are altered, the surrounding tissues undergo hypoxia. In such an environment, fibroblasts change to a fibrotic phenotype. Fibrosis is induced by factors that are released by damaged ECs [99], as well as by other unknown mechanisms [96, 100]. Insulin and hypoxia exert a synergic effect on self-assembled tissues. They increase collagen deposition as demonstrated by tests on human and animal cell cultures [101] (unpublished data). Nevertheless, long-term effect of hypoxia exposure (more than 2 weeks) induced acidification of the cell culture medium and a thinning of the engineered tissues [102]. Hence, cyclic hypoxia seems a better alternative than constitutive hypoxia because it produces thicker tissues in vitro.

4.3.3. Adenosine

Adenosine and other derivatives have been used to enhance the rate of wound healing [103]. Their receptors were also found to be involved in fibrosis. Activation of A_{2B}-adenosine receptors resulted in an increase of collagen synthesis and a decrease in MMP-9 activity [104, 105]. This molecule was successfully tested to produce rabbit tissues by the self-assembly approach [101]. Effects of adenosine on human cultures remain to be evaluated.

4.3.4. Lysophosphatidic acid

Lysophosphatidic acid (LPA) is a bioactive lipid found in blood. LPA binds to its receptors at the surface of many cells and activates pathways leading to proliferation, migration, and secretion of cytokines. LPA expression is upregulated in disease conditions such as in fibrosis and cancer or cancer [106, 107]. As LPA is naturally present in human blood, it was used in vitro and approved by regulatory agencies. LPA-cultured fibroblasts showed increased collagen type-I and fibronectin deposition in a dose-dependent manner that could be completely reversible. No adverse effects were noted: alpha-smooth muscle actin was not overexpressed and cell proliferation rates remained normal [108]. Thicker stroma and enhanced collagen deposition kinetics suggested that the production time could be reduced by 25% when LPA was added to the cell culture medium.

5. Bioengineering substitutes that resemble native tissues

Classical self-assembly technique involves sheet stacking in order to generate a tissue with sufficient mechanical strength. The superimposition of sheets Influences cell distribution. Although fusion of all sheets occurs following sheet stacking, a pattern at the site of each sheet fusion remains visible. Epithelial cell seeding has been noted to reduce sheet demarcations after sheet stacking. Nevertheless, different layers are visible in the 3D self-assembled tissue, Which does not correspond to native stroma architecture and weakens tissue mechanical strength [31, 66, 85, 88]. To outwit this issue, a newly reseeding self-assembly protocol was elaborated and allowed a more uniform distribution of cells throughout the tissue without delineation marks [88].

5.1. Reseeding self-assembly technique

Ascorbic acid triggers collagen deposition that reaches a plateau level after 2 weeks of fibroblast culture [88]. This time period also correlates with the thickness reached by self-assembled tissue [85]. When fibroblasts reach confluence, the cells begin to secrete and deposit collagen to form the ECM, a step that lasts 2 weeks before collagen synthesis rate decreases. These observations led to the generation of engineered tissues by reseeding of cells instead of sheet stacking (**Figure 1C**). The new reseeding approach is based on the fact that a second layer of fibroblasts seeded onto the first sheet will concomitantly induce a transitory peak of MMP activity and a boost of collagen secretion. The fibroblasts in the first sheet play a role in this

remodeling, and after an additional 2 weeks of culture, the reseeding process results in the generation of a stroma with the same thickness as the one obtained by classical stacking of three sheets produced without reseeding. This dense stroma supported the development and maturation of the epithelium [88]. The reseeding technique offers a remarkable alternative to the classical self-assembly protocol because it is faster and it reduces costs associated with extensive culture medium consumption as well as material [88] (**Figure 3**).
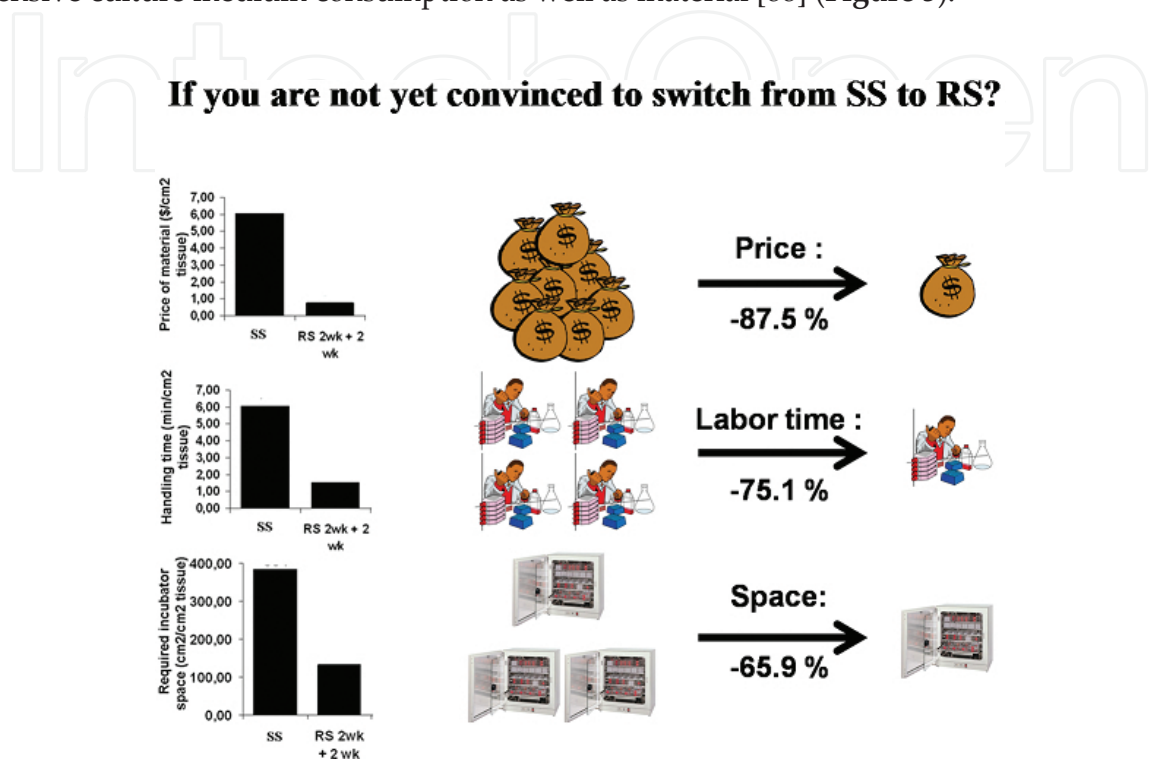

Figure 3. Graphical illustration of the improvements to the self-assembly technique (SS) when reseeding of cells (RS) is applied. Reseeding allows the reduction of costs, handling time, and incubator space requirements throughout the steps of cell culture.

5.2. Organ-specific stroma

Over the years, it has been shown that the origin of mesenchymal cells has a direct impact on the quality of bioengineered tissues. Carrier *et al*. [109] showed that reconstructed human cornea substitutes had great macroscopic and histological differences; especially in the corneal epithelium thickness and differentiation whether dermal fibroblasts or keratocytes (corneal fibroblasts) were used to produce the ECM. Constructs made with dermal fibroblasts were less transparent and lacked ultraviolet absorption characteristics compared to corneal tissues which were produced using autologous keratocytes [109]. The importance of this cross talk was further evaluated by mass spectrometry analyses performed on human stromal and epithelial layers of corneal substitute. Not only is the origin of mesenchymal cell important, but a fully differentiated and stratified epithelium is required for appropriate ECM synthesis and organization. This reciprocal regulation between the ECM and epithelial components is initiated as epithelial cells adhere to the stroma, they emit a continuous signal that mediates ECM remodeling accordingly [63].

Modifications to the original protocol have generated near-to-native self-assembled bladder and urethral human tissues. Many improvements include mechanical stimulation [68], the use of autologous human stromal cells, urothelial cells, urine[110, 111], and a new proposed reseeding technique of stromal cells [88]. In particular, the absence of an air/liquid interface and the presence of urine allowed the new bladder mucosa model [110] to be continuously cultured in submerged conditions. Consequently, these modifications generated a bladder model that preserved the best urothelial cell properties and uroplakin distribution [110, 111].

5.3. Importance of blood vessels for tissue homoeostasis

Blood vessel formation can occur through two distinct mechanisms: angiogenesis and vasculogenesis. Vasculogenesis involves the recruitment of progenitors of ECs from the bone marrow, which leads to the formation of a vascular plexus *de novo*, whereas angiogenesis occurs when pre-existing vessels form new branches or sprouts [112, 113]. Finally, inosculation of a tissue is the anastomosis of the pre-established capillary-like network within the graft or engineered tissue with the host's vasculature [114].

ECs are in a stable quiescent state, however, they can become activated upon angiogenic stimuli in engineered tissues [115]. For instance, in response to conditions, such as tissue ischemia or chronic hypoxia, new collateral vessels can grow. Endothelium proliferation is stimulated by growth factors such as vascular endothelial growth factor (VEGF) that induce sprouting of new blood vessels. Additionally, proangiogenic signals increase MMP activity that prompts ECs to break apart their basement membrane allowing sprouting [116].

5.3.1. Vascularization strategies in tissue engineering

ECs are a promising angiogenic cell source for therapeutic vasculogenesis because they have the potential to proliferate and rearrange themselves into functional capillary-like networks. Human umbilical vein endothelial cells (HUVEC) are an important source of ECs widely used in vasculogenesis [117]. Advantages of this cell type use are the noninvasive cell source, the profusion of medical wastes, and the impressive source of ECs in umbilical cords and placental tissues. Although the therapeutic use of HUVECs is limited because of their allogeneic nature, they remain a valuable EC source for basic and applied research needs [117].

To overcome this issue, the potential of ASCs for their differentiation into ECs was explored. Isolated adipose stromal vascular fraction (SVF) from white human adipose tissue is rich in adult stem cell populations, including EC progenitors. Freshly harvested SVF containing mixed white adipose stromal cells and white adipose ECs was cultured in 3D collagen hydrogels. Within the first week, the culture showed a formation of capillary network with continuous lumen [118], and after 3 weeks it gave rise to a functional 3D vascularized skin substitute that responded well to implantation in mice. This experiment demonstrates the synergy of vascular and stromal cells in blood network formation de novo. Hence, white ASCs Demonstrate promising results with minimal cell handling. Finally, human microvascular endothelial cells (HMVEC), which originate from small superficial capillaries, also represent a promising avenue for tissue endothelialization. These cells have been incorporated in in vitro

models using the self-assembly method and formed vascular networks with lumen [115]. HMVECs are an easily accessible source because they can be derived from a skin biopsy or any other tissue. The use of HMVECs could be particularly suitable for therapeutic application because it is best adapted for organ specific reconstructs.

5.3.1.1. Vascularization of self-assembled tissues

The co-culture of dermal fibroblasts and keratinocytes with HUVEC on a chitosan/collagen sponge showed the establishment of a capillary-like network similar to the microvasculature found in vivo [119]. Prevascularization of tissues prior to implantation has yield impressive improvements in regenerative medicine. In 2005, human endothelialized reconstructed skin models revealed an important reduction in the delay of functional vascularization after implantation in mice. Early signs of vascularization were observed in the endothelialized human skin grafts within 4 days following tissue implantation, as opposed to 14 days in the nonendothelialized reconstructed skin. Mouse blood vessels were only detected after 14 days in both models demonstrating that neovascularization is a latter process. The uniform distribution of ECs across the reconstruct ensures adequate perfusion of the entire graft. The colocalization of human and host mouse ECs inside a human capillary within the graft suggests the formation of chimeric microvessels and confirms inosculation between both microvascular networks [114] (later confirmed in Gibot *et al.* [120] generated self-assembled tissue).

The progress of endothelialized tissue–engineered dermal substitutes lead to the introduction of a new in vitro model of capillary-like network formation in self-assembled skin substitutes without the use of an exogenous scaffold. In this approach, stromal sheets, formed by culturing dermal fibroblast during 4 weeks, were seeded with ECs. To generate the 3D skin, two endothelialized stromal sheets were stacked and allowed to fuse [121] (**Figure 1B**). Although a capillary network was observed, the fact that ECs were seeded in a single plane orientation, on top of the stromal sheets, resulted in a vascularized skin model with mainly a 2D vascular network rather than a 3D network. In order to provide the reconstructed skin with the optimal 3D capillary network, ECs were co-seeded with fibroblasts (**Figure 1D**). Incorporation of ECs in the reconstructed model using the reseeding technique produced a capillary-like network with increased tissue elasticity and mechanical strength [88]. Moreover, because fibroblasts were seeded at high density, ECM was readily generated and allowed the dermal stroma to be rapidly embedded with ECs [88].This vascularized stroma had pericyte-like cells that expressed the neuron-glial 2 (NG2) marker, which characterizes the surrounding of capillary-like structures.

6. Conclusions

The self-assembly approach is used to generate several tissues for fundamental and clinical research applications. Over the years, adjustments to the stroma elaboration protocols and especially the ECM generation were proposed to improve the quality of the bioengineered

substitutes. As one of the main objectives is to reduce the production time and costs, mechanical, biological, and chemical modifications were also introduced. Organ-specific ECM was associated with a better epithelial differentiation and an overall tissue architecture that closely mimics native tissues. To improve clinical applications, endothelialized tissues were generated and grafted with better survival and functions compared to nonvascularized substitutes.

Author details

Ingrid Saba[1], Weronika Jakubowska[1], Stéphane Chabaud[1] and Stéphane Bolduc[1,2*]

*Address all correspondence to: stephane.bolduc@fmed.ulaval.ca

1 Experimental organogenesis research center of Laval University /LOEX, University Hospital (CHU) of Quebec - Laval University, Enfant-Jésus Hospital, Quebec City, QC, Canada

2 Department of Surgery, Faculty of Medicine, Laval University, Quebec City, QC, Canada

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