The Basement Membrane: Key to the Reverse Engineering Biological Tissues

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ABSTRACT

We reveal the basement membrane, a specialized connective tissue structure found in all tissue systems, as a framework in an adaptive computer aided design (CAD) strategy for the reverse engineering of 3 dimensional (3D) tissue structures. Our approach to the creation of functional 3D tissue structures is centered on our previous models of vascular supply systems which included complete and accurate replications of capillary bed systems, the circulatory interface necessary to sustain 3D tissue structures. By using the basement membrane as a guide, we seek to design models for the reverse engineering of the other extracellular connective tissue structures and their matrix elements. We demonstrate the basement membrane as a platform for the design and engineering of tissue scaffolding for vascularized alveolar systems in the lung and for the vascularized dermal skin layer. The resulting structure will support the 3D growth and differentiation of cells and their products. The biomedical industry stands to be greatly impacted by this CAD approach to the engineering of 3D tissue structures.

Keywords: basement membrane, tissue scaffold, reverse engineering.
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1 INTRODUCTION

The main barrier preventing the successful engineering of 3-dimensional (3D) tissue structures is the inability of researchers to mimic the microscopic architectures that are essential to the, support, growth, maintenance and function of tissue structures. The approach traditionally taken towards

engineering 3D tissue structures first began as what we will refer to here as the developmental approach. Here researches use the knowledge acquired in developmental, cell and molecular biology to attempt to initiate and sustain tissue generation without the aid off externally fabricated devices [1-6]. The inability to initiate the spontaneous, yet controlled formation of vascular growth caused most researchers to abandon any approach that did not first develop some sort of vascularization [7-12]. Difficulties arising with the bioengineering of blood vessels [13] caused many researchers to focus strictly on the engineering of vascular tissues [14, 15]. Initially polymeric systems, developed with polymer scaffolds that released angiogenic factors, were used to recruit precursor cells into porous scaffolding [16-18]. But these so called biocompatible and biodegradable materials lacked specific form and induced thrombosis, chronic inflammation or rejection [17, 19]. Recent use of synthetic materials such as Dacron or expanded polytetrafluoroethylene (ePTFE), have produce varying degrees of success when use in applications for large vessels such as the aorta, but fail to be useful in vessels smaller than 6mm [20] because of inefficient mechanical properties. Other approaches have use decellularized xerographic blood vessels for vascular scaffolds [21], used flat sheets of collagen and elastin prepared by freeze drying [22] or from 2 dimensional (2D) decellularized fibroblast cultures [19], rolled into tubes. These rolled tubes were limited to 3mm and only remained open for a limited time. The continued failure to produce 3D microvascular systems has some researchers creating 2D sheets of tissues and layering them creating a 3rd dimension [23]. This approach produces only limited functionality and lacks the vascular organization[23] necessary to produce the design variations found in our different tissue types.

The second type of approach, we will refer to as the guided approach to tissue engineering. This approach uses implants that act as a structural scaffold, that through mechanical and chemical support, aid tissue development. This guided approach to tissue engineering began with the use of splints for the immobilization [24-26] encouraged healing of large bone defects [27]. Technologies for the design of scaffolding for bone development have become an area of enormous study [28-33]. Having achieved some early success [26, 34, 35], the use of scaffolding in bone healing [25, 36] generated an interest in designing scaffolding for use in soft tissue regeneration [37-42]. Scaffolds for soft tissues [43-51] as well as boney tissues [29, 31, 52-57] have become extremely advanced, but the structural designs which are essential to tissue growth and functionality have yet to be incorporated into scaffold designs. Most of the work in the engineering of bone scaffolding in bones centered on its mineralized extracellular matrix. The extracellular matrix of bone, with the exception of bone marrow, comprises the overwhelming majority of its structure [58]. In contrast, the soft tissues, which representing the other organ systems of the body plan, are more served by the configuration of its cellular components. As in bone these cellular configurations are supported by an extracellular matrix which, opposite to that of bony tissues, comprises a very small percentage of the soft tissues' structure.

The extracellular matrix in soft tissues can be seen as a scaffold supporting its various functional configurations of cells and their luminal spaces [59, 60]. The microscopic design and biochemical properties of the extracellular matrix are as vital to the maintenance of a tissues' functionality and the health of the organism as the cells themselves [61]. This has been demonstrated with the re-functionality observed in the recellularization of decellularized tissue [62, 63].

Of all the extracellular matrix components, we identify the basement membrane or basal lamella as the core extracellular support structure. Here is where our approach to tissue engineering becomes unique. Given the proper image data, computer aided design (CAD) tools can be used to create models that accurately replicate this system's interfacing of the cellular boundaries in animal tissues. Where other approaches to tissue engineering seek to design scaffolds with physical characteristics that support and maintain cell growth, our approach is to replicate from medical imaging data, the structural designs of the basement membrane and its interconnective cellular support structures that are naturally found supporting the cells in the tissue structures sought to be engineered.

Basement membranes are generally composed of two layers, one glycoprotein rich, and the other rich with collagen proteoglycans. This structure originates from embryonic mesenchyme and surrounds all

stationary cell units. This association is created during the organ forming invaginations formed by primary germ layer during embryonic development. Through hemidesmosomes, intergrins and glycoprotein laminins, the basement membrane interfaces the cellular elements of the body with its connective tissue framework. Together these structures embrace and support cells in the organ forming units which are ultimately joined by connective tissues into multicellular organisms.

2 MATERIALS AND METHODS:

2.1 Extracellular Matrix Consideration

To make our framework for 3D scaffold constructions complete and accurate we looked for additional structural information from the tissues for which the scaffold is intended. We sought to utilize structural features that could be found in tissues which could be set apart from its cellular structures. Using standard light microscopy and transmission electron microscopy we analyzed the regions within cellular tissues that are found structurally supporting the cells.

2.2 Designing Basement Membrane Supported Tissue Scaffolding

In designing the framework for tissue scaffold production we used models from image data previously collected using both micro computer tomography (Micro-CT) [64, 65] and the 3 dimensional (3D) serial reconstruction of histological sections[66].Models were imported into the CAD software Rhinoceros™ 4.0 (Rhino) for tissue scaffold considerations and basement membrane design.

The lung, an extremely difficult tissue for the body to regenerate, was designed using a model acquired from images of histological section [66]. Model was modified for 3D prototyping using Rhino, first by scaling it to fit the Zprinter™ 450's fabrication stage, secondly by formatting the model for compatibility with the design software Solidworks™ in order to interface the design directly with the controls of the Zprinter™ 450.

In order to support the of vascularized, hair, nerve and gland containing, skin scaffold for vascularized dermal skin layer was modeled in Rhino using non uniform rational B-splines (NURBS) as extracellular support units fashioned around a selected region taken from a vascular tree system created using CAD derived from Micro CT image data as reported [71]. This CAD represents the basement membrane of this vascular system and was model with the CAD software GeoMagic™ using 3D image data collect from a corrosion cast replicating the lumen of the vascular tree system found in the dorsal skin of rabbit [64, 65].

2.3 Isolate Image of Support Structures

In order to increase the accuracy of the 3D serial histological sections we looked at way to characterize and distinguish specific support structures found to be structurally significant to the tissue organization characteristically patterned to support the unique functions in the different organs. Using special staining procedures sometime is helpful in distinguishing cell structures. The staining methods that best distinguishes the extracellular support structures call for the use of harsh and sometimes toxic chemical. These chemicals must be disposed of by costly hazardous waste support infrastructures. Instead we used image filtering software from Adobe Photoshop, stylize glowing edges, to distinguish structures uniquely characterized by their location and by the way their crystalline structure transmits light.

3 RESULTS

3.1 Framework for Multi Scaffolds Tissue Scaffolding

The framework for a vascularized tissue scaffold can be created using our previously reported results on the computer aided designing of complete and accurately replicated vascular tree systems[64, 65], and CAD tools, (Figure 1). Included in this framework is a design for the basement membrane

replicated from a vascular tree system which support all the layer of hair containing mammalian skin. This design was obtained as shown in our recent publications[64, 65].

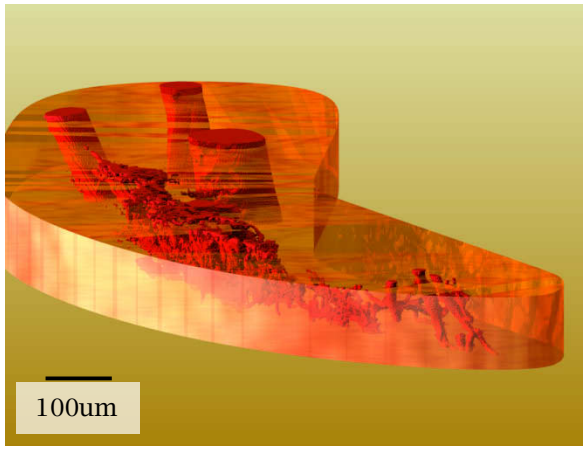

Fig. 1: A 3D computer aided design (CAD) of framework for tissue scaffolding that will support the genesis of dermal and subdermal tissues. This scaffolding framework supports further design modifications.

3.2 Search for Support Structures Using Transmission Electron Microscopy

Preparing samples for this procedure requires sectioning through a single plane creating a 2 dimensional representation of the sectioned plane (Figures 2-4). 3D analysis of the basement membrane has shown that a single membrane make its way not only throughout kidney glomeruli but is continuous throughout the kidney. The basement membranes' continuity is consistent in all cellular tissue types making this connective tissue structure our candidate for the main support structure used scaffolding.

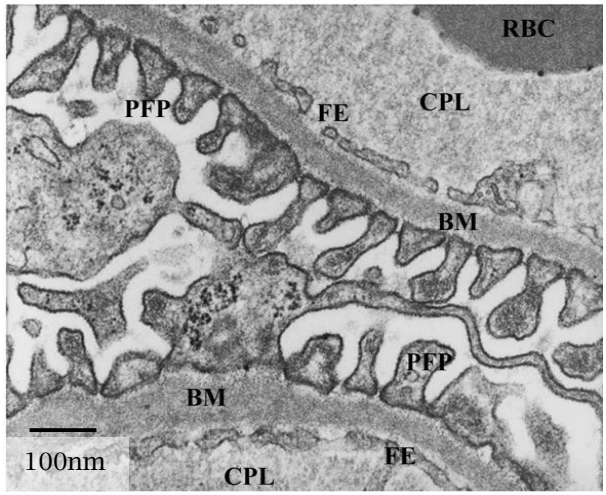

Fig. 2: In this electron micrograph of a kidney glomerulus there appears to be two separate basement membranes structures. BM basement membrane; CPL capillary lumen; PFP podocyte foot process; FE fenestrated endothelial cell cytoplasm; RBC red blood cells.

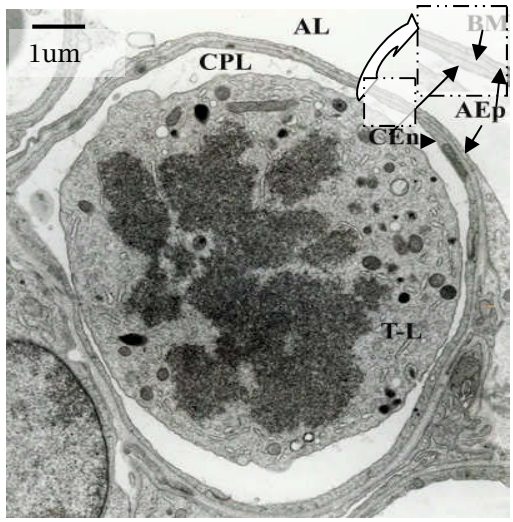

Fig. 3: A cross section through a lung alveolar capillary. The basement membrane shown here interfaces both the capillary endothelium and the alveolar epithelial cells and as with other tissues is continuous throughout the lung. AL alveolar lumen; CPL capillary lumen; CEn capillary endothelium; AEp alveolar epithelium; T- lymphocyte in pro-metaphase.

The basement membrane is the outer most layer of connective tissue which supports cells that interface the body with its outer environment. In Figure 2 we see the basement membrane interfaces with the urinal tract and in Figure 3 the respiratory tract. In the lung (Figures 3 & 4) both the capillary endothelium and the alveolar epithelial cells are supported by the basement membrane as are cellular components found deeper in the lungs structure.

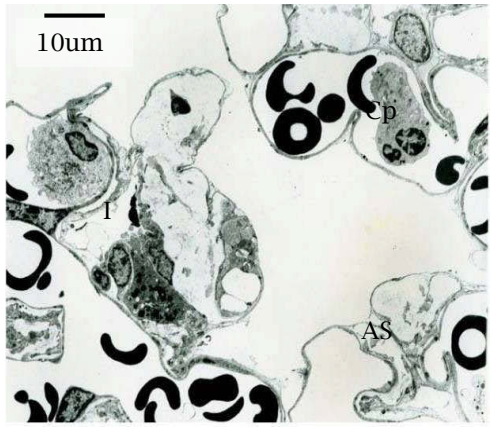

Fig. 4: An electron micrograph of a section of plastic embedded lung tissue, similar to the sections used in the 3D serial reconstruction of the avian lung tissue modeled in Figure 5. Cp capillary; AS alveolar space; I interstitium connective tissue.

The lung's volume consists mostly of air filled alveoli spaces lined by epithelium and blood filled vascular spaces lined by endothelium (Figure 4). The endothelium and epithelium that line these two passages are interface by a single basement membrane (Figure 3). This basement membrane branches into various directions and remains continuous throughout. Fibrous, mostly elastic, supportive

connective tissues occurs in the interstitium between these two cell layers where additional structural supports is needed, such as a support medium for larger blood vessels, nerves, and lymphatic ducts, all of which have their own basement membrane systems.

3.3 Fabrication of Computer Aided Designed Tissue Scaffolding

Ultrastructurally accurate tissue scaffold fabrication is demonstrated with CAD. Figure 5A shows the 3D mess layout created using CAD software as a tool for tissue scaffold design and production. Note the empty space between the two tissue lumen representations. It is in this continuous space that the basement membrane supported cells of the alveoli and capillary structures reside. Figure 5B demonstrates the ability of CAD software to separate these two representations of the blood capillaries and the alveoli spaces for individual, structural specific, nano-feature designing.

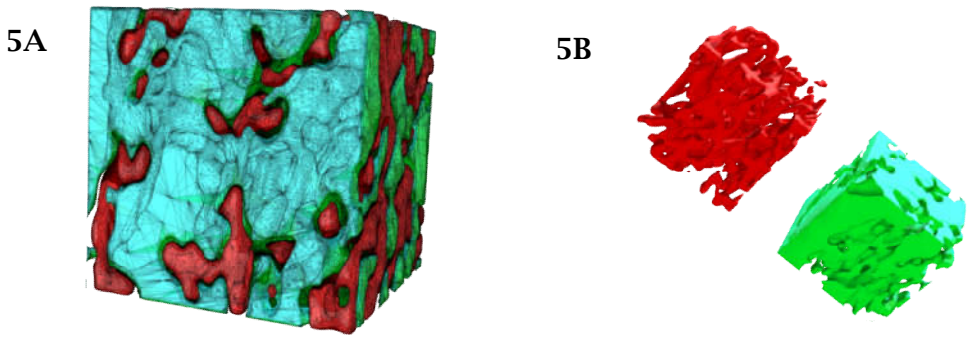

Fig. 5: 3D reconstruction of serial plastic section demonstrating the luminal space within the blood (red) and air (blue/green) capillaries in tissue taken from avian lung.

Fig. 6: Tissue scaffold prototype fabricated from CAD model in figure 5. Spaces in this scaffold design are for endothelial, epithelial, interstitial cell and their corresponding extracellular matrixes.

Prototype for a tissue scaffold was successfully fabricated using a rapid prototype devise. We see that our CAD models can be designed to support cell structures using image data replicating the structural patterns of functional respiratory tissue samples prepared using 3D image acquisition of serial tissue section [66].

3.4 Locating Basement Membrane with Image Filters

Using an image filter for edge detection and demonstration we were able to produce 2D images capable of defining the basement membrane containing regions of sectioned tissues. In Figure 7B the basement membrane regions of the functional unit of the liver, a liver lobule, with its central vein and its surrounding sinusoids, have been distinctively distinguished from the surrounding cellular tissues. In Figure 8 we used the same image filtering used in Figure 7. The filter produced different colors for edges of different light densities, reflecting the location, and composition of different extracellular connective tissue structures.

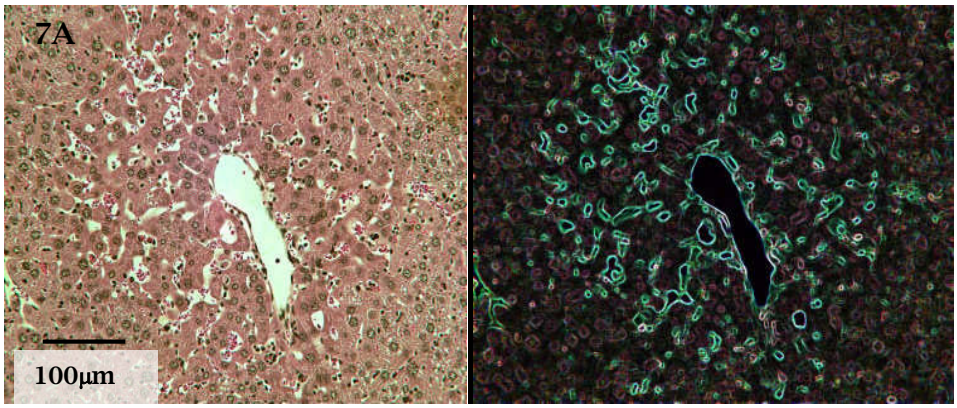

Fig. 7: A liver lobule with its central vein. The top photomicrograph is routine haematoxylin - eosin stained light microscopy image. The bottom image shows the top photomicrograph computer processed to highlight areas in the lobule where the basement membrane resides.

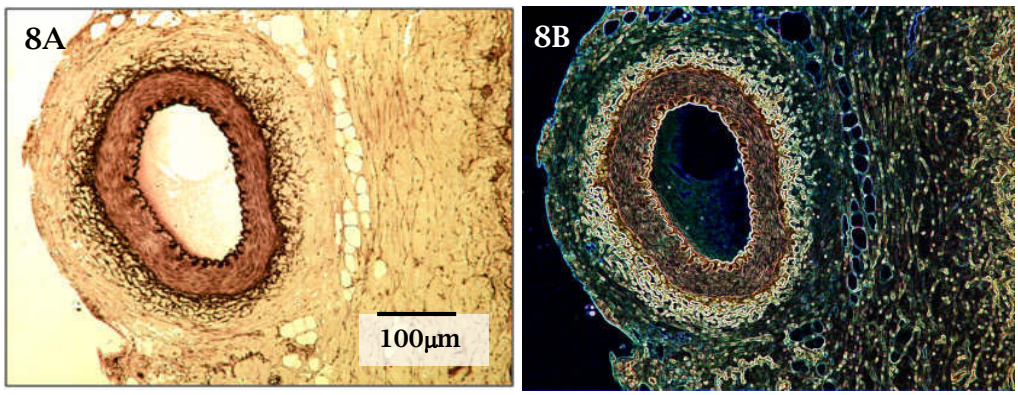

Fig. 8: Photomicrograph of a large artery which supplies blood to neighboring muscle tissue. The lower filtered image demonstrates using color variations the different connective tissue types that support this structure.

4 DISCUSSION

As occurred in the aircraft, automotive and most recently the micro electronics industries technological integrations through the use of CAD and CAM will revolutionalize tissue engineering and its ability to impact regenerative medicine, wound healing and transplant therapy strategies. The key tissue structure that this revolution will be seated around is the basement membrane.Through

itsinterconnectivenesswith interstitial connective tissues,basement membranes are the central interfacing component of connective tissue networks that serves as a natural cellular scaffold in multicellular animal tissues. This connective tissue network supportsthe cellular and luminal patterns responsible for the functional properties whichcharacterize tissue and organ systems. This information makes the basement membrane an ideal structural system to use as the backbone for the computer aided designing of tissue scaffolding.

Using 3D image data acquired using methods describe here and in previous works[64, 65, 67], we have shown that CAD tools can produce models designed for use in computer aided manufacturing (CAM) of tissue scaffolding (Figure 6), not only for capillary bed systems and their connecting vasculature (Figure 1)[65], but to replicate as imaged from a sample from a specific organ system and manufacture using CAM a structural representation of unique cellular boundaries for specific tissue structures (Figures 5&6).

By applying special staining or image filtering techniques to a 3D reconstruction of serial section protocol we can create a well defined representation of basement membrane locations (Figure 7B). The differentiation of connective tissue boundary types by color (Figure 8B) can produce in a single 3D reconstruction a CAD model with surfaces differentially representing (Figure 5B) the different connective tissue elements. Points fitting these surfaces can be differentially weighted with fabrication data reflecting the connective tissue structuresdifferentbiochemical composition. Other structurally relevant points can be weight with manufacturing data pin pointing peptide attachment protocol creating the molecular cues necessary to orchestrate the scaffold's cellularization and tissue development during subsequent computer integrated tissue morphology manufacturing protocols.

To suit the needs of our morphological manufacturing process this computer aided tissue morphology manufacturing protocol must integrate manufacturing techniques such as the 3D photo patterning of hydrogels, the multiphotoncrosslinking of protein matrixes and surface modifications with signal inducing peptides and enzyme responsive bio-smart material. This method is designed to enhance a variety of therapeutic protocols including, but not limited to, theengineering of intact, functional vascular networks that include the capillary structures The engineering around these vascular networks the structural tissue units (Figure 7B) [68] supported by their mimicked design.

Addition experiments we are planning are the analysis of cellularization results of tissue scaffolds from our preliminary CAD's using CAM. Serial microtomy and micro-CT and image reconstruction of tissues staining in-bloc with silver for basement membrane identification and the serial reconstruction of images filtered to color characterize connective tissue boundaries in photomicrographs of microtome sectioned tissues.

What is it going to take to truly begin the successful engineering of 3 dimensional (3D) vascularized tissue structures that biologically mimic our own tissues? With today's technology we can surly conceive many ways to approach the task. First, it is clear that not one single lab exist today which has all the tools necessary to accomplish this goal. Furthermore no single discipline can within itself perceive the realization of this goal and in all reality there may not be a single institution currently prepared to take on this challenge.

The integration of cutting edge technologies have resulted in the development of computer aided design (CAD) tools and computer integrated manufacturing strategies which have recently reached microscopic dimensions [69-71] and are beginning to impact the field of tissue engineering [72, 73]. We must form cooperatives from different institutions to produce our proposed tissue scaffold design. These cooperatives must strategically tie together the technologies developed with the integrated manufacturing systems used to revolutionizethe design and production of aircraft, automobiles and most recently, the micro electromechanical systems (MEMS) [74, 75].These cooperatives must use to create novel tissue scaffolding that integrates technologies which link the photo-crosslinking of protein matrixes and stimulatory/inhibitory peptides surface modifications. Cooperatives must assimilate

these results with nano release technologies that coordinated the release of regulatory proteins such cytokines for use in enzyme responsive bio-smart materials.

Coordinate stem cell technology with phenotypical cellular morphological responses to integrated cell matrix scaffolding, cell seeding technology and stimulatory bioreactor technology. Finally, for the 'in site' manufacturing of tissue scaffolds cooperatives must integrate surgical technology with computer aided manufacture (CAM) tooling paths. Here the multi laser 3D CAD patterning of electromagnetic wave polymerizableinjectable matrixes derived using the technological integrations discussed above will support and guide host cell in the regeneration of tissue structures on the body's surface or within a body cavity.

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