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Bioengineering liver tissue by repopulation of decellularised scaffolds

INTRODUCTION

Chronic liver disease is a major health concern, with 1.5 billion individuals affected worldwide, and associated with an annual global mortality of 2 million people[1]. In the United Kingdom, liver disease is the third commonest cause of premature death[2], and is associated with societal and health care costs measured in the billions of pounds per annum[3]. In the United states, 44000 people die of chronic liver disease each year[4], with an estimated annual hospitalisation costs demonstrating an increasing trend and measured at 18 billion dollars per year in 2016[5], to which must be added similar magnitude financial costs of pre-hospital healthcare and social care burden[3].

Although vaccination programs and antiviral therapy may result in decreasing prevalence in chronic liver disease of viral aetiology, the consequences of alcohol and hepatic steatosis has resulted in a gradually increasing incidence of chronic liver disease[6-8]. Despite the enormous scope for prevention of progression to chronic liver disease through vaccination, antiviral therapy, and lifestyle interventions, the only treatment for end-stage liver disease remains liver transplantation. However, due to the shortage of available organs, 10% of patients die whilst on the waiting list for an organ[9], and many more are never considered for transplantation because of the need to optimise graft usage. Moreover, transplanted patients face the short and long-term side effects of immunosuppression.

These challenges have motivated the investigation of bioengineering liver tissue with a view to delivering bioengineered organs for transplantation. Despite progress in the generation of biogels and 3D bioprinting, reproducing the immensely complex 3D microarchitecture of liver parenchyma remains a major challenge. By decellularizing

tissues with surfactant detergents, it is possible to remove the cellular component of tissues, leaving behind the 3D extracellular matrix (ECM) providing not only a scaffold but also cell fate instructions to appropriate repopulating cells. In the context of liver transplantation, many deceased organs are discarded because of inadequate cellular function[10]. As an aspirational objective, such organs could be decellularised, and repopulated with cells of recipient origin with a view to bioengineering immunologically syngeneic organs. The theoretical benefits would include timely generation of organs, transplanted in an elective manner, without the need for immunosuppression.

This review describes current progress in the field of bioengineering liver tissue from decellularised matrix and repopulating cells. To orientate the reader, the further review sections will deal with the following areas: Section 2 (ECM structure and role in cell fate) provides a summary of the structure and function of the extracellular matrix, describing its paramount influence in cell fate and bioengineering, as well as an account of the evolution of synthetic and ECM substrate components to enhance tissue culture; Section 3 (General concepts in decellularisation and non-hepatic applications) provides an account of decellularization of tissues in general and non-hepatic applications, as a background context in which to consider liver decellularisation and repopulation; Section 4 (Scaffold sterilisation) discusses scaffold sterilisation; Section 5 (Liver decellularisation and recellularisation) provides an account of decellularisation and repopulation of liver tissue with subsections dealing with the variety of cellular components of liver parenchyma; Section 6 (Recellularisation of extra hepatic blood vessels) discusses the recellularisation of extra-hepatic blood vessels; Section 7 (Immunogenicity of decellularised scaffolds) provides an account of scaffold immunogenicity; and Section 8 (Conclusion) concludes the review with a discussion of the remaining challenges in the field.

ECM STRUCTURE AND ROLE IN CELL FATE

Introduction

Whilst a full account of the role of ECM in cell biology is beyond the scope of this review, its fundamental role in influencing cell behaviour requires emphasis in the context of the use of decellularised ECM scaffolds. This section describes the structure of the ECM and provides an overview of ECM cell interactions as well as the evolution in the use of ECM based substrates to enhance tissue culture.

ECM content and structure

Although the structure of ECM varies immensely between tissues in terms of proportion and layout of its constituents, common components can be identified and include Glycosaminoglycans, water, 4 major classes of extracellular proteins (the collagens, elastin, proteoglycans, and glycoproteins), and numerous growth factors as well as other bioactive cell behaviour influencing species.

Glycosaminoglycans such as chondroitin sulphate, heparan sulphate and hyaluronic acid[11] are long, negatively charged macromolecules consisting of linear repeats of uronic and amino disaccharide units. In isolation or when combined with proteins to form proteoglycans[12], Glycosaminoglycans bind water, which is critical for imparting compressive resistance to tissue.

Collagens imparts tissue tensile strength and structural integrity. They consist of 3 alpha chains, the various combinations of which make up the 28 known collagen types. In broad structure, Fibrillar collagen is assembled in triple helical structures which combine to form fibrils of varying size and thickness. Non fibrillar collagen does not form fibrils but rather a mesh like network, such as that in in basement membrane by collagen type 4[13].

Elastin complements collagen's tensile strength properties to provide elasticity[14].

Glycoproteins[15,16] are peptide units covalently bound to carbohydrate groups, but not in a linear or repeating pattern, as in proteoglycans. The glycoproteins are described as connecting molecules, in that they carry binding sites to multiple other molecules including other ECM molecules, secreted growth factors, and extra-cellular membrane receptors on cells including cell adhesion molecules. The principal glycoproteins are

fibronectin and laminin. Cell attachment to glycoproteins is mediated through distinct peptide domains[17] such as the Arg GlyAsp (RGD) and Arg GluAsp Val (REDV) sequences in fibronectin[18, 19], as well as Val-Al -Pro-Gly domain in elastin[20], which binds integrins on cell surface. Binding motifs may be overtly apparent of may be revealed after unfolding of ECM proteins by fibroblasts, or following the action of ECM degrading enzymes, thus introducing further complexity in the interplay between the ECM and multiple cell types in the control of cell behaviour[21]. Laminin is composed of alpha, beta and gamma heterotrimeric chains arranged in cross or Y shapes[12]. It is found in basement membrane and connects ECM components, with different forms and modifications resulting in specific controls on cell behaviour[22].

ECM cell interactions

The ECM is much more than simply a 3D scaffold which houses resident cells. It is also a source of critical biochemical and physical signalling which influences fundamental processes of cell survival, organization and differentiation[23].

ECM in development, cell migration, stem cell niche, and adult tissue fate: The importance of the ECM in cellular organisation is apparent from its synthesis and secretion in the very earliest stages of development, exemplified by the assembly of laminin and collagen 4 in mouse embryos as early as the blastocyst stage[24]. Moreover major developmental defects are caused by ECM proteins[25], with ECM mutations resulting in wide ranging anomalies affecting body shape[26], as well as development of neural tube[27], and muscle[28].

In addition to broad control of development, the ECM acts as a regulator of the extent and direction of cell migration. Thus, laminin chain knockout results in uncontrolled and undirected neural crest cell migration[29], whilst fibronectin mutations result in impaired migration of cardiac precursor cells[30].

The ECM controls stem cell fate not only in development, but also in adult tissues where it plays a major role in the definition of the stem cell niche, keeping stem cell in a

quiescent state until appropriate circumstances trigger a requirement for their proliferation[31].

The ECM also influences the behaviour of differentiated cells in adult tissues. Following cues from multitude factors (physical, chemical, oxygen partial pressure, and numerous others which together define physiological niche)[32], the ECM is altered and remodelled by resident cells in adult tissues. ECM remodelling is much more than a reconfiguration of local 3D scaffold shape: the ECM is a reservoir of multiple biologically active species which impact on cell behaviour, and which are recruited and released upon remodelling. Thus, although resident cells produce and deposit their local ECM, they are also influenced by it in a process referred to as 'dynamic reciprocity' or 'bidirectional crosstalk' between cells and their environment[33,34].

ECM – cell biochemical and biophysical signalling: The ECM interacts with cells *via* multiple receptors in the cell membrane including integrins, discoidin domain receptors, syndecans, CD44, and receptor for hyaluronic acid. Of these, the most studied are the integrins. Distinct alpha and beta subunits combine to make 24 different known integrins, which act specifically on defined cell types in a contextual manner to determine cell growth and survival, promote invasion and migration, and direct cell differentiation and stem cell fate[35]. Mechanistically, the importance of binding motifs is emphasised by experiments demonstrating that blocking the integrin binding site of fibronectin (the RGD motif) by competitive inhibition with RGD peptides resulted in major embryonic symmetry anomalies[36].

In addition to direct communication with cells *via* cell membrane receptors, the ECM influences cell fate by acting as a reservoir of growth factors, morphogens and enzymes, which may be released as active forms in defined circumstances, in a manner that has been most studied in relation to many growth factor families including the transforming growth factor beta, platelet derived growth factor, fibroblast growth factor and insulin like growth factor superfamilies of growth factors[14]. These growth factor signals are added to and complemented by those of other bioactive species including,

matrix cryptic peptides[37], matrix bound vesicles containing bioactive molecules (RNA, lipids, proteins)[38], with wide-ranging roles including impacts on cell differentiation[31] chemotaxis[39], mitogenesis[40], angiogenesis[41,42], and wound healing[43].

The ECM signals to cells and influences cell fate in biophysical ways as well as *via* biochemical mechanisms. For example, by determining cell shape with microprinted fibronectin islands, McBeath *et al*[44] showed that mesenchymal stem cells would differentiate to adipocytes if they assumed a rounded shape, and to chondrocytes if allowed to assume a spread shape.

The ECM also influences cell proliferation[45] and cell fate *via* its stiffness and elasticity. Thus, mesenchymal stem cells differentiation may be directed towards either neurological, muscle or bone phenotypes by varying the elasticity of the underlying substrate to mimic the corresponding tissue types[46], *via* mechanisms involving mechano-sensitive ion channels, and Yes-Associated Protein and Transcriptional Coactivator With PDZ-Binding Motif[14].

The evolution of complex substrates for cell culture

The powerful influence of the ECM in the control of cell fate has motivated the use of alternatives to 2D plastic cell culture with a variety of complex substrates to minimise the loss of functional specificity that is otherwise frequently observed. Thus, there has been a gradual evolution in the use of materials to mimic the ECM *in vitro*, culminating in the recent development of decellularised scaffolds, representing to date the most accurate version of native ECM.

Substrates for enhanced cell culture include synthetic or naturally occurring chemicals. The synthetic substrates are man-made polymers such as polycaprolactone, polyethylene glycol (PEG) and polyglycolic acid[23], or hydrogels composed of hydrophilic polymers such as polyacrylic acid, polyethylene glycol and polyvinyl alcohol[47]. These have the advantages of reliability, consistency, reproducibility, low variability, but tend to produce host inflammatory responses[48] and fundamentally lack

the complexity of native ECM. The naturally occurring substrates are components of ECM, either in single form or in combinations of varying complexity.

Synthetic substrates may be bioengineered to include biological entities in a number of ways: (1) by crosslinking cell adhesion peptides (for example, the RGD domain of fibronectin or VPVGV domain of elastin) to synthetic polymers like PEG to promote cell interaction[49]; (2) By incorporation of specific growth factors to favour desired cell behaviour, for example neuronal[50], bone[51], and vascular[52] differentiation, with the option of positioning of boundary forming signals[53], or temporal control by determining the mechanism of release of the bioactive species[54]; and (3) by incorporating enriched ECM components into poly-ethylene-glycol hydrogels[55].

Hydrogels are hydrated polymers or materials with ≥ 30% (v/w) water content that maintain their structural integrity through crosslinks between their constituents[56], which can be synthetic polymers, or from ECM components in single form[57] or multi component form[58]. Cell derived hydrogels such as Matrigel, or hydrogels generated from specific decellularised tissues are more complex and have been used for organoid culture[59], as 2D substrates, or cell medium additives.

Whether synthetic, naturally occurring or combined, the deposition of substrate components on a given surface has evolved to high level of precision, achieving resolutions of fractions of micrometres, with micro-patterning techniques such as photolithography[60], elastomeric stamping[61], nanofiber lithography[62], electrospinning[63], and 3D bioprinting using 'bio ink' (ECM derived from specific tissue in hydrogel and colloid form)[56].

Nevertheless, despite the wide range of available substrates, be they synthetic, naturally occurring or combined, the complexity of specific tissue microarchitecture combined with the multitude of growth factors within the ECM, means reproducing ECM by the techniques mentioned above remains elusive. Hence the concept of decellularization, whereby the cells of a specific tissue are removed, thereby leaving behind a native cell free ECM scaffold, theoretically maintaining both 3D micro-architecture and the ECM associated biological signalling.

GENERAL CONCEPTS IN DECELLULARISATION AND NON-HEPATIC APPLICATIONS

Introduction

The objective of whole organ decellularisation has 2 components: (1) to completely remove the cellular component, whilst (2) leaving the 3D microarchitecture and vital growth factor content of the ECM intact, such that repopulating cells have an environment which favours regeneration of the native tissue.

The first objective is necessary as there is evidence that residual cellular debris is not only toxic to repopulating cells, but also triggers inflammatory and destructive responses *in vivo*[64-67] (discussed in section 7 "Immunogenicity of decellularised scaffolds") rather than the desired regenerative events. The second objective is necessary to retain the vital physical and biochemical ECM properties by which it influences cell fate.

Herein lies a fundamental difficulty in decellularization techniques, in that whilst both necessary, these 2 objectives are in conflict, as the stringent conditions required to clear toxic debris of decellularization will also inflict some damage to the ECM. Small molecules like growth factors will be particularly susceptible to being washed away[68], but even large macromolecules, though less vulnerable because of size and cross linking, may also be damaged[69].

The following section outlines the techniques used for decellularisation and discusses their merits and disadvantages.

Decellularisation techniques

A multitude of decellularisation techniques have been developed using physical, chemical, and enzymatic, methods either singly or in combination, and adapted to suit the differing requirements of the native tissue being treated.

Physical methods: (1) Sonication.Sonication utilises an ultrasound emitting device to transfer acoustic energy in a solvent containing tissues to be decellularised[70]. Cell membranes are disrupted by the sonication waves, and resultant debris requires removal by other methods[71-74]. Sonification process may significantly increase temperature of the solvent and tissues, risking denaturisation, and therefore may need to be combined with a cooling mechanism[70]. Sonication is typically used with detergents to decellularize dense tissues such as tendons, ligaments[75], and cartilage[71], although has also been used in kidney decellularisation[76]; (2) Freeze-Thaw. Freeze-thaw achieves cell lysis through rapid thermal change, though debris requires additional methods for clearance[70]. The technique has been used in combination with detergents to reduce to the quantities of chemical reagents for decellularisation[77,78]. The formation of ice crystals may be detrimental to the ECM, leading some researchers to advocate the use of cryoprotectants to mitigate the detrimental effects without affecting cell lysis[79]; and (3) Immersion and agitation. The decellularisation effects of chemical reagents may be enhanced by agitation in instances where decellularisation is achieved by immersion in chemical reagents[70]. The length of immersion, and intensity of agitation depend on the tissue[80], and this approach is usually only appropriate for epidermal tissues and smaller organs, such as small intestine submucosa[81], trachea[82], other cartilaginous tissues[83,84], and thyroid gland[85].

Chemical methods: (1) Detergents. Detergents have been used extensively to decellularize large vascular organs by vascular perfusion[70]. Ionic detergents like sodium dodecyl sulphate (SDS) and sodium deoxycholate solubilise cell membranes and denature proteins[86-88]. Non-ionic detergents, of which Triton X-100 is the most frequently and successfully used, disrupt lipid-lipid, lipid-protein, and DNA-protein interactions[89,90]. Detergents are frequently combined in decellularisation techniques, with variations in concentration and perfusion time, and require washing steps to remove residual traces after decellularisation[91,92]; (2) Hypertonic and hypotonic solutions. Hypertonic saline causes dissociation of DNA protein interactions[93], which,

combined with cell shrinkage and swelling, causes cell lysis[94]. Debris clearance further steps to achieve full decellularisation[95]; (3) Acids and bases. Bases, such as ammonium hydroxide, have been used as an adjunct to detergent based decellularisation techniques to enable clearance of DNA which, in alkali solution, denatures to low viscosity single stranded nucleic acid, facilitating its removal by perfusion[96,97]. Acids such as peracetic acid have been used predominantly for sterilisation of scaffolds (see section 4 "Scaffold sterilization"). However, both bases and acids have significant detrimental effects on the ECM, by damaging collagen and other structural proteins, as well as by denaturing key growth factors[81,98]; (4) Alcohols. Alcohols diffuse into cells and cause cell lysis by a dehydrating mechanism, and thus have been used as decellularizing agents[99,100], but also as sterilising agents either alone or in combination with acids; (5) Chelating agents[101-104]. Chelating agents such ethylenediaminetetraacetic acid and ethylene glycol tetra acetic acid bind metallic ions that are essential for protein interaction[105,106], resulting in the disconnection of intercellular integral proteins and disruption of cellular adhesion in the ECM. Full decellularisation requires additional agents such as detergents[107]; and (6) Enzymatic treatments[108-111]. A variety of enzymes have been utilised for tissue decellularisation, with trypsin and nucleases being the most frequently used. Trypsin is a serine protease that hydrolyses proteins involved in cellular attachment[112], thus dissociating cells from the ECM[113]. Nucleases (DNases and RNases) cleave phosphodiester bonds between nucleotides in nucleic acids and have been used to improve the removal of remaining nucleic acid debris in conjunction with other decellularisation agents[114, 115].

Implications of decellularisation technique heterogeneity: The above summary of techniques provides an insight into the enormous heterogeneity of approaches. In addition to the variety of methods above, many protocols use varying combinations of 2 or more methodologies. Furthermore, techniques vary in other factors including temperature of decellularizing process, flow rates of perfusion agents. Such considerations may be quite subtle, yet critically important: for example, one study of

tracheal decellularization involving repeated cycles of decellularizing agent reported that the number of cycles critically altered the integrity of the scaffold between cycles 18 and 22[116].

This technique heterogeneity reflects the differing requirements of different tissues. Tissues with obviously different macroscopic structures require different methodology: for example, perfusing a decellularising agent *via* the circulation in vascular tissue such as liver may be effective, but unlikely to be so in tough avascular structure of tendon. In this regard, a tissue classification of laminate, amorphous, composite, whole organ suggested by Keane[117]. Moreover, the matrisome (the protein content profile of the ECM) is subtly tissue dependant[118] such that even tissues of similar consistency may behave differently. For example, similar tissues such as tendon and ligament may behave quite differently despite exposure to same decellularizing agent[119,120]. Furthermore, even within a defined tissue type, individual variation with factors including age and sex may affect matrisome content[121-124].

Though not intended to be comprehensive given the scope of this review, Table 1 provides examples of the breadth of tissues in which decellularisation has been studied, and range of decellularisation protocols. This reflects the fact that the field is at an empirical stage where methodology is in an assessment phase with multiple criteria to be considered. The optimal method of decellularization may be difficult to determine and define particularly in the context of tissues with multiple cell types, as the optimum decellularizing method for one cell type may not coincide with requirements for others. Attempts at decellularization must perhaps be seen as producing an inevitably imperfect result, which may be corrected and refashioned by repopulating cells *in vitro* and in the host.

This high level of heterogeneity in tissue samples and technique raises the question of how to assess success in decellularisation. In this regard, Crapo *et al*[125] has suggested that successful decellularization should be determined on the basis of producing ECM which (1) does not contain more than 50 ng of DNA per mg dry weight; (2) with residual DNA fragments no longer than 200 bp; and (3) with no visible nuclear

components, based on observations of *in vivo* adverse effects of these biochemicals[64,67,126]. The area of research is rapidly changing, and thus it is anticipated that new criteria of scaffold quality are likely to arise, as suggested by other authors[127-129]. Ultimately, the success of decellularisation is surely defined by the matrix to accept repopulating cells and whether those cells survive and collectively allow physiologically significant neo-organ function. These aspects will be discussed in detail as they pertain to liver function in section 5 (Liver decellularisation and recellularisation).

SCAFFOLD STERILISATION

Introduction

In vitro culture of mammalian cells provides ideal conditions for survival of cells of interest, but also for unwanted micro-organisms. Moreover, the potential for culture infection in decellularised scaffold experiments is higher than in standard cell culture given the non-sterile tissue of origin. Thus, not only for the success of *in vitro* scaffold repopulation, but also in terms of safety in the context of scaffold *in vivo* reimplantation, there is a need to eradicate microorganisms from decellularised scaffolds.

The ideal requirements for decontaminating agents would be (1) the ability to remove all microorganisms and spores; (2) to be removeable or non-toxic to repopulating cells or potential host; and (3) to leave the scaffold ECM unaltered. Thus, the end product could be tested in terms of its sterility, toxicity, and preserved biological properties.

In addition, there is a distinction to be made between sterilisation (killing or removing all microorganisms, including bacterial spores and disinfection (killing or removing all pathogenic microorganisms but not bacterial spores). Most protocols use disinfection techniques, but these may be deemed insufficient in the clinical context, should current experimental methodology progress to that stage. The section below provides an account of techniques used to remove micro-organisms from decellularised scaffolds prior to cell repopulation, as well as a summary of the studies that have compared the efficacy of these techniques.

Sterilisation and disinfection techniques

Irradiation: Irradiation using Gamma rays or electron beam act by inflicting direct damage to DNA and proteins, and by generation of oxidative species and free radicals. The advantages of irradiation are its delivery at room temperature, with no residual chemical toxicity, but with disadvantage of matrix denaturation with increasing dose[130]. To date, the main applications have been in bone and tendon[131] decellularisation.

Ethylene oxide: Ethylene oxide is a toxic organic compound which reacts with sulfhydryl, amino and carboxyl groups in proteins and nucleic acid molecules[132]. It is a gas at room temp and very permeable so penetrates tissues well, but is very adsorbent to decellularised ECM so difficult to clear, and may form toxic species with water such as ethylene glycol[133].

Peracetic acid: Peracetic acid is produced by the reaction of hydrogen peroxide and acetic acid, with antimicrobial activity resulting from the peroxide group (O-O) oxidation of sulfhydryl groups in proteins[134], and with activity against viral particles when combined with ethanol[135]. Although its advantages are that its decomposition molecules (acetic acid, water, and oxygen), are non-toxic and water soluble, it does result in chemical alteration of ECM[136]. There have been wide ranging applications including many examples in liver, with some favourable outcomes in comparative studies (Table 2).

Hydrogen peroxide and hydrogen peroxide low-temperature plasma: Hydrogen peroxide is a powerful oxidant which reacts with cell membranes and causes the denaturation of nucleic acids and proteins[137]. The plasma form of Hydrogen peroxide, generated by magnetic excitation of gas at low temperature, contains many charged and reactive species which also denature proteins and nucleic acids and cell membranes.

Despite the advantage that the end decomposition products (water and oxygen) are non-toxic, the highly reactive original species do result in chemical alteration of proteins[138].

Alcohol: Alcohol disinfects by denaturing proteins. Although it does not eradicate spores, it has been found to be relatively sparing of ECM structure, allowing its use in a wide range of decellularised tissues tissues[131,139]. In the case of liver decellularisation, its use has been mostly in relation to processed ECM[140-142], such as ECM based hydrogels.

Ultra-violet light: Ultraviolet light in the 200–300 nm wavelength range is associated with the strongest disinfection properties, produced by direct DNA damage and generation of ozone as a reactive species. Its advantages are the relative ease of delivery, and the absence of toxic residue, but its limitations are its superficial penetration only, reflected in its use restricted to thin dimension tissues such as small intestine[136], or in case of liver, used for slices of tissue[143].

Antibiotics: Antibiotics use has been reported[131] for treatment of decellularised ECM, including liver[144,145], but their limitations are the restricted spectrum of activity and inability to eradicate spores.

Conclusions

Thus, there are numerous microorganism eradication options, and, based on the properties of sterilisation methods and suitability for specific tissue types, some authors[131] have suggested guidelines to recommend particular methods of sterilisation. In practice, whether these theoretical recommendations deliver the desired microbiological outcome is uncertain, and therefore experimental comparisons of methods seems indicated.

In this regard, only a few comparative studies have been carried out for different organ systems including liver and are summarised in Table 2. Drawing confident conclusions from these studies is difficult because of heterogeneity in the range of techniques used, range of tissues examined, in different animal species.

However, from the studies where comparisons were made, there appears to be some degree of consistency favouring the use of peracetic acid, in achieving sterility with minimal ECM damage in sheep liver[96], porcine kidney[139], porcine temporo-mandibular joint disc[146], rabbit kidney[147], porcine liver[97,148], and mouse lung[149].

LIVER DECELLULARISATION AND RECELLULARISATION

Introduction

Since the first report of successful decellularisation and repopulation of liver tissue carried out in rat liver by Uygun *et al*[86], there have been significant developments with further reports in other models, and evolution in many aspects including the challenge of sizing up technology for larger species livers, investigation of optimal decellularisation method, progress in the variety, delivery, and functional assessment of repopulating cells, culminating in recent reports providing the first evidence of physiologically significant function in large animal bioengineered organs. This section provides an account of areas of advance, highlighting studies which have contributed incremental progress in the field, and for which additional information is given in Table 3.

Liver decellularisation

Similar to the situation in the non-hepatic context, numerous protocols for liver decellularisation have been reported[150,151], varying in nature of decellularizing agents, technique, and time required ranging from hours[86,152] to days[153], to weeks[154] (rat, pig, human respectively) correlating with organ size. Perfusion of decellularising agents *via* the vasculature is the only means of reaching whole parenchymal space in a large organ such as the liver and has been used in all such studies.

The vessels available for infusion of decellularising agents are the portal vein, hepatic artery, and hepatic veins. Of these options, perfusion *via* the portal vein has been used most frequently although some authors report infusion *via* the hepatic veins *via* the inferior vena cava[155,156], the hepatic artery[157], and the hepatic artery and portal vein in combination[158]. Determining whether infusion route is an important factor in decellularisation quality is difficult as almost all studies report one particular technique, presumably arrived at empirically. Two studies suggest pulse flow *via* the hepatic artery provided better quality decellularisation, though whether this improved recellularisation potential with repopulating cells not assessed[159,160].

Choice of detergent for decellularisation is equally varied though protocols using SDS and/or triton X-100 are the most frequently used, with SDS more effective at removing cellular debris, but at the expense of greater detriment to ECM structure. There are few comparative studies, with the exception of those of Ren *et al*[161], Wu *et al*[162], and Kajbafzadeh *et al*[96], showing lesser matrix degradation (with better structural protein, growth factor and glycosaminoglycan retention) and better repopulating cell function with triton X-100 in rat, porcine and sheep liver decellularisation models respectively.

In addition to biochemical content, mechanical structure of ECM important in contributing to signals which influence cell function[46]. In studies comparing protocols in sheep liver, Triton X-100 and SDS resulted in scaffolds with similar tensile strength, but Triton X-100 based protocols resulted in better retention of elasticity[96,163].

Disinfection and sterilisation of scaffold

Diverse methods have been used to eradicate micro-organisms from decellularised liver scaffolds. Once again there are few studies directly comparing the available methods, but those that exist provide some consensus in favour of perfusion with peracetic acid with reports in sheep[96] and porcine[97,148] liver models, suggesting that peracetic acid was optimal in the dual objective of achieving sterility and maintenance of matrix structure, albeit with protocols varying in concentration and time of exposure.

Characterisation of decellularised scaffold

As a result of the many decellularisation and sterilisation techniques, arises a need for some means of assessing the resultant scaffold to enable comparisons of scaffold quality not only for comparative research but also in view of future clinical applications. Ultimately, although the most meaningful quality criterion is how successfully a scaffold accommodates repopulating cells to generate a neo-organ with useful function, this high-level objective has proved difficult to achieve, resulting in the use of intermediary scaffold assessment methods. It is likely that as research advances, new criteria will emerge, with those which best predict end function becoming dominant.

DNA content: Some of the earliest scaffold quality criteria were put forward by Crapo *et al*[125], who suggested that successful decellularization should be determined on the basis of producing ECM which (1) does not contain more than 50 ng of DNA per mg dry weight; (2) with residual DNA fragments no longer than 200 bp; and (3) with no visible nuclear components[125], based on observations of *in vivo* adverse effects of these biochemicals[64,67,126]. In addition to gel electrophoretic methods to determine DNA fragment length, light microscopy with hematoxylin and eosin stain and DAPI stain have been used to demonstrate absence of residual DNA and supplemented by electron microscopy to visualise cell free matrix microarchitecture[86].

Protein and complex polysaccharide content: In contrast to nucleic acids which must be removed, there is a need to preserve structural proteins, growth factors and other complex molecules in the matrix. Many studies report qualitative and quantitative measures of the structural proteins collagen, laminin, elastin, fibronectin as well as glycosaminoglycans [86,158,161], whilst others quantify pre and post decellularisation content for known ECM associated growth factors including hepatocyte growth factor[161], basic fibroblast growth facto[164], vascular endothelial cell growth factor (VEGF) and insulin-like growth factor 1[165], and many others described by Park *et al*[166].

Non-destructive scaffold assessment: The above methods of scaffold assessment require physical sampling and destruction of the decellularised scaffold, preventing its subsequent use for recellularisation. Thus, pursuing the need to establish methods of scaffold assessment that leave the scaffold intact for further experimentation, Geerts *et al*[167] describe non-destructive methods of scaffold assessment by computerised tomography and biochemical analysis of decellularisation effluent perfusate.

Vascular tree structural integrity: The vasculature has a particular importance in the intended aim of recellularisation as parenchymal cell populations are critically dependant on a reliable blood supply. Thus many authors report preservation of ECM scaffold which define vessels as demonstrated by injection of coloured Dextran[90], radio-opaque dye[165], and corrosion casts[168].

Liver scaffold recellularisation

Repopulating cell heterogeneity: With recellularisation of scaffolds comes the choice of repopulating cells. Many different cell types have been investigated including cell lines, induced pluripotent stem cells (IPSCs), mesenchymal stem cells, foetal stem cells, primary adult cells, and their propagated form after culture in organoids -all with associated advantages and shortcomings.

Immortalised cell lines are useful experimental work tools in that they offer a homogeneous population with a stable phenotype, which can be easily propagated in large numbers. However, there is little or no scope for a role beyond experimentation and into clinical applications given the risk of unchecked proliferation and malignant transformation. IPSCs[166] are also very powerful experimental tools with all the advantages of cell lines, and the added benefits of phenotypic versatility, but are similarly limited in clinical applications because of malignant transformation concerns. Mesenchymal stem cells offer a potentially clinically relevant cell type in terms of sourcing, propagation and safety, with possible beneficial immune modulation effects[169], but are probably limited in their range of differentiation end points[170].

Hepatic foetal cells[90] offer advantages of propagation and differentiation plasticity, but have little clinical application potential because of ethical, availability, and immuno-allogeneicity issues. Primary cells[164] offer the advantages of stable, mature phenotype without concerns for malignant transformation, but present difficulties in terms of sourcing, and propagation to clinically relevant cell numbers during which loss of function is often observed. Organoid cultured primary cells (discussed in more detail in the section on cholangiocyte recellularisation below) may offer a realistic solution to expanding primary cells *in vitro* without loss of desirable phenotype.

Thus, there are a multitude of studies reporting hepatic scaffold repopulation using a variety of cell types, introduced into scaffolds *via* different routes, and using various cell combinations, and reporting different means of assessing the repopulated scaffold. The sections below deal with this heterogeneity by describing progress in recellularisation by considering each main hepatic cell type. It is entirely acknowledged however, that optimal function will be achieved by simultaneous co-recellularisation of a variety of cell types, as cell interactions are critical for optimal cell function[171]. Key examples of this concept in the liver recellularisation literature include the reports of (1) Baptista *et al*[90] showing that human foetal liver cells and human umbilical vein endothelial cells (HUVECs) exhibited better function when infused together in scaffold than individually; (2) Barakat *et al*[168], showing that human foetal stellate cells and human foetal hepatocytes together resulted in the generation of mature hepatocyte phenotype; and (3) Kojima *et al*[172], showing that co-seeding of hepatocytes with liver sinusoidal endothelial cells (LSECs), but not HUVECs, improved hepatocyte function.

Hepatocyte recellularization: The first report of liver tissue decellularisation and repopulation by Uygun *et al*[86] in a rat model was followed by others in rodent models[172-174], and thereafter on a larger scale in pig[153] and human livers[154].

These and other models have used a variety of hepatocyte sources for recellularisation including mostly primary hepatocytes[175,176], but also primary

hepatocytes after spheroid propagation[174], foetal hepatocytes[90] and hepatocyte carcinoma cell lines[87].

The mechanism of re-introduction of hepatocytes has been by means of infusion *via* the portal vein in the vast majority of studies, though infusion *via* multiple vascular routes (Hepatic artery, Portal vein, supra and infra hepatic vena cava)[89] and *via* the *via* bile duct[175] have also been reported. There are few comparative studies to determine whether one or other route is optimal, though one study reports significantly higher parenchymal engraftment of hepatocytes after infusion *via* the biliary tree in comparison to the portal vein[173]. For portal vein infusion of hepatocytes, multiple sequential infusions result in better cell engraftment efficiency, cell proliferation, and cell function than infusion of the same number of hepatocytes in one single infusion[86,164].

Many indicators of function have been used to assess the function of hepatocytes reintroduced into decellularised scaffolds, including: (1) Albumin and urea production[86,90]; (2) elimination of ammonia, consumption of glucose and expression of cytochrome p450 metabolic enzymes[161,164,175]; (3) Immunofluorescence demonstration of expression of hepatocyte-specific marker fumarylacetoacetate)[175]; (4) Immunostaining demonstration of hepatocyte viability enzymes such as UDP glucuronosyltransferase 1, glucose6phosphatase[86]; (5) Expression of dipeptidyl peptidase- 4, a bile canaliculus marker, demonstrating hepatocyte polarity[172]; and (6) Immunofluorescence demonstration of hepatocytic lineage markers α-fetoprotein, CYP2A and CYP3A[90].

Ultimately, however, the most meaningful measure of hepatocyte function is whether a repopulated scaffold can exhibit significant function in the harsh test of *in vivo* physiological environment. Two studies have reported the early stages of such function: Bao *et al*[174] repopulated decellularised and heparin treated rat liver scaffolds with primary rat hepatocytes from spheroid culture. Repopulated scaffolds were implanted heterotopically in rats having undergone 90% hepatectomy, with control animals undergoing 90% hepatectomy without scaffold implantation. At 72 h post-operation,

hepatocytes in the implanted scaffolds expressed liver specific genes, including coagulation factor X, albumin, and cytochrome P450. In contrast to control rats whose ammonia levels rose substantially, scaffold implanted rats had significantly slower ammonia increases, and mean survival in this acute liver failure model was increased from 16 h to 72 h.

Anderson *et al*[175] repopulated a decellularised porcine liver using HUVECs infused *via* the vena cava and portal vein and porcine hepatocytes *via* bile duct infusion.

In vitro assessment of the repopulated scaffolds showed increasing production of Von Willebrand factor over time, albumin production, ammonia detoxification and urea production. The presence of HUVEC repopulated vasculature was essential to sustain blood flow in an *ex-vivo* blood circuit. The authors also investigated a porcine heterotopic liver transplant model of acute liver failure. Thus, scaffold portal vein and vena cava were anastomosed to native portal vein and inferior vena cava respectively, and native liver blood flow was entirely abolished by ligation of native portal vein branches and arteries to native liver. The scaffolds sustained flow for 48 h during which intracranial pressure (ICP) and ammonia levels (indicators of acute liver failure) were monitored. Control animals underwent portocaval shunt and liver devascularisation without scaffold implantation. Although no definite differences were seen in ICP measurements, the scaffold transplanted animals showed clear evidence of ammonia level stabilisation in contrast to inexorable increase in control animals. The authors suggested that scaffold functionality was limited by the small size of the grafts (required by the heterotopic implantation) which restricted the number of implanted hepatocytes and resulted in significant small for size syndrome.

Cholangiocyte repopulation: In comparison to hepatocyte repopulation, there are to date few if any reports of repopulation of the biliary tree using primary cholangiocytes. In a rare report in this category, Chen *et al*[177] repopulated a decellularised rat liver scaffold with primary rat cholangiocytes *via* the bile duct and hepatocytes *via* the portal vein, and perfused the repopulated scaffold for 48 h *in vitro*, with assessments showing

expression of a number of cholangiocyte genes including cytokeratin 7, Cystic Fibrosis transmembrane conductance regulator (CFTR), hepatocyte nuclear factor-1 alpha (HNF-1α), gamma glutamyl transferase (GGT).

The reason for the relative absence of studies reporting repopulation with primary cholangiocytes is the longstanding challenge of propagating and maintaining cholangiocytes in conventional cell culture with loss of essential phenotype[178]. As an alternative, driving pluripotent stem cells towards cholangiocytic differentiation requires extensive manipulation[179] and the clinical applicability of such cells remains in doubt in terms of the risk of malignant change[180]. The evolution of organoid culture, however, offers possible opportunities.

Organoid cultures are 3D cell culture systems whereby cells of choice, when placed in the 3D environment of a supporting substrate (typically Matrigel) undergo cell differentiation, self-organization, whilst retaining the ability to propagate[181]. Thus, organoid culture has provided a potential solution to the supply of biliary epithelial cells, allowing expansion of cholangiocytes from small adult tissue samples whilst retaining cholangiocyte phenotype[182] such expression of such as cytokeratins 7 and 19, and epithelial cell adhesion molecule. The technique, first achieved with intra-hepatic human cholangiocytes from a liver biopsy by Huch *et al*[183], was then confirmed subsequently using extrahepatic bile duct cholangiocytes[184], and bile derived cholangiocytes[185,186], with demonstrable transcriptomic and phenotypic differences between cholangiocytes of different origin within the biliary tree[187].

The availability of cholangiocytes provided by organoids has allowed their use in repopulation of decellularised biliary tissue in several models. Thus, Willemse *et al*[188] repopulated decellularised human bile duct tissue with intra hepatic, extra hepatic, and bile derived cholangiocytes from organoid culture and analysed expression of cholangiocyte markers and biliary function of the tissue engineered constructs. In contrast to intra hepatic counterparts, extra hepatic and bile derived cholangiocytes repopulated decellularised bile duct efficiently, exhibited tight junctions and polarity with apical cilia, showed a gene expression profile suggesting maturation of

cholangiocytes, as well as appropriate expression cholangiocyte - specific transporter genes such as CFTR, which was active in a functional assay. Similarly, Roos *et al*[189] isolated cholangiocytes from human bile collected from gall bladders after cholecystectomy, percutaneous trans-hepatic cholangiography, and endoscopic retrograde cholangio-pancreatography (ERCP), and demonstrated efficient and long-term organoid culture (passage > 15 over > 5 months). The cholangiocytes in organoids showed transcriptomic patterns consistent with native cholangiocytes, expressed functional ion channel protein MDR1, and efficiently repopulated decellularised human bile duct scaffolds.

The potential of organoid cultured cholangiocytes was further emphasised by Sampaziotis *et al*[184] who cultured biliary organoids using human cholangiocytes from deceased donors as well as ERCP brush samples. Transcriptomic analysis showed maintained genetic stability over passages and expression of key biliary markers, including cytokeratins 7 and 19, HNF-1β, GGT, secretin receptor, sodium-dependent bile acid transporter (SLC10A2), CFTR and SRY-box 9. Electron microscopy revealed the presence of ultrastructural features characteristic of cholangiocytes, including cilia, microvilli, and tight junctions. Finally, several assays demonstrated key functionalities: (1) Rhodamine 123 accumulated in the ECO lumen only in the absence of the MDR1 antagonist verapamil; (2) fluorescent bile acid cholyl-lysyl-fluorescein was actively exported from cholangiocyte organoids; and (3) Secretin promoted water secretion, resulting in distension of the bile duct lumen, whereas somatostatin negated the effects of secretin. Moreover, *in vivo*, the cholangiocytes self-organized into bile duct–like tubes after transplantation into nude mouse kidney capsule. Finally, the cholangiocytes maintained their phenotype in biodegradable polyglycolic acid scaffolds discs and densified collagen cylinders. Respectively, the repopulated structures were used in mouse *in vivo* models to successfully repair gall bladder wall and reconstitute a functional extra-hepatic biliary tree.

In a further analysis of the potential of organoid cultured cholangiocytes, Sampaziotis *et al*[186] isolated human cholangiocytes for intrahepatic, extrahepatic and gall bladder

bile. Transcriptomic analysis showed that cholangiocytes from different sites expressed a core of similar genes but differed in others. The cholangiocytes displayed a gradual shift in their transcriptional profile along the biliary tree, suggesting a response to region-specific microenvironments. Thus, when grown in organoid culture, cholangiocytes of different regions of the biliary tree reverted to a single common expression profile but, when exposed to gall bladder bile adopted the expression profile corresponding to the site of origin of bile. Using a mouse model of cholangiopathy induced by 4,4' methylenedianiline, intraductal delivery of human gallbladder organoids resulted in engraftment of cholangiocytes, correction of cholangiopathy and phenotype rescue, in comparison to 100% fatality amongst the control group. In a human liver model using discarded deceased donor livers with ischaemic biliary injury, injected organoids engrafted in areas of denuded biliary epithelium, and corrected cholangiopathy.

Thus, in conclusion, whilst there has been a deficit in reports of biliary tree repopulation for decellularised liver scaffolds since the first report of this approach in 2010 from Uygun *et al*[86], the advent of organoid culture appears to have provided a novel means of propagating stable, functional cholangiocytes in sufficient numbers. This would appear to be the best current way of progressing with biliary repopulation of decellularised liver scaffolds.

Hepatic vascular recellularization: Reconstitution of a viable vasculature in a decellularised liver scaffold is of paramount importance, to allow not only function but survival of the other liver cell populations. The objective is complicated in the case of the liver because of its dual blood inflow supply *via* hepatic artery and portal vein, the immensely complex architecture of liver sinusoids, and the uniquely specialist functions of the sinusoidal endothelial cells. This area of research has progressed in terms of the range of cells used, attempts to optimise the quality of endothelial cover to minimise thrombosis, and advancement in large animal blood perfusion models.

In the first report relating to liver scaffold repopulation, Uygun *et al*[86] used commercially sourced rat cardiac microvascular cells to create an endothelial lining, allowing the repopulated scaffold to be perfused in an *ex-vivo* rat blood circuit for 24 h, and in an *in vivo* heterotopic implantation model to renal vessels for an 8 h perfusion period. Subsequent reports have used a variety of cell types to create vascular cover including Ms1 cells[90], HUVECs[90,172,175,176], human EA.hy926 endothelial cell line[87,88], immortalised endothelial cells[190], and primary liver sinusoidal endothelial cells[172].

Functionality of these repopulated vascular cells has been assessed by various criteria including (1) light microscopy to show vascular cover[90], and supplemented with electron microscopy to demonstrate the presence of sinusoidal cell fenestrae[172]; (2) demonstrating the expression of endothelial cell gene product such as of Von Willebrand factor[90,175], endothelial nitric oxide synthase (eNOS)[90], Lymphatic vessel endothelial hyaluronan receptor 1 and stabilin 2 expression[191], Platelet endothelial cell adhesion molecule 1 (PECAM-1), CD34, VE-cadherin (vascular endothelial cadherin), eNOS, VEGF expression[87], sinusoidal endothelial marker (SE- 1) and stabilin-2[172]; (3) platelet adhesion studies[90]; (4)Transcriptomic analysis of infused HUVECs assuming an LSEC phenotype[191]; and (5) Glucose consumption rate[175,191] of infused endothelial cells.

Given the prime importance of preventing thrombosis in the scaffold, several approaches have explored treating the scaffold with anticoagulants and enhancing endothelial cell cover of the decellularised vascular network. Thus, Bao *et al*[174] investigated layer by layer deposition of heparin in decellularised scaffolds, with hepatocyte repopulation, and reported sustained blood perfusion up to 72 h in a heterotopic rat implantation model, in comparison to rapid thrombosis in un-heparinised scaffolds. In a later study, the same group[176] optimised the layer-by-layer technique and showed that heparinisation did not interfere with hepatocyte or endothelial cell repopulation.

Whilst interesting as a possible method of improving initial thrombogenicity, maintaining heparin deposition is not achievable in the longer term, and could present

undesirable consequences. Thus, some authors have investigated the use of heparin to maximise endothelial cell cover, rather than chemically bonding it to scaffold. Studies reporting better endothelial cell repopulation in the presence of heparin preparations include that of Hussain *et al*[87], who reported that exposing scaffold to heparin-gelatin mixture improved endothelial cell ability to migrate and cover vessel discs, perhaps by exploiting gelatin's multiple integrin binding sites which facilitate endothelial cell binding. Scaffolds repopulated with Hep G2 hepatocytes and endothelial cells after heparin gelatin coating showed improved *ex vivo* blood perfusion, in comparison to uncoated scaffolds. Similarly, Meng *et al*[190] 2019 used immortalized endothelial cells to repopulate decellularized rat liver scaffolds. Gelatin hydrogels-based perfusion significantly increased the number of cells that were retained in the scaffolds, and Doppler ultrasound detected active blood flows within the re-endothelialised liver scaffolds 8 d post-transplantation.

Adopting a different approach, some groups have investigated the manipulation of endothelial cell attachment to scaffold to improve vascular cover. Devalliere *et al*[88] covalently coupled the cell-binding domain REDV to the vasculature of decellularized rat livers before seeding endothelial cells *via* the portal vein. REDV coupling increased cell attachment, spreading and proliferation of endothelial cells within the scaffold resulting in uniform endothelial lining of the vasculature, and a reduction in platelet adhesion and activation. Ko *et al*[89] conjugated anti-endothelial cell antibodies to liver scaffolds resulting in uniform endothelial attachment and reduced platelet adhesion upon blood perfusion *in vitro*. The re-endothelialised livers, withstood physiological blood flow *in vivo* for up to 24 h in a porcine implant model. Kim *et al*[192] used aptamers (short, single-stranded DNA or RNA molecules that selectively bind to specific targets) with CD31 specificity. Aptamer coated scaffolds showed higher endothelial cell coverage, enabled perfusion with blood for 2 h with reduced platelet adhesion *ex vivo*, and restored liver function in a hepatic fibrosis rat model.

In the most significant advances to date in the area of successful hepatic vascular perfusion of repopulated scaffolds, at least in terms of length of *in vivo* perfusion,

Shaheen *et al*[191] seeded decellularized whole porcine livers with HUVECs and showed successful perfusion of the heterotopically implanted scaffolds into for up to 20 d. The same group[175] later co-seeded primary porcine hepatocytes after HUVEC reendothelialization. Repopulated scaffolds were implanted heterotopically in a pig model and produced improved biochemical function in an acute liver failure model.

In conclusion, the difficult problem of repopulating the vasculature of decellularised scaffolds has seen significant progress, with reports of *in vivo* blood perfusion lasting many days. Whist encouraging, there remain advances to be made in the development of clinically relevant cell populations for this purpose, and the repopulation of the highly specific liver sinusoidal endothelial cells.

Conclusions

The field of hepatic scaffold recellularisation has advanced from *in vitro* rodent liver scaffold models to large animal *in vivo* blood perfusion. Whilst this represents much progress, significant areas of development remain to be investigated. Of the different liver cell types, even in the case of hepatocytes where repopulation results are the most advanced, the degree of hepatocyte function observed to date is still limited. Cholangiocyte recellularisation is far behind, though organoid sourced cells may help with this challenge. Intra-hepatic vascular recellularisation has allowed impressive *in vivo* perfusion but using cells which have limited application beyond experimental models. Minority cell groups such as Kupffer cells and stellate cells, though important in their influence on other cell types, have not been repopulated decellularised scaffolds.

RECELLULARISATION OF EXTRA HEPATIC BLOOD VESSELS

Introduction

If the objective of whole liver recellularisation is the bioengineering of neo-organs is implantation to provide useful function, neo-livers will need to be fully reconnected to the recipient circulation, with both hepatic arterial and portal venous inflow, and

hepatic vein outflow. To date, because the focus of investigation has understandably been to achieve viable blood circulation through the sinusoidal network, extra-hepatic vascular inflow has relied exclusively on portal reperfusion of recellularised grafts. Whilst much progress has been made with sustained portal perfusion of up to 20 d in large animal models[191], recellularisation and perfusion of the hepatic artery has not been reported. This gap in the field will need to be addressed, as, unlike hepatocytes which may survive on portal flow alone, the biliary tree is critically dependant on hepatic arterial supply.

Arterial scaffold recellularisation precedents

In addition to thrombogenicity, the hepatic artery presents considerable other difficulties stemming from the biophysical demands of withstanding arterial pressure in the short and long term. In the short term, a recellularised artery and its arterial anastomosis needs to be able to tolerate pressures of 3000 mmHg[193], and then do so in the long term without accelerated atherosclerosis.

The challenges of bioengineering viable arterial conduits[193] is an entire field in itself, with much research motivated by the clinical need represented by the immense burden of cardiac, cerebrovascular and peripheral vascular disease. The research trajectory of vascular biologists and clinicians in vessel bioengineering has followed much the same path as those studying the liver. As a result of the drawbacks of synthetic[194] and allogeneic and xenogeneic grafts[195] (long-term patency issues due to thrombosis, inflammation, and stenosis), there has been an evolution towards cellular repopulation of scaffolds of various types. Thus, following pioneering reports by Weinberg *et al*[196] of early bioengineered vessels containing collagen, Dacron and a combination of smooth muscle and endothelial cells, L'Heureux *et al*. reported the use of extracellular matrix with vascular cells to bioengineer a blood vessel[197], with subsequent reports of successful bioengineered grafts in clinical practice[198,199].

Arterial vessel anatomy is complex and consists of three concentric layers (1) the intima layer, composed of endothelial cells resting on an internal elastic lamina layer of

type 4 collagen and elastin, which separates it from media; (2) the media, composed of smooth muscle cells (SMC), type I and type III collagen; and (3) the adventitia, containing fibroblasts embedded in a loose collagen matrix of type I and type II collagen. The ability of arteries to withstand arterial pressure waves stems from the complex tri-layer of cells and ECM above, which therefore likely requires recapitulation to achieve similar function in recellularised grafts. In the context of tubular grafts, this challenge has been investigated using a variety of biofabrication techniques including biomaterial moulding[200], cell sheet engineering[201], bio-ink applications, with tissue maturation[202] under fluid flow[203] in purpose designed bioreactors[193].

In the specific case of arterial scaffolds obtained by decellularisation techniques, followed by repopulation with appropriate cells, there are many examples of successful long term outcomes in a variety of experimental models, reviewed by Krawiec *et al*[204], and including (1) Cho *et al*[205] who used canine bone marrow mononuclear cells differentiated under different culture conditions to generate smooth muscle and endothelial phenotypes. These were reintroduced into decellularised dog carotid arteries sequentially to create media and intimal layers in neo-vessels, which were reimplanted in a canine carotid model. Seeded grafts were patent at 8 wk compared to thrombosis at 2 wk in unseeded controls; (2) Similarly, Zhao *et al*[206] used ovine bone marrow stem cells and differentiated them into endothelial and smooth muscle phenotypes, before seeding them onto decellularized carotid artery scaffolds. Seeded scaffolds were mechanically stable and patent at 5 months, in comparison to unseeded controls, which all occluded at 2 wk or less; (3) Kaushal *et al*[207] isolated endothelial precursor cells from peripheral blood of sheep, expanded them *ex vivo* and then seeded them on decellularized porcine iliac vessels. Seeded grafts remained patent for 130 d as a carotid interposition graft in sheep, whereas non-seeded grafts occluded within 15 days; (4) Borschel *et al*[208] repopulated decellularised rat femoral arteries with primary endothelial cells, which were implanted as interposition grafts. Patency rates at 4 wk were 89% and 29% recellularised grafts and control grafts respectively; (5) Ma *et al*[209] repopulated decellularised foetal pig aortas with canine endothelial cells and

demonstrated 6-month patency after reimplantation in a canine carotid model; and (6) Dahan *et al*[210] repopulated decellularised pig carotid artery with autologous endothelial and smooth muscle cells and demonstrated 6-week patency in a carotid interposition graft model.

Conclusion

Thus, the problem of arterial recellularisation brings very significant and specific challenges, but with some promising possible solutions suggested by long term successful perfusion bioengineered repopulated decellularised arterial neo-vessels in several animal models.

IMMUNOGENICITY OF DECELLULARISED SCAFFOLDS

Introduction

In its most ambitious objective, bioengineering neo-organs by decellularisation and recellularisation would involve the use of allogeneic or even xenogeneic scaffolds repopulated with appropriate cells originating from the intended recipient. The resultant neo-organ would thus in theory be immunologically syngeneic, at least from the perspective of the repopulating cells. The question remains, however, whether non-self scaffold, even if covered by syngeneic cells may elicit an adverse immune or inflammatory reaction.

Scaffold immunogenicity studies

Overwhelmingly, *in vivo* animal studies and human clinical studies examining implantation of decellularised scaffold show non pathological and constructive, functional tissue remodelling with the partial restoration of tissue appropriate to the site of implantation[211]. Examples of such animal studies include that of Mirmalek-Sani *et al*[157], who observed no local or systemic adverse host response to decellularised porcine liver scaffold introduced into rats, and similar report of studies involving further xenogeneic introduction of decellularised scaffolds of goat into mouse[212], rat

into rabbit[213] and mouse into rat[214]. These results are matched by successful use of decellularised scaffolds in the clinical setting without adverse effect, such as that used by Lawson *et al*[199] who constructed bioengineered vascular grafts for dialysis in patients with chronic renal failure, and other clinical reports describing favourable results with the use of decellularised scaffolds in oesophageal tissue[215], tendon[216], major cardiac vessel[217], and chronic wound management[218]. However, there have also been reports, albeit in a small minority, of scaffold related inflammatory reactions[219,220], thus raising questions relating to the immunogenicity of decellularised ECM.

Depending on the nature of an implanted material into a host, the host response may broadly be characterised as either (1) pro-inflammatory, eventually leading to the deposition of non-functional dense scar tissue, or, in contrast; and (2) 'constructive remodelling', leading to the controlled incorporation/degradation of the implanted material and its replacement with functional tissue consistent with the site of implantation[221,222].

The factors that determine which of these responses prevails are incompletely understood, but involve the interaction of the implanted material with innate[223] and adaptive immune system cells[224] such as the natural killer cells, macrophages, and lymphocytes, which can be directed to assume very different phenotypes, resulting in either a reconstructive or inflamatory reaction. The constructive remodelling response is characterised by the directing of macrophages towards the M2 (reconstructive) rather than M1 (inflammatory) phenotype, and the presence of T helper cells of Th2 phenotype, with cellullar upregulation and downregulation of anti-inflammatory and proinflammatory genes respectively[225].

In relation to the reaction ellicited by the implantation of decellularised ECM, investigation suggests that decellularised ECM per se does not ellicit an inflamatory reaction, but does stimulate a strong pro-healing phenotype of the innate and adaptive immune systems[66,225,226]. Adverse reactions do result, however, as a consequence of retained cellular products from incomplete decellularisation[67,227], post decellularisation

processing of scaffolds such as cross linking[100,228], or remnants of decellularising cells[229], or sterilising agents methods in the implanted scaffold[222].

The mechanism whereby decellularised ECM ellicits a reconstructive response is incompletely understood but likely relates to molecular homology, the effect of bioactive molecules within the ECM, and the influence these biomolecules have on host immune and regenerative cells.

Thus, the constituent biochemicals of ECM, including laminin, collagens, fibronectin,and glycosaminoglycans are amongst the most highly conserved molecules in mamalian species[230]. As a result of this high degree of conservation, allogeneic and even xenogeneic ECM implants ellicit similar 'self' recognition and constructive cell responses[225,231]. The infiltration of implanted decellularised scaffold by host cells results in the exposure and release of bioactive molecules inluding cryptic peptides, which modulate the immune response and direct innate and adaptive immune cells towards a reconstructive phenotype[232]. These, and other bioactive molecules within the ECM also act as chemotactic agents for stem and progenitor cells *in vitro* and *in vivo*[233]. Indeed, cryptic peptides from collagen III can reproduce progenitor cell chemotaxis[40,234].

Conclusion

In conclusion, although there are some reports of adverse reactions to implantation of decellularised ECM, these examples are due to retained cellular products or decellularising agents, rather than the ECM itself, which ellicits a favourable remodelling response, even if xenogeneic. This allows some optimism for the prospect of recellularising appropriate animal ECM scaffolds for clinical use in humans.

CONCLUSION

In the 12 years since the first report of liver decellularisation[86] and repopulation to the present, there has been much progress in the field, which has moved from predominantly *in vitro* small animal models to *in vivo* large animal models sustaining

bioengineered liver perfusion for up to 20 d *in vivo*[191]. Despite this, many challenges and areas of investigation remain.

Firstly, even in the restricted domain of a single organ such as the liver, decellularisation protocols remain varied, and more often arrived at in empirical rather than comparative ways. Assessment of the quality of the decellularised scaffold is described according to numerous criteria with only some having been validated in terms of recellularisation efficacy. Standardisation of technique and quality assessment will need to progress significantly not only to facilitate experimental investigation, but also in future to meet clinical application standards. In the anticipation of sourcing human scaffolds from decellularised deceased donor livers, such considerations would apply particularly given the likely variability of scaffold quality, in contrast to the relative reproducibility of scaffolds originating from experimental animals. Should xenogeneic scaffolds ever be considered and repopulated with human cells, zoonotic as well as immunological concerns would have to be addressed.

In the area of recellularisation, the first hurdle remains the establishment of a viable vasculature, as no parenchymal function or survival is possible without it. In the liver, this is a particularly difficult problem because of the dual blood supply, and the uniquely specific functions of the sinusoidal endothelial cells. Thus, hepatic arterial recellularisation, and the fashioning of a neo-hepatic artery capable of withstanding arterial pressure has not been attained, but will be essential, as survival of the biliary tree will not be achieved without it.

Although recellularisation of portal sinusoidal and hepatic venous compartments has much progressed, with the achievement of *in vivo* perfusion albeit with portal hypertension[175], these results have been achieved with cells (often HUVECS) which, whilst providing excellent experimental tools, raise barriers to progress to the ultimate aim of recellularising scaffolds with cells from the intended recipient, and generating a syngeneic organ obviating the need for immunosuppression.

Immune considerations aside, the diversity of cell function in the vasculature of the liver is another area requiring investigation. Whilst HUVECs seem to assume some

characteristics of liver sinusoidal cells when introduced into decellularised scaffolds, it remains to be shown that they can carry out the numerous, unique, and vital functions of LSECs. If they do not, a more refined recellularisation population will be required.

Assuming that a viable and fully functional vasculature is achieved, recellularisation of the main parenchymal elements, the hepatocytes and cholangiocytes, is also far from attained. In terms of the former, repopulation of decellularised scaffolds with primary hepatocytes has proved reproducible, but has only provided the beginnings of significant function, with temporary stabilisation of serum ammonia in the most successful *in vivo* models[175]. Amongst many others, endocrine, synthetic, detoxifying, and bile metabolic functions have not yet been demonstrated. Repopulation of the biliary tree is also unattained, till now largely due to the difficulty of propagating cholangiocytes in sufficient numbers, though this challenge may be alleviated by the advent of organoid culture. Other cell types, such as Kupffer cells and stellate cells, present as minorities in terms of numbers but significant in their influential interaction with hepatocytes and cholangiocytes, have not been investigated at all in recellularisation.

Although currently very distant, matters relating to clinical applications will also need much consideration. Thus, the entire process, decellularisation agents and methods, the resultant scaffold, and repopulating cells would need to meet stringent clinical grade standards. Concerns regarding scaffold immune response on the part of the host, though thus far not an observation in the context of experimental models, would have to be addressed more rigorously, as would zoonosis in the scenario of xenogeneic scaffolds.

Finally, it seems difficult to envisage that a clinical grade neo-organ could be generated entirely *in vitro*. More likely a partially recellularised scaffold may be produced, and require completion of repopulation *in vivo*, implying, at least temporarily, an auxiliary role for such neo-organs, rather than the prospect of transplantation in the manner that is practised with retrieved donated organs.

In summary, bioengineering of organs by decellularisation and repopulation remains a fascinating area still in an early phase of investigation, where the last decade has produced major advances but also left vast opportunity for research and development.

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Table 1 Examples of non-liver decellularisation protocols

Orga	Species	Decellularisation technique	Recellularization	Significant outcome	Ref.
Heart					
	Rat	SDS + Triton X-100	Neonatal cardio-myocytes	(1) Maintained eight constructs for up to 28 days by coronary perfusion in a bioreactor that simulated cardiac physiology; (2) Macroscopic contractions were observed by day 4; and (3) By day 8, under physiological load and electrical stimulation, constructs could generate pump function in a modified working heart preparation.	Ott *et al*[235]
	Pig	Freeze and Thaw + hypotonic solution + trypsin/EDTA/NaN3 + Triton X-100/EDTA/NaN3 + deoxycholic acid	Chicken embryonic cardio-myocytes	Cardiac extracellular matrix supported the formation of organized chicken cardiomyocyte sarcomere structure	Wainwright *et al*[236]

			in vitro.	
Rat	SDS *vs* POETE	Not performed	SDS decreased DNA and GAG and enriched the collagen content 10-fold.	Bruyneel *et al*[237]
Pig	SDS *vs* Triton X-100 *vs* CHAPS *vs* OGP	Not performed	3% SDS as a detergent showed optimal decellularization.	Ferng *et al*[238]
Rat	SDS + Triton X-100	Induced cardiac progenitor cells	(1) Optical mapping of recellularised scaffolds shows field-stimulated calcium transients that propagate across islands; and (2) Bipolar local stimulation demonstrated cell-cell coupling within scaffolds.	Alexanian *et al*[239]
Kidney				
Rat	Saline + SNP + Triton X-100, DNAse + SDS	Murine pluripotent embryonic stem cells	(1) Primitive precursor cells populated and proliferated within the glomerular, vascular, and tubular structures; and (2)	Ross *et al*[240]

			Cells lost their embryonic appearance and expressed immunohistochemical markers for differentiation.	
Monkey	1% SDS *vs* 1% Triton X-100	Not performed	SDS at 48C to be most effective in preserving the native architecture.	Nakayama *et al*[241]
Pig	0.5% SDS *vs* 0.25% SDS *vs* 1% Triton X-100 with 0.1% ammonium hydroxide	Not performed	0.5% SDS was the most effective detergent.	Sullivan *et al*[242]
Pig	SDS	Not performed	(1) Kidney decellularized scaffolds implanted in Yorkshire pigs easily re-perfused, sustained blood pressure; (2) Scaffolds maintained renal ultrastructure; and (3) However, presence of inflammatory cells in the pericapsular	Orlando *et al*[243]

			region and complete thrombosis of the vascular tree were evident.	
Rat, pig, and human	SDS	HUVECs + Rat Neonatal kidney cells	(1) The resulting grafts produced rudimentary urine *in vitro* when perfused *via* their intrinsic vascular bed; and (2) Transplanted orthotopic grafts in rats, perfused by the recipient's circulation, produced urine *via* the ureteral conduit *in vivo*.	Song *et al*[244]
Pig	Sonication + SDS + Triton X-100	Not performed	(1) Significant decrease in decellularization time with sonication; and (2) Sonicator power proved to have significant effect on the microarchitecture integrity of the scaffold.	Manalastas *et al*[76]

Lung				
Rat	Heparinized PBS + SDS + Triton X-100	HUVECs	Orthotopic Transplantation of grafts with 6 h of perfusion *in vivo*.	Ott *et al*[245]
Rat	PBS + SNP + CHAPS + EDTA + Benzonase	Rat neonatal lung epithelial + lung vascular endothelial cells	(1) *In vitro,* the mechanical characteristics of the engineered lungs were like those of native lung tissue; and (2) *In vivo* gas exchange for short time intervals (45 to 120 min).	Petersen *et al*[246]
Mice	Triton X-100 + SDS + DNase	Embryonic stem cells	Demonstrated growth of foetal alveolar type II cells.	Price *et al*[247]
Rat	Heparinized PBS + SDS + Triton X-100	HUVECs + rat foetal lung cells	Orthotopic transplantation of grafts with 7 d of perfusion *in vivo*.	Song *et al*[248]
Trachea				
Rabbit	Freeze/thaw + Sonication + SDS	Not performed	(1) Orthoptic transplantation od decellularized scaffolds into segmental tracheal	Hung *et al*[249]

			defects in rabbits; (2) Respiratory epithelium regeneration on the inner surface; and (3) Cartilaginous tubular structures could not maintain structural integrity.	
Pig	Freeze and Thaw + Agitation/immersion + SDS	Not performed	Successful decellularization.	Guimaraes *et al*[82]
Rabbits	Sonication + 1 % SDS	Not performed	Orthotopic transplantation of partially decellularized trachea with no immunosuppression treatment resulted in 2 months of survival in two rabbits and one long-term survival (2 years) in one rabbit.	Dang *et al*[71]
Nerve				
Human	Triton X-100 + SDS + EDTA + sonication	Not performed	Detergent and sonication more effective than	Suss *et al*[74]

			detergent only.	
Small intestinal submucosa				
Pig	SDS/Triton X-100/DNase *vs* Agitation and immersion	Not performed	SDS/Triton X-100 combination for decellularization proved superior.	Syed *et al*[81]
Thyroid				
Rabbit	SDS + immersion/agitation	HTFC	The scaffolds exhibited good cytocompatibility, supported HTFCs growth, and proliferation.	Weng *et al*[85]

HTFC: Human thyroid follicular cells; SDS: Sodium dodecyl sulphate; HUVECs: Human umbilical vein endothelial cells.

Table 2 Summary of studies comparing different sterilization techniques used for decellularised scaffolds

Species	Organ	Sterilization technique	Outcome	Ref.
Sheep	Liver	Compared 6 different sterilization methods: (1) Freeze drying; (2) Ethylene oxide gas; (3) Gamma irradiation; (4) Gamma	(1) Peracetic acid or ethylene oxide + gamma irradiation was associated with the best outcome; and (2) Freeze drying and Gamma irradiation completely sterilized the liver, but also reduced the mechanical properties.	Kajbafzadeh *et al*[96]

		irradiation + Peracetic acid; (5) Gamma irradiation + Ethylene oxide gas; and (6) Gamma irradiation + Freeze drying		
Porcine	Liver	Compared 3 different sterilization methods: (1) Peracetic acid; (2) Ethanol; and (3) Slightly acidic electrolyzed water	(1) Ethanol caused a significant loss in collagen content; (2) The retained glycosaminoglycan content decreased in all treatments; and (3) Peracetic acid and slightly acidic electrolyzed water treatments achieved the highest efficiency of sterilization.	Hussein *et al*[148]
Mouse	Lung	Compared 2 different sterilization methods: (1) Gamma irradiation; and (2) Peracetic acid	(1) Irradiation produced significant structural distortion; and (2) Peracetic acid had less effect on the resulting architecture.	Bonenfant *et al*[149]
Porcine	TMJ Fibro-	Compared 3 different	(1) Gamma irradiation and Ethylene Oxide caused	Matuska *et al*[146]

	cartilage disc	sterilization methods: (1) Peracetic acid; (2) Gamma irradiation; and (3) Ethylene oxide.	structural damage leading to inferior cell adhesions; and (2) Peracetic Acid caused minimal structural damage but also induced chemical modifications leading to better cell attachments.	
Porcine	Kidney	Compared 4 different sterilization methods: (1) 70% Ethanol; (2 0.2% Peracetic acid in 1 M NaCl; (3) 0.2% Peracetic acid in 4% Ethanol; and (4) Gamma irradiation	(1) All four methods were successful in decontamination; (2) Gamma-irradiation was very damaging to collagen fibres and glycosaminoglycans, leading to less proliferation of human renal cortical tubular epithelium cells; and (3) 0.2% peracetic acid in 1 M NaCl was found to be the best method as it completely decontaminated the renal tissue and demonstrated to have preserved essential components of the ECM.	Poornejad *et al*[139]
Porcine	Liver	Compared 2 different sterilization methods: (1	(1) ECM treated with Acetic acid showed higher initial attachment and albumin and urea production in	Coronado *et al*[97]

		Hydrochloric acid; and (2) acetic acid.	HepG2/C3A cell cultures compared to Hydrochloric acid; and (2) Acetic acid preserved bioactive moieties compared to Hydrochloric acid.	
Rabbit	Kidney	Compared 4 different sterilization methods: (1) Antibiotics (Penicillin G, Amphotericin B and Gentamicin; (2) Peracetic acid (0.5 %, 1% and 1.5 %); (3) Gamma irradiation 5 KG; and (4) 3 UV-irradiation 20-480 nm	(1) UV-irradiation is not able to sterile; (2) Gamma irradiation resulted in reduced mechanical strength and altered microstructure; and (3) 0.5 % Peracetic acid was the most efficient method to completely decontaminate rabbit decellularized kidney while preserving the mechanical properties and main components of the matrix.	Moradi *et al*[147]

ECM: Extracellular matrix.

Table 3 Liver decellularisation recellularisation studies

Species	Decellularisation method	Recellularisation cell type and route	Outcome	Ref.
Female Lewis rats	SDS + Triton X-100	(1) Primary rat hepatocytes *via* the Portal vein; and (2) Rat cardiac microvascular endothelial cells *via* portal vein	(1) Demonstrated Successful decellularization/Recellularization with cell viability and function; (2) Demonstrated the feasibility of transplanting these recellularised liver grafts *in vivo* with minimal ischemic damage; and (3) The recellularised graft supports liver-specific function including albumin secretion, urea synthesis and cytochrome P450 expression at comparable levels to normal liver *in vitro*.	Uygun *et al*[86]
Fisher 344 rats	Triton X-100 + SDS	Rat liver progenitor cell line WB344 through the inferior vena cava	(1) Perfusion with 0.1% SDS for 1 hour completely cleared all DNA; and (2) Supplementation of all perfusion solutions with antibiotics/antimycotics prevented microbial growth, and the IDL could be stored at 4°C for several weeks.	Shupe *et al*[156]
Male Sprague Dawley	Trypsin + EGTA + Triton X-100	Primary mice hepatocytes *via*: (1) Direct	Systematic comparison of three different reseeding methods showed that a multistep strategy	Soto-Gutierrez *et al*[164]

rats		parenchymal injection; (2) Continuous perfusion *via* the portal vein; and (3) Multistep infusion *via* the portal vein	provides the greatest seeding efficiency and the presence of functional hepatocytes.	
Male Lewis rats	SDS + Triton X-100	Primary rat hepatocytes *via* the portal vein (from spheroid culture)	(1) Layer-by-layer heparin deposition was used to avoid thrombosis, followed by repopulation of hepatocytes, and successfully implanted as a TEL into the portal system; (2) Treatment of extended hepatectomized rats with a TEL improved liver function and prolonged survival; mean lifespan was extended from 16 to 72 h; and (3) At 72 h post operation, the TEL sustained functional and viable hepatocytes.	Bao *et al*[174]
Ferret	Distilled water + Triton X-100 + ammonium hydroxide	Human foetal liver cells + human umbilical vein endothelial cells co-infusion *via* the portal	Demonstrated delivery of cells to different compartments of the liver tissue *via* different pathways EC delivered through the vena cava selectively seeded larger and smaller blood vessels up to the	Baptista *et al*[90]

		vein	pericentral area of the liver lobule and cells seeded through the portal vein reached predominantly the periportal area of the liver lobule.	
Adult male Sprague-Dawley rats	SDS or Triton X-100 + sodium hydroxide	Primary rat hepatocytes *via* the portal vein	Decellularised scaffolds constructed by perfusion of Triton X-100 were of superior quality and can provide a more effective and ideal scaffold for tissue engineering and regenerative medicine.	Ren *et al*[161]
Porcine	SDS + DNase	Porcine hepatocytes *via* the portal VEIN	Demonstrated a protocol to decellularise rapidly a full-size porcine liver with small detergent volumes within 24 h.	Bühler *et al*[153]
Human	Distilled water + SDS + Triton X-100	Human cell lines hepatic stellate cells (LX2), hepatocellular carcinoma (Sk-Hep-1) and hepatoblastoma (HepG2) *via* suspension	Decellularised human liver cubic scaffolds were repopulated for up to 21 d using human cell lines with excellent viability, motility and proliferation and remodelling of the extracellular matrix.	Mazza *et al*[154]
Piglet	Triton X-100 + ammonium hydroxide	Murine endothelial cells (MS1) with	(1) Developed an effective method for re-establishing the vascular network within decellularised	Ko *et al*[89]

		combination of static and perfusion techniques (*via* the portal vein)	liver scaffolds by conjugating anti-endothelial cell antibodies to maximize coverage of the vessel walls with endothelial cells; (2) This procedure resulted in uniform endothelial attachment throughout the liver vasculature extending to the capillary bed of the liver scaffold and greatly reduced platelet adhesion upon blood perfusion *in vitro*; and (3) The reendothelialized livers, when transplanted to recipient pigs, were able to withstand physiological blood flow and maintained for up to 24 h	
Porcine	SDS + Triton X-100	Rat primary hepatocytes and human umbilical vein endothelial cells (cells cultured in scaffolds, but not in a perfusion circuit)	(1) The heparinized scaffolds showed improved anticoagulation and cytocompatibility compared to the control scaffold both *in vitro* and *in vivo* test; and (2) The layer-by-layer technique showed that heparinisation did not interfere with hepatocyte or endothelial cell repopulation.	Bao *et al*[176]
Porcine	SDS	Human EA.hy926 endothelial cells	(1) The study demonstrated, exposing scaffold to heparin-gelatin mixture improved	Hussein *et al*[87]

		and HepG2 hepatic carcinoma cells *via* the portal vein	endothelial cell ability to migrate and cover vessel discs, perhaps by exploiting gelatin's multiple integrin binding sites which facilitate endothelial cell binding; and (2) Scaffolds repopulated with Hep G2 hepatocytes and endothelial cells after heparin gelatin coating showed improved *ex vivo* blood perfusion, in comparison to uncoated scaffolds.	
Male Lewis rats	Trypsin + EGTA + Triton X-100	Primary rat hepatocytes *via* the bile duct and the portal vein	The study results suggest that biliary tree cell-seeding approach is promising, and that liver progenitor cells represent a good cell source candidate.	Ogiso *et al*[173]
Male Lewis rats	Trypsin + EGTA + Triton X-100	(1) Primary rat hepatocytes *via* the Bile duct; and (2) LSECs *via* the portal vein	(1) Hepatocytes co-seeded with LSECs retained their function compared with those seeded alone; (2) LSECs maintained hepatic function, and supported hepatocyte viability under blood perfusion in the engineered liver graft owing to their antithrombogenicity; and (3) Successfully achieved continuous blood flow into the vascularized liver graft by extracorporeal	Kojima *et al*[172]

			perfusion for at least 8 hours	
Female Lewis rats	SDS + Triton X-100	Human EA.hy926 endothelial cells *via* the portal vein	(1) Coupled the cell-binding domain REDV to the vasculature of decellularised rat livers; and (2) REDV coupling increased cell attachment, spreading and proliferation of endothelial cells within the scaffold resulting in uniform endothelial lining of the vasculature, and a reduction in platelet adhesion and activation	Devalliere *et al*[88]
Female Lewis rat	SDS	(1) Rat cholangiocytes *via* the common bile duct; and (2) Rat hepatocytes *via* the portal vein	(1) Demonstrated for the first time, whole liver grafts co-populated with hepatocytes and cholangiocyte; (2) Cholangiocytes formed duct-like structures, with the viable hepatocyte mass residing in the parenchymal space, in an arrangement highly comparable to the native tissue; and (3) Both albumin and urea assay results confirmed hepatocyte functionality and the gene expression analysis of cholangiocytes in recellularised liver grafts indicated viability and sustained gene expression of	Chen *et al*[177]

			functional proteins.	
Adult Sprague-Dawley rats	Triton X-100 + NH4OH	Rat sinusoidal endothelial cells were perfused *via* the Portal vein in either RPMI media or in 5% gelatin hydrogel solution	(1) Used immortalized endothelial cells to repopulate decellularised rat liver scaffolds; (2) Gelatin hydrogels-based perfusion significantly increased the number of cells that were retained in the scaffolds; and (3) The Doppler ultrasound detected active blood flows within the re-endothelialised liver scaffolds 8 d post heterotopic transplantation.	Meng *et al*[190]
Male Lewis rats	Trypsin/EGTA solution + Triton X-100/EGTA	Human induced pluripotent stem cells derived hepatocyte-like cells *via* bile duct	(1) The first study to generate a recellularised liver model with human hepatic function using human induced pluripotent stem cells; and (2) This result suggested that the BD was an appropriate recellularization pathway regardless of the hepatocyte type.	Minami *et al*[250]
Porcine	SDS + Triton X-100	Human umbilical vein endothelial cells *via* the superior vena cava followed by *via* the portal vein	Decellularised whole porcine livers revascularized with human umbilical endothelial cells and implanted heterotopically into immunosuppressed pigs whose spleen has been removed sustained perfusion for up to 20 d.	Shaheen *et al*[191]
Porcine	Triton X-100 +	(1) Human	(1) Co-seeded primary porcine	Anderson

	SDS	umbilical vein endothelial cells *via* the vena cava and the portal vein; and (2) Porcine hepatocytes *via* the bile duct	hepatocytes after human umbilical vein endothelial cell reendothelialization; and (2) Repopulated scaffolds were implanted heterotopically in a pig model and produced improved biochemical function in an acute liver failure model.	*et al*[175]
Female Sprague-Dawley rats	SDS + DNase	Human umbilical vein endothelial cells *via* the Portal vein	(1) Used aptamers (short, single-stranded DNA or RNA molecules that selectively bind to specific targets) with CD31 specificity; and (2) Aptamer coated scaffolds showed higher endothelial cell coverage, enabled perfusion with blood for 2 h with reduced platelet adhesion ex vivo, and restored liver function in a hepatic fibrosis rat model.	Kim *et al*[192]

TEL: Tissue-engineered liver; SDS: Sodium dodecyl sulphate; LSECs: Liver sinusoidal endothelial cells; REDV: Arg GluAsp Val.

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