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Extracellular matrix hydrogel therapies: *in vivo* applications and development

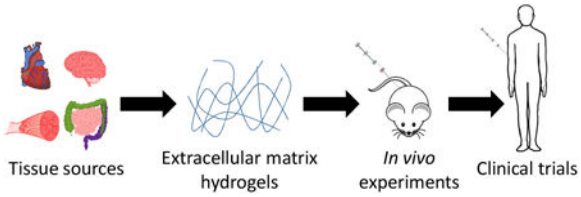
Martin T. Spang and **Karen L. Christman***

Department of Bioengineering, Sanford Consortium for Regenerative Medicine, University of California, San Diego, La Jolla, CA 92037, USA

Abstract

Decellularized extracellular matrix (ECM) has been widely used for tissue engineering applications and is becoming increasingly versatile as it can take many forms, including patches, powders, and hydrogels. Following additional processing, decellularized ECM can form an inducible hydrogel that can be injected, providing for new minimally-invasive procedure opportunities. ECM hydrogels have been derived from numerous tissue sources and applied to treat many disease models, such as ischemic injuries and organ regeneration or replacement. This review will focus on *in vivo* applications of ECM hydrogels and functional outcomes in disease models, as well as discuss considerations for clinical translation.

Graphical abstract



Keywords

Extracellular matrix; Hydrogel; Decellularization; Regenerative medicine; Tissue engineering

1. Introduction

Decellularized scaffolds have become increasingly prevalent in the field of tissue engineering. Historically, decellularized extracellular matrix (ECM) has been used as patches or sheets for surgical applications. For example, some of the most widespread clinical uses include soft tissue repair, such as burn wounds and diabetic ulcers, in which

*Corresponding author at: Dr. Karen L. Christman, Department of Bioengineering, Sanford Consortium for Regenerative Medicine, University of California, San Diego, 2880 Torrey Pines Scenic Drive, La Jolla, California, 92037. christman@eng.ucsd.edu; Office Phone: 858-822-7863; Fax: 858-534-5722.

products derived from decellularized human skin, porcine small intestine submucosa (SIS), and porcine urinary bladder are used [1]. In preclinical models, these have been used in a range of applications, including myocardial [2-5], liver [6], tracheal [7, 8], and esophageal repair [9, 10]. However, there are limitations with these patches, namely they require surgical access for implantation and are not amenable to minimally invasive delivery, including injections and catheter-based procedures.

Decellularized ECM can be further processed to generate an injectable material through two main approaches, as seen in Figure 1. In the first approach, the ECM is simply ground or milled into a fine powder and then suspended in liquid for subsequent injection. A few clinical products exist in this powder form, including demineralized bone matrix [11], bladder matrix (MatriStem), and micronized acellular dermis [12, 13] (micronized AlloDerm), and others such as human lipoaspirate [14-16], liver [17], and cardiac tissue [18] have been examined in preclinical models. In the second approach, the particulate ECM is enzymatically digested, most commonly by pepsin, as first described by Fryetes *et al.* [19]. Following digestion, the ECM is brought to physiological pH and salt conditions, to match *in vivo* conditions as well as to inactivate pepsin. This digested ECM can then self-assemble into a nanofibrous hydrogel upon incubation at 37°C or injection *in vivo*.

When compared to using the powder form as a therapy, the hydrogel has disadvantages of increased manufacturing time and includes an additional foreign protein for digestion; however, ECM hydrogels also have several advantages, allowing for new clinical applications. ECM hydrogels have been used in different ways and combinations, including as coatings [20-24], with cells [22, 25-33], with growth factors [27, 34, 35], as hybrid/modified materials [26-28, 31, 36-48], and, more recently, as bioinks for tissue 3D printing [49-55]. While ECM hydrogels can also be used as *in vitro* cell culture platforms [56, 57], this review will focus on state-of-the-art decellularized ECM-based hydrogels with an emphasis on *in vivo* applications and functional outcomes in disease models (Figure 2). The subsections below cover how different ECM hydrogels, either from tissue specific or non-tissue-specific sources, have been used to treat each type of tissue.

2. Myocardium

The most common cause of heart disease and where ECM hydrogels have been applied most extensively is myocardial infarction (MI). Other cardiac pathologies that are in need of a tissue engineering solution include heart failure and congenital heart disease. As shown in Table 1, porcine-derived myocardial ECM hydrogels have progressed the furthest in terms of translation with a product currently in clinical trials for post-MI patients (clinicaltrials.gov Identifier: NCT02305602). This hydrogel was designed to prevent and/or treat heart failure post-MI by recreating a myocardial-specific extracellular microenvironment to facilitate endogenous cell infiltration and repair. This myocardial ECM hydrogel has the ability to go through a cardiac injection catheter as well as has no hemocompatibility issues, likely because of the digestion process [58], as opposed to ground ECM which contains a number of high molecular weight collagens that are known agonists for platelet activation [59, 60]. Multiple *in vivo* models, including rat and porcine MI models, displayed the feasibility,

safety, and efficacy of the injectable ECM hydrogel therapy, showing increases in cardiac muscle, neovascularization, and regional and global cardiac function [58, 61, 62].

To further investigate this mechanism of repair, Wassenaar *et al.* performed whole transcriptome analysis on RNA isolated from the infarct region of rat hearts injected with the myocardial ECM hydrogel as compared to saline [63]. Pathways associated with cardiac repair (e.g. neovascularization, cardiac transcription factors) were upregulated, and pathways associated with negative left ventricular remodeling (e.g. inflammation, apoptosis, fibrosis) were downregulated. Improvements in cardiac function, as demonstrated in animal models, could suggest a stimulation in cardiac muscle formation that is reminiscent of developmental stages. Increases in cardiomyocytes and cardiac muscle were in fact observed in both rat and porcine MI models [58, 62]. As an alternative to porcine myocardium, rat [38, 64, 65], goat [66], and human myocardium [67-70] have been tested *in vitro*. Additionally, porcine small intestine submucosa (SIS) [71, 72] and human placenta [73] has been explored *in vivo* as a non-tissue-specific source as well as human and porcine pericardium as a potentially autologous tissue sources [34, 35, 74-76]. SIS hydrogels demonstrated functional improvements in a mouse MI model alongside increased angiogenesis and decreased infarct size [71, 72], whereas the human placenta hydrogel did show functional improvements in a rat MI model [73]. When compared to porcine pericardium, human pericardium performed comparably with regards to neovascularization and c-Kit+ cellular infiltration when injected into non-ischemic rat myocardium [74]. However, both human pericardial and myocardial ECM showed significant variability in composition across patient sources [67, 76].

Several groups have explored modification of the myocardial ECM, including as a chitosan-ECM hybrid patch [43, 44, 48], an injectable silk-ECM hybrid material [45, 46], through cross-linking via genipin [48]. The chitosan-ECM hydrogel patch was tested in a rat heart full-thickness defect model, representative of a congenital heart disease, Tetralogy of Fallot [44]. The chitosan-ECM patch showed significant improvements in right ventricular ejection fraction over a commercially available bovine pericardium-derived patch [44]. A silk-ECM hybrid was developed to allow for greater control of the mechanical properties of an ECM hydrogel. This scaffold was implanted [46] and injected subcutaneously [45] in a rat model, allowing for cellular infiltration and vascularization of the biomaterial. Furthermore, cardiac ECM hydrogels have been tested for 3D printing applications [50, 52]. A 3D printed cardiac ECM hydrogel patch was combined with human cardiac progenitor cells and was printed with a pattern to promote vascularization of the construct [52]. Following implantation in a Balb/c nude mouse MI model, the patch showed significant improvements in cardiac function [52]. The pericardial ECM hydrogel has also been modified with the addition of growth factors including basic fibroblast growth factor (bFGF) [34] and an engineered hepatocyte growth factor (HGF) fragment [35]. When bFGF was delivered with the pericardial ECM hydrogel in a rat MI model, there was significantly enhanced neovascularization compared to delivery alone or in collagen, and the new vasculature anastomosed with the existing vasculature [34]. Delivery of HGF fragments in the pericardial hydrogel in a rat MI model improved fractional area change and increased arteriole density [35]. These studies show how ECM hydrogels can be modified to improve mechanical properties, cell infiltration, neovascularization, and functional outcomes.

3. Musculoskeletal tissues

3.1. Skeletal muscle

Skeletal muscle can be damaged acutely from blunt injuries, resulting in scar tissue formation and volumetric muscle loss. Alternatively, skeletal muscle can undergo ischemic damage and atrophy in cases of peripheral artery disease (PAD). An injectable porcine-derived skeletal muscle ECM hydrogel was developed to treat PAD and was initially tested in rat hindlimb ischemia models [32, 77, 78]. The skeletal muscle ECM significantly promoted endothelial cell infiltration, arteriogenesis, muscle cell proliferation, and muscle progenitor cell infiltration [77]. Following this initial study, the porcine skeletal ECM was compared alongside decellularized human umbilical cord ECM, which served as a young developmental non-tissue-specific experimental group [78]. As a functional outcome, perfusion of the ischemic limb was measured and normalized against the healthy limb. Both the umbilical cord and skeletal muscle ECM significantly increased perfusion versus the saline control. However, through muscle fiber morphology assessment, the skeletal muscle ECM hydrogel more closely matched native healthy skeletal muscle in terms of average muscle fiber area, circularity, and roundness, as compared to human umbilical cord ECM. This skeletal muscle ECM was further tested as a cell delivery platform. In healthy mice and a hindlimb ischemia model, the skeletal muscle ECM hydrogel significantly increased skeletal myoblast survival, particularly when co-delivered with fibroblasts to better mimic the muscle-specific microenvironment [32]. As similarly demonstrated in MI models, ECM hydrogels increase the formation of vessels and perfusion, illustrating a possible mechanisms of repair that can be observed across multiple tissue applications.

In addition to treating PAD, ECM hydrogels have also been examined for more traditional skeletal muscle tissue engineering using an abdominal wall defect model in which a portion of skeletal muscle is removed [37, 79, 80]. Hong *et al.* first developed a porcine dermal ECM hybrid with poly(ester urethane)urea [37]. The materials were interwoven through a concurrent electrospin/electrospray technique and subsequently incubated at 37°C for gelation. The scaffold was implanted into a rat abdominal wall defect model, harvested 4 weeks later, and assessed histologically and mechanically [37]. The resulting tissue had significantly increased stiffness, almost matching native tissue stiffness [37]. Furthermore, staining showed extensive macrophage (CD68 positive) and smooth muscle actin positive cell infiltration and increased muscle area as indicated by Masson's trichrome [37]. Wolf *et al.* compared a porcine-derived dermal ECM hydrogel with a urinary bladder matrix (UBM) hydrogel in a rat abdominal wall defect model [79]. Both the dermal and UBM hydrogels provided an environment for CD68 positive and myosin heavy chain positive cell infiltration, but UBM hydrogels degraded more quickly and led to greater amounts of myogenesis [79]. A porcine colon ECM hydrogel was also tested in a rat abdominal defect model, showing biocompatibility and pro-regenerative macrophage polarization ratios (M2:M1) [80]. The research of ECM hydrogels in an abdominal wall defect models demonstrates a wide range of non-tissue-specific sources as a contrast to the use of skeletal muscle ECM hydrogels in PAD models.

3.2. Tendon

Ligament and tendon injuries can result from overuse and accumulation of microtears, leading to eventual full-thickness tearing and loss of mechanical function. An injectable hydrogel could provide a scaffold for repairing both types of injuries, as it can fill a cavity and disperse throughout the tissue. Farnebo *et al.* developed an injectable hydrogel therapy derived from human cadaveric tendon that can be injected intramuscularly [81]. The tendon ECM hydrogel was capable of inducing human adipose-derived stem cell proliferation *in vitro* alongside macrophage and fibroblast invasion *in vivo* when subcutaneously implanted and intramuscularly injected in rats. This material was tested in a rat Achilles tendon full-thickness defect model, where the hydrogel caused a significant increase in ultimate failure load of *ex vivo* tendons over saline controls at 4 weeks post-injection [82]. Further studies explored supplementation of the material with rat-derived platelet-rich plasma and rat adipose-derived stem cells [25]. At 2, 4, and 8 weeks post-injection, tendons were harvested and mechanically tested *ex vivo*. The elastic modulus was significantly increased over saline controls at weeks 2 and 4 for all the hydrogel groups (hydrogel alone, hydrogel with platelet-rich plasma, hydrogel with adipose-derived stem cells, and hydrogel with both platelet-rich plasma and adipose-derived stem cells). At week 8, only the hydrogel with both plasma and stem cells had a significantly increased elastic modulus [25].

While there were promising initial *ex vivo* functional studies with human tendon sources, others looked to transition to more commercially sustainable sources, including porcine [83] and equine [41]. Crowe *et al.* studied the effects of lyophilization and reconstitution of the hydrogel, showing that reconstituted hydrogels provided greater rat adipose-derived stem cell viability and proliferation *in vitro* compared to non-lyophilized hydrogels stored at 4 °C [84]. Furthermore, reconstituted hydrogels maintained cell survival, as monitored via bioluminescence, when implanted subcutaneously in rats as compared to fresh non-lyophilized hydrogels [84]. The ability of hydrogels to be lyophilized and stored long-term in addition to coming from xenogeneic sources could greatly increase their commercialization potential.

3.3. Articular cartilage

As cartilage is known to have limited regenerative capacity, invasive microfracture surgery is the current clinical standard to facilitate cartilage regeneration through recruitment of stem cells. An injectable hydrogel could accompany or replace this procedure if delivered with the appropriate stem cells or growth factors. Kwon *et al.* first described a porcine articular cartilage ECM hydrogel drug delivery system that was initially injected subcutaneously in rats [85]. The hydrogel was monitored in real time through *in vivo* fluorescent imaging, and sustained release of fluorescein labeled bovine serum albumin, which served as model protein, was observed over 20 days. Various hybrid *in vitro* studies of devitalized cartilage and decellularized cartilage have been performed, aimed at optimizing the cellular response and mechanical properties of the gels [40, 86, 87]. Devitalized cartilage only kills the cells in the cartilage through freeze-thaw cycles and does not necessarily remove cellular debris, whereas decellularized cartilage includes additional chemical processing after devitalization to remove cellular content, specifically detergent rinses [86]. When mechanically tested as *ex vivo* gels, devitalized cartilage possessed a greater yield stress as compared to

decellularized cartilage [86]. Another study demonstrated that when cultured with rat bone marrow-derived stem cells, methacrylated porcine-derived decellularized cartilage hydrogels demonstrated a stress-strain profile similar to native porcine cartilage [40]. Moreover, sox-9, aggrecan, and collagen II expression increased, whereas collagen I expression decreased on methacrylated cartilage hydrogels as compared to methacrylated gelatin controls [40]. The combination of these studies suggests that decellularization and digestion may be too harsh for some tissues, resulting in gels that may be too mechanically weak for a load bearing tissue and necessitating material modification.

3.4. Bone

The nonunion of fractured bone can result from the lack of blood supply and appropriate growth factors to the fracture region. An ECM hydrogel can be injected in the fracture region to deliver exogenous growth factors or sequester them endogenously. Bone ECM is unique in that it requires demineralization through hydrochloric acid agitation, as described by Peterson *et al.* in a commercially available demineralized bone matrix [11]. Sawkins *et al.* expanded upon this with further processing of decellularization and digestion of the demineralized bone matrix, resulting in an injectable hydrogel derived from bovine tibiae [88]. This demineralized and decellularized bovine-derived bone matrix was mixed with alginate and explored as a delivery vehicle of growth factors in an *ex vivo* chick femur defect model for bone repair [26, 36]. When seeded with human adult Stro-1+ bone marrow stromal cells supplemented with bone morphogenetic protein-2 and then placed in the defect, this resulted in increased bone matrix formation and collagen deposition [26]. This hybrid delivery vehicle with human adult Stro-1+ bone marrow stromal cells was subcutaneously implanted into immunodeficient mice, and it correlated with bone formation through micro-CT assessment and Von Kossa staining, regardless of growth factor supplementation [27]. This study reiterates the versatility of ECM hydrogels as delivery platforms for stem cells and growth factors; however, the diversity of stem cells and growth factors requires much optimization and careful selection as they become increasingly complex therapies.

3.5. Meniscus

Similar to articular cartilage, meniscus injuries can result from acute tears or gradual degradation. An injectable hydrogel with the appropriate mechanical properties could replace the meniscus, or a hydrogel could be remodeled to match the native tissue if able to recruit endogenous cells or if delivered with the appropriate cells. Wu *et al.* described an injectable porcine meniscus ECM hydrogel, in which the hydrogel supported primary bovine chondrocyte and mouse fibroblast survival up to 7 days and infiltration at 14 days *in vitro* [89]. Additionally, this materials was subcutaneously injected in a female Balb/c mouse and showed rapid degradation but no severe immune response by 7 days post-injection [89]. A bovine meniscus ECM hydrogel was also explored for the delivery of human mesenchymal stem cells (MSCs) in an orthotopic rat model of meniscal injury [33]. Meniscal regeneration was observed macroscopically, with MSCs retained out to 8 weeks post-injection. This suggested that the MSCs may have played a role in reducing hydrogel degradation, which may have led to long-term mechanical improvements of the injured meniscus. Visser *et al.* described a protocol to functionalize equine cartilage, tendon, and meniscus ECM hydrogels

with photo-crosslinkable methacrylamide groups to react with methacrylated gelatin [41]. This study explored chondrocyte and mesenchymal stem cell gene expression, matrix formation, and hydrogel stiffness up to 6 weeks *in vitro* as compared to methacrylated gelatin controls [41]. They noted increased hydrogel stiffness following methacrylation but decreased survival of chondrocytes [41]. Specifically, chondrocytes displayed inflammatory pathway upregulation in tissue-derived gels [41]. Even though methacrylation improved mechanical properties and decreased degradation rates, the study suggests that methacrylation may reduce the cellular survival and differentiation effects of decellularized ECM, reducing the bioactivity of the ECM hydrogel, contrary to improvements seen in articular cartilage hydrogels. It is important to balance the bioactivity of an ECM hydrogel with any modification, as this can negate the utility of the material as compared to less bioactive alternatives.

4. Neural tissues

4.1. Brain

The brain can become injured following cortical impact or due to a lack of blood supply, resulting in traumatic brain injury or an ischemic stroke lesion, respectively. Whereas a stroke cavity can be targeted for injection(s) of a hydrogel therapy, traumatic brain injury may require a series of injections sites as the damage may be diffuse. DeQuach *et al.* first reported a method to process porcine brain into a hydrogel for culture of neurons [90], and others have described similar techniques for developing porcine brain hydrogels for *in vitro* studies [91, 92]. Bible *et al.* tested a hydrogel mixture of porcine UBM and porcine brain ECM, which was delivered with neural stem cells into a rat stroke model [28]. The cells were tracked through (19)F- and diffusion-MRI over 7 days, but they did not form fiber tracts or integrate with the cellular network [28]. Further studies examined the effects of UBM hydrogel concentration in the stroke cavity in a rat model and its effects on gelation and host cell infiltrate [93, 94]. Gelation only occurred above 3 mg/mL, whereas lower concentrations were seen permeating in the peri-infarct area [93]. Based on the increased infiltration of microglia, neural/oligodendtrocyte progenitors, and M2 phenotype macrophages, it was suggested that a UBM hydrogel concentration of 8 mg/mL would result in the highest endogenous repair response [94]. In another study, a human umbilical cord ECM hydrogel demonstrated significantly increased proliferation of human MSCs over UBM, spinal cord, and brain ECM hydrogels, whereas all hydrogels promoted differentiation of MSCs towards neural stem cells [95]. Similar to UBM, the umbilical cord hydrogel was injected into a stroke cavity in a rat model, demonstrating *in situ* gelation and macrophage infiltration.

Further studies tested injections of a UBM hydrogel following traumatic brain injury in rats [29, 96]. The UBM hydrogel alone and with mouse neural stem cells decreased neuron loss and white matter injury as compared to saline vehicle and sham controls [29]. However, only rats that received an injection of UBM hydrogels with neural stem cells reduced memory and cognitive impairments in the Morris water maze test as compared to the saline vehicle and UBM hydrogel alone [29]. In another study, UBM hydrogel injections alone decreased lesion volume and myelin disruption upon histological examination as compared to the

saline vehicle control [96]. This was accompanied by vestibular motor improvements in the beam balancing test but did not result in cognitive improvements in the Morris water maze test as compared to the vehicle control [96]. These studies suggest that a non-tissue-specific approach can decrease the damage resulting from an ischemic injury, but tissue-specific components (i.e. neural stem cells) may be required for true functional improvements.

4.2. Spinal cord

Spinal cord injury results in a loss of function and sensation below the injury, but hydrogel therapies are aimed at bridging the gap in the spinal cord. Methods have been published for preparing porcine spinal cord hydrogels and testing *in vitro*, demonstrating the proliferation, migration, and differentiation of neoplastic rats cells derived from neural crest tissue [91] and mouse neuroblastoma cell neurite formation [92]. The porcine spinal cord hydrogel and UBM hydrogel were tested in a rat spinal cord injury model [97]. Both materials provided a bridge across the lesion cavity, both displaying integration, neovascularization, and axonal growth, even though the hydrogels degraded rapidly [97]. Both hydrogels were also tested as a delivery platform for human MSCs derived from Wharton's jelly, but did not show any significant differences in blood vessel or neurofilament formation [97]. Modification of these hydrogels in the future may reduce degradation and improve the effects of exogenously delivered cells.

5. Other tissues and organs

5.1. Dermis

As dermal products are usually sheets for treating burn wounds and diabetic ulcers, ECM hydrogels can allow for pre-molding into irregular shapes, similar to injecting into an irregularly shaped cavity. Mechanical strength is an important design criterion for dermis, where hydrogels are typically lacking. Pilipchuk *et al.* aimed to improve the poor mechanical properties of dermal ECM hydrogels through glutaraldehyde crosslinking after gelation and subsequent subcutaneous implantation [42]. These hydrogels were formed via the methods described by Uriel *et al.* for adipose tissue and were pH induced [98]. Crosslinking increased mechanical stiffness and reduced *in vitro* pepsin degradation, and following rat subcutaneous implantation, crosslinked hydrogels better maintained their shape and volume as compared to non-crosslinked hydrogels [42]. Dermal ECM via the Uriel method was also tested in a rat full thickness wound healing model [99]. Dermal hydrogels were pre-molded and fixed within a silicone splint ring to prevent wound contraction, but due to fast degradation and weak mechanical properties, no significant wound healing was observed with the dermal ECM hydrogel. The dermis is unique in that an ECM hydrogel therapy can be easily applied, whereas other tissues require invasive procedures or injections. As these hydrogels were pre-molded, new and innovative delivery strategies may emerge, such as spraying or direct application to the wound.

5.2. Pancreas

In cases of type 1 diabetes, the pancreas does not produce enough insulin, resulting in a lack of blood glucose control. However, a pancreas-derived ECM hydrogel could provide a favorable environment for stem cell differentiation and support. In a study by Chaimov *et al.*,

porcine pancreatic ECM hydrogels were developed and tested in a diabetic mouse model as a proof of concept [31]. Induced pluripotent stem cells derived from human liver cells and human MSCs were differentiated into glucose-regulated insulin-producing cells. These cells were then encapsulated in a pancreatic ECM hydrogel and injected subcutaneously in a diabetic mouse model. The pancreatic ECM hydrogel encapsulated with differentiated cells demonstrated significantly improved glycemic control when mice were administered glucose as compared to injected cells or ECM hydrogels alone [31].

5.3. Vocal fold

The vocal fold can become scarred by trauma, resulting in a loss of function, specifically an inability to vibrate and produce sound. An injectable ECM hydrogel can provide a scaffold in the damaged region to prevent scar formation/progression. A porcine SIS hydrogel was studied as a delivery vehicle for cells and growth factors in a rabbit vocal fold injury model [100, 101]. When delivering rabbit bone marrow derived MSCs in an SIS hydrogel, harvested vocal folds had a reduced scar index and enhanced vibratory capacity through videokymographic analysis, when compared to the SIS hydrogel or MSCs alone at 8 weeks post-injection [100]. When delivering HGF in an SIS hydrogel, mucosal wave oscillations were significantly increased, and elastic and viscous moduli were significantly decreased as compared to saline and HGF alone at 3 months post-injection [101]. Both studies showed increased hyaluronic acid deposition and functional improvement over respective controls, as the SIS hydrogel provided greater targeting and retention of the respective therapy.

5.4. Adipose tissue

An adipose-derived hydrogel would most commonly be used for reconstructive surgery. An injectable filler material would be ideal, but many current clinical biomaterials do not match the native microenvironment, degrade too quickly, and/or do not actually generate adipose tissue. Many studies have investigated an injectable decellularized adipose tissue powder [16, 102], but few have developed a decellularized injectable hydrogel. Young *et al.* first developed and characterized an injectable decellularized adipose tissue hydrogel derived from human lipoaspirate, showing hydrogel formation following subcutaneous injection in athymic mice alongside *in vitro* growth and survival of patient-matched adipose-derived stem cells [103]. This material was tested with adult adipose-derived stem cells and a crosslinking agent, transglutaminase, in athymic mice [104]. At 4 weeks post-injection, the ECM hydrogels showed tissue integration, whereas the ECM hydrogels with stem cells and/or crosslinking agent showed new adipocyte formation greater than the ECM hydrogel alone. This was compared against the current clinical standard, Juvederm (Allergan), which showed no tissue integration or new adipocyte formation at 4 weeks post-injection. Poon *et al.* developed a human and porcine adipose ECM hydrogel demonstrating >90% efficiency of differentiating adipose-derived stem cells into adipocytes [30]. The porcine adipose ECM hydrogel seeded with rat adipose-derived stem cells and fibroblast growth factor displayed significant adipogenesis 8 weeks post-subcutaneous implantation in a rat [30]. Even though the hydrogels were injectable in this study, they were pre-molded and implanted within silicone rings for stability [30]. In another study, a human lipoaspirate ECM hydrogel was combined with silk fibroin to improve mechanical properties [47]. This hydrogel was

subcutaneously implanted with autologous pre-differentiated adipose stem cells and demonstrated increased vascularization over hydrogel alone controls [47].

As a contrast to typical pepsin digestion, Uriel *et al.* described a method for decellularization and digestion of rat adipose tissue using a dispase enzyme, homogenization, and urea extraction [23, 98]. In addition to thermally induced gelation, these hydrogels also formed when pH was reduced to ~4.0. Hydrogels created from both temperature and pH induction supported adipocyte differentiation and adipogenesis when pre-molded and subcutaneously implanted in a rat model. Furthermore, a rat dermis-derived ECM hydrogel was explored using the digestion method described by Uriel *et al.* [105]. When pre-molded and subcutaneously implanted in a rat model, the dermis-derived ECM hydrogel also encouraged similar levels of adipogenesis through both methods of induction when compared to the adipose-derived ECM hydrogel.

Following invasive surgery, adhesions can form between organs and surrounding connective tissue restricting organ function. One strategy to prevent this is to generate adipose tissue, which is non-adherent. In particular, following spinal cord surgery, epidural fibrosis can result in scar tissue adhering to the dura. An injectable hydrogel surrounding the spinal cord could limit this adhesion by guiding the surrounding tissue to form adipose tissue. Lin *et al.* developed a decellularized porcine adipose hydrogel, performing decellularization through freeze-thaw cycles as a detergent-free method [106]. This hydrogel was combined with hyaluronic acid, loaded with rat adipose stromal cells, and injected into a post-spinal cord injury (laminectomy) rat model of epidural fibrosis. After 6 weeks, the hydrogel displayed an ability to block fibrous tissue formation within the ECM hydrogel with a preference toward adipose tissue development, resulting in lower adhesion scores. This demonstrates how ECM hydrogels can guide the regeneration of tissue, specifically adipose tissue, possibly due to tissue-specific cues. This could lead to combination therapies in the future to guide regeneration of complex tissue systems, at the interface of different tissues.

5.5. Colon

Inflammatory bowel disease, specifically ulcerative colitis, can result in disruptions of the gastrointestinal tract. An ECM hydrogel delivered via enema can gel within these defects, creating a scaffold for a continuous gastrointestinal tract. A porcine SIS hydrogel was administered during a daily enema for 7 days to treat a rat ulcerative colitis model, resulting in a thin hydrogel coating of the colon [107]. Harvested colons had significantly lower inflammation and ulceration scores as compared to the vehicle control, which was neutral buffered pepsin. Furthermore, monolayers of intestinal epithelial cells from harvested colons were assessed through transepithelial electrical resistance measurements, where the SIS hydrogel group showed increased resistance, indicating an improvement of the epithelial barrier [107]. This demonstrates how some ECM hydrogels can be easily and repeatedly applied, specifically daily, to potentially improve the effects of the therapy. For injectable therapies, this could translate to injections every couple weeks, potentially addressing fast degradation issues observed in many applications.

5.6. Dental tissues

During an endodontic procedure, most commonly a root canal, necrotic dental pulp is removed, and the remaining cavity is filled with a bioinert material. Instead, a hydrogel could be placed in this cavity to assist in dental pulp/dentin tissue repair to result in a functional tissue. The culture of dental pulp cells and stem cells have been studied on porcine UBM [108, 109] and bovine bone ECM [110]. A hybrid UBM hydrogel containing bioactive glass with silver ions has also been developed [108, 109]. Silver was specifically chosen for its antibacterial properties, as there is a high risk of infection in the dental cavity [108, 109]. The material promoted proliferation of primary human dental pulp cells in culture and prevented bacterial growth *in vitro* [108, 109]. This material was implanted subcutaneously with human dental pulp stem cells in immunodeficient mice showing dentin-like morphology and an odontoblast-like layer reminiscent of dental tissue [109]. Another study investigated the odontogenic differentiation of dental pulp stem cells in bovine bone ECM, showing significantly upregulated markers for bone and increased mineral deposition as compared to collagen I gel controls [110]. As dental applications work at the interface of different tissues, different sources of ECM hydrogels may be necessary to regenerate respective tissues. ECM hydrogels from varying sources are showing promise with regards to bone and dental pulp.

5.7. Liver

In cases of liver disease, the liver can undergo chronic cirrhosis, which may progress to liver failure if untreated, as the liver has become too severely damaged to regenerate. However, transplantation of functional hepatocytes in an ECM hydrogel platform could assist in liver regeneration. Sellaro *et al.* described the first porcine liver ECM hydrogel, which demonstrated preservation of primary human hepatocyte function, specifically albumin secretion, hepatic transport activity, and ammonia metabolism [111]. Studies by Skardal *et al.* mixed liver ECM, both fresh and decellularized, with collagen type I, hyaluronic acid, or heparin-conjugated hyaluronic acid hydrogels to study the survival and function of primary human hepatocytes, showing both synthesized and secreted steady levels of albumin and urea alongside sustained drug metabolism of ethoxycoumarin [112]. Takeda *et al.* studied tunable sheets and 3D porous structures derived from liver ECM hydrogels, showing trends of optimal HepG2 (human liver carcinoma) cell proliferation at 1% ECM w/v [113]. Others have studied how liver ECM affects hepatocyte function [114], morphology [114] and phenotype [115] *in vitro,* with only one *in vivo* study to date [22]. Lee *et al.* investigated a decellularized rat liver ECM hydrogel as a delivery platform for primary hepatocytes in athymic mice, and upregulated albumin and hepatocyte nuclear factor 4-alpha expression at 1 week following subcutaneous injection [22]. As the liver already possesses regenerative capacity, ECM hydrogels could rescue and enhance this property.

5.8. Lung

A number of chronic respiratory diseases are difficult to treat with a regenerative medicine approach since delivery to the lungs can be challenging, as typical aerosolized liquids rapidly clear from the lungs as well as undergo shear forces that are too harsh for stem cell delivery. Hydrogels can typically be injected or pre-molded, but the digested liquid pre-gel

ECM can also be inhaled to reach and form a gel in the lungs. A porcine lung ECM hydrogel was developed as a delivery system for human bone marrow derived MSCs in the lungs [116]. The ECM hydrogel was delivered in a rat emphysema model as an orotracheal solution instillation of pre-gel lung ECM mixed with rat MCSs. Lungs were harvested 24 hours later and histologically assessed for the presence of MSCs. The ECM demonstrated MSC viability and attachment *in vitro*, as well as increased cell retention *in vivo*. In another study, a lung ECM hydrogel was tested in a rat hyperoxia model and was delivered via instillation or nebulization [117]. Both methods of inhalation reduced cell apoptosis and oxidative damage, which was assessed histologically. This unique delivery method of inhalation is promising as a noninvasive therapy, as it can not only increase the retention of stem cells, but may also increase retention and distribution of other therapeutics, including antibiotics or bronchodilators.

6. Translational considerations

6.1. Tissue source

Tissue source species can impact the properties of an injectable ECM hydrogel, specifically its mechanical properties as well as its scalability. Source species can be xenogeneic, typically from porcine or bovine sources, allogeneic from cadaveric donor tissue sources, or autologous in rare cases. A few studies directly compared porcine and human sources [68, 74, 83]. It was shown that human myocardial sources had decreased mechanical stiffness compared to porcine [68]. However, for tendon, human-derived hydrogels were mechanically stronger than porcine sources [83]. There are possibilities for autologous tissue sourcing, as demonstrated with the pericardium [74] and omentum [118]-based tissue sources, but these sources require invasive surgeries and delayed processing time with an inability to be off-the-shelf. Additionally, another issue is the patient-to-patient variability of human sources, as this has been demonstrated in sulfated glycosaminoglycan and collagen content in human myocardium [67] and pericardium [74], as well as difficulties in processing some samples of human myocardial ECM into the hydrogel form [68]. Xenogeneic sources are readily available, as compared to human autologous or cadaveric tissue, and can allow for increased scale of production and batching of tissue, which can ameliorate the variability demonstrated on the individual scale.

6.2. Tissue age

Tissue source age also plays an important factor as tissue ECM typically becomes more cross-linked and stiffens with age alongside increasing adipose tissue deposition and fibrosis [119, 120]. In the comparison of human cadaveric hearts versus porcine hearts, tissue source age may have played a role, as porcine sources were from young adult pigs, while cadaveric sources were from much older human donors [68]. This variation in age was directly studied by Sood *et al*. in applications of neuron cell culture, where porcine fetal brain ECM outperformed porcine adult brain ECM in regards to neuronal network formation, calcium signaling, and spontaneous spiking activity with primary rat neuron cells [121]. In another study, neonatal rat cardiomyocytes cultured on fetal cardiac ECM showed greater proliferation, as compared to neonatal and adult rat cardiac ECM [64]. These observations are reinforced with studies on decellularized ECM that is produced from cell cultures *in*

vitro. Cell-secreted matrices from young and old mouse MSCs showed compositional differences in the secreted ECM [122]. Young MSC-derived ECM was able to rescue the phenotype of old MSCs, improving replication and osteogenic capacity. Additionally, it was shown that cell-secreted ECM from human fetal bone marrow MSCs improved proliferation and maintained differentiation potential of late-passage human adult MSCs, as compared to cell-secreted ECM matrices from adult human MSCs [123]. These studies suggest that younger tissue may be a better source for tissue engineering applications, given that younger tissue sources, with the exception of fetal tissues, are readily available.

6.3. Tissue specificity

Each ECM is different in terms of structure and composition, providing unique microenvironments and biochemical cues. ECM can retain a number of tissue-specific components following decellularization, including proteins [124], growth factors [125] and nanovesicles [126], suggesting that some ECM hydrogels may provide an increased therapeutic effect by matching components of the target tissue's native ECM. There are only a handful of studies that have directly compared tissue-specific to non-tissue-specific ECM hydrogels. For example, French *et al.* showed that coatings of cardiac ECM significantly increased expression of early cardiomyocyte markers in cardiac progenitor cells compared to both collagen and adipose ECM [127]. Viswanath *et al*. also compared hydrogels derived from spinal cord, bone, and dental ECM showing a significant increase in human apical papilla derived MSCs differentiating towards the neural lineage on spinal cord ECM [128]. These studies show the potential of tissue-specific ECM hydrogels to guide stem cell differentiation towards the appropriate lineage, which would be important for either exogenous stem cell delivery or endogenously recruited stem cells *in vivo*. Even fewer studies have directly examined tissue specificity *in vivo.* In particular, a porcine skeletal muscle ECM hydrogel was compared against a human umbilical cord ECM hydrogel in rat hindlimb ischemia model [78]. Both materials improved hindlimb perfusion over the saline control; however, muscle treated with the skeletal muscle ECM hydrogel more closely matched native healthy skeletal muscle histologically, as compared to human umbilical cord ECM, suggesting the importance of tissue-specific ECM cues.

While a majority of ECM hydrogel applications contain at least one tissue-specific approach in a functional model (e.g. cardiac [58, 62, 63], skeletal muscle [32, 78], and tendon [25, 82]), there are a number of non-tissue-specific *in vivo* applications, most notably with porcine UBM and SIS hydrogels, likely because sheets of these ECM are already commercial products. A UBM hydrogel in a traumatic brain injury rat model showed decreased neuron loss and white matter injury [29], as well as decreased lesion volumes, myelin disruptions, and vestibular motor improvements over respective controls [96]. SIS hydrogels demonstrated functional improvements in a mouse MI model [71], as well rabbit vocal fold injury models [100, 101]. A direct comparison of an esophageal hydrogel versus a UBM hydrogel was studied in a rat mucosal resection model [129]. Differences were observed *in vitro*, as the esophageal hydrogel promoted greater migration and organoid proliferation; however, both treatment groups fully recovered [129].

These improvements seen across non-tissue-specific ECM treatments may occur because ECM contains components that are shared across tissues, in particular, collagens. Non-specific ECM may support mechanisms necessary for regeneration (i.e. neovascularization), which complements the enhanced perfusion observed in the hindlimb ischemia model, but does not support tissue-specific regeneration (i.e. muscle stem cell recruitment and fiber development) [78]. Standard ECM components may be sufficient in certain applications, but, as illustrated in the hindlimb ischemia study evaluating a skeletal muscle ECM hydrogel, tissue specificity may promote enhanced regeneration. Future studies are needed to fully address this question, particularly those that evaluate ECM hydrogels from the same species and use the same decellularization methods. These variables can both affect residual ECM components, which will affect *in vivo* outcomes.

Beyond tissue specificity, regional specificity may also be important. For example, O'Neill *et al.* compared ECM from regions of the kidney, specifically the cortex, medulla, and papilla, on kidney stem cell growth and metabolism in various physical forms, including sheets, hydrogels, and solubilized ECM [130]. Papilla ECM in solubilized and hydrogel forms showed notable differences on kidney stem cells, including lower proliferation and higher metabolic activity, relative to ECM derived from the cortex, medulla, and whole kidney. These results were echoed in a study comparing ECM from the inner and outer meniscus [131]. Collagen and aggrecan expression of human bone marrow MSCs varied between the inner and our meniscus ECM hydrogels [131]. Tissue is typically processed to ensure a homogeneous material through removal of vessels, nerves, and adipose tissue, but these studies highlight that regions of a tissue may play a role in tissue-specific approaches.

6.4 Tissue processing

In addition to the challenges associated with tissue source, tissue processing offers a unique set of challenges for ECM hydrogels. In particular, there is a high degree of variability in the decellularization techniques that are used between groups, including sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid, and Triton X-100 [132]. These techniques are not rigorously optimized and/or criteria for optimization are not clear with the exception of DNA content, as lower concentrations are generally desirable for yielding a sufficiently decellularized materials. Many techniques are tissue-specific, and no decellularization technique is considered standard for each tissue. Cell dense tissues (e.g. myocardium [61]) require harsh detergents such as SDS, whereas more fragile tissues (e.g. lung [116], pancreas [31]) use mild detergents such as Triton. Challenges remain with regard to finding techniques that are stringent enough to rigorously remove the cellular components yet mild enough to produce ECM of sufficient quality. Solubilization is less varied, with the majority of solubilization techniques being performed by partial enzymatic digestion with pepsin [19]. There are a number techniques to characterize and assess the quality of ECM hydrogels, including histological analysis, polyacrylamide gel electrophoresis, a 1,9-dimethylmethylene blue dye (DMMB) assay, mass spectroscopy, electron microscopy, mechanical testing, and cell culture [133]; however, each group often only performs a few of these techniques. One of the more promising newer modalities is to use ECM targeted mass spectrometry, which can quantify ECM components as well as residual cellular proteins [67, 134]. A recent review by Saldin *et al.* discusses in more detail the decellularization and

solubilization protocols for various ECM hydrogels in addition to hydrogel characterization methods [135].

6.5. Sterilization

Though not explicitly stated in most studies, aseptic technique is the most widely used method for ensuring sterility of ECM hydrogels. Decellularization steps typically include harsh detergents, acids, and antibiotics that limit bacterial exposure and growth following the tissue harvest. Furthermore, the digestion step, most commonly including pepsin in low pH, mediates proteolysis that can kill most bacteria [136]. Therefore, if aseptic technique is employed during digestion and all subsequent processing, the ECM can be effectively considered as sterile. Briefly discussed in a review by Badylak [137], terminal sterilization is an option for ECM scaffolds, but tends to have detrimental effects on mechanical properties of the scaffolds. Ethylene oxide, gamma irradiation, and electron beam irradiation were shown to significantly decrease the uniaxial and biaxial mechanical stiffness of UBM sheets [138]. With regards to dermal hydrogels, electron beam and gamma irradiation increased the *in vivo* degradation of the hydrogels in a dose-dependent manner, whereas ethylene oxide did not significantly alter the mechanical properties of the scaffold [139]. Another sterilization protocol, supercritical carbon dioxide, has been tested on UBM hydrogels, specifically sterilizing the lyophilized pre-gel. This technique still permitted hydrogel formation following sterilization, which was not possible following a high dose of gamma irradiation [140]. This suggests it may be a possible method for terminal sterilization of ECM hydrogels, but further studies are needed to ensure that bioactivity is not affected *in vivo* [140].

7. Future outlook on ECM hydrogels

Decellularized ECM hydrogels have demonstrated their potential as scaffolds to induce mechanisms of repair across multiple target tissues; however, these mechanisms are not completely understood. Many studies show that ECM hydrogels promote cell infiltration, particularly progenitor cells [62] and macrophages [94, 141], neovascularization [142], and positive functional remodeling (Table 1). Future studies should further elucidate these mechanisms, providing further insight to the selection, processing, and modification of an appropriate ECM hydrogel for an intended regenerative application. There is much room for optimization of ECM hydrogel therapies, as the breadth of ECM hydrogel research provides many options for tissue source, processing, and application.

Current research suggests there will be an expansion of this platform into new and more complex tissues, as seen with pancreas [31] and neural applications [28, 96], which are still in early stages. With the advent of new technologies, including 3D printing [53, 54] and modification protocols [143], ECM hydrogels are becoming increasingly complex, progressing towards the complexity of the target tissues. Other ECM hydrogels are laying the foundation for *ex vivo* characterization and *in vitro* testing, allowing for quicker application in functional models. The platforms that are closest to clinical application tend to be acellular, but there are opportunities to develop combination therapies, including delivery of stem cells [27] and growth factors [27, 34, 35] which may improve the therapeutic effects

of an ECM hydrogels, or in some cases, such as the pancreas, may be required for a regenerative therapy. These additions, however, add complexity in terms of translation and increase cost, and therefore, investigators should closely evaluate the cost-benefit ratio of such combined therapies.

Many ECM sheet products (AlloDerm, Lifecell Corp.) and injectable ground ECM products (ACell's Cytal™ Wound Matrix, MicroMatrix®, CorMatrix®) have reached commercialization, and injectable ECM hydrogels may soon follow this trend, with one product reaching clinical trials. In parallel, work has shown that digested ECM and hydrogels can be lyophilized and stored for long-term use [83, 84], supporting future translation and commercialization efforts. Trends have emerged regarding tissue sources, as the species [68], age [64, 121], and specificity [130] can not only impact the performance of an ECM hydrogel, but can have strong implications concerning scalability, a current challenge in the tissue engineering industry. As ECM hydrogels are approaching the clinic, standards and good manufacturing practices will be developed for the field, providing more uniform characterization methods and validation to guide future therapies and allow for improved comparisons between studies.

Due to the breadth of research across various tissue sources and targets, ECM hydrogels offer a wide array of approaches to complex disease and injury models, particularly as minimally invasive injectable therapies. The foundation for translational efforts are in development, as ECM hydrogels offer new opportunities and demonstrate potential in regenerative medicine applications.

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Statement of Significance

Extracellular matrix (ECM) hydrogel therapies are being developed to treat diseased or damaged tissues and organs throughout the body. Many ECM hydrogels are progressing from *in vitro* models to *in vivo* biocompatibility studies and functional models. There is significant potential for clinical translation of these therapies since one ECM hydrogel therapy is already in a Phase 1 clinical trial.

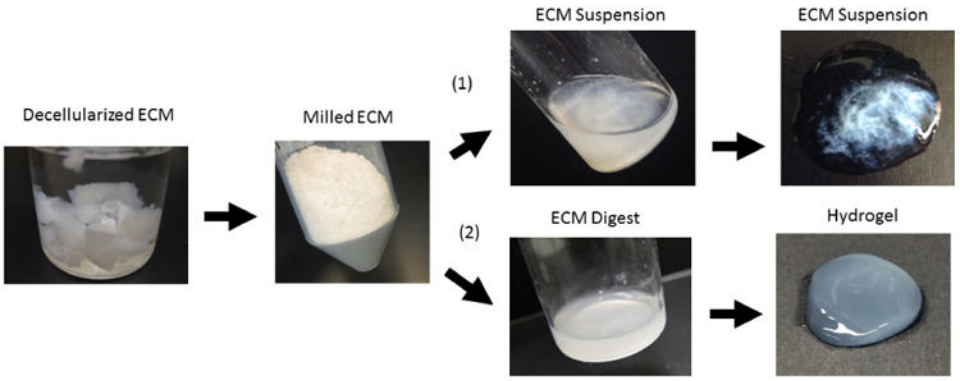

Figure 1.

Images of the workflow for developing injectable decellularized hydrogels. After continuous agitation in detergent solution, decellularized tissue is lyophilized and milled to yield only the extracellular matrix (ECM). Milled ECM can either be (1) suspended in solution and remain a non-free-standing solution, or (2) undergo enzymatic digestion to form an inducible non-flowing hydrogel. Images were reprinted from [62] with permission from Elsevier.

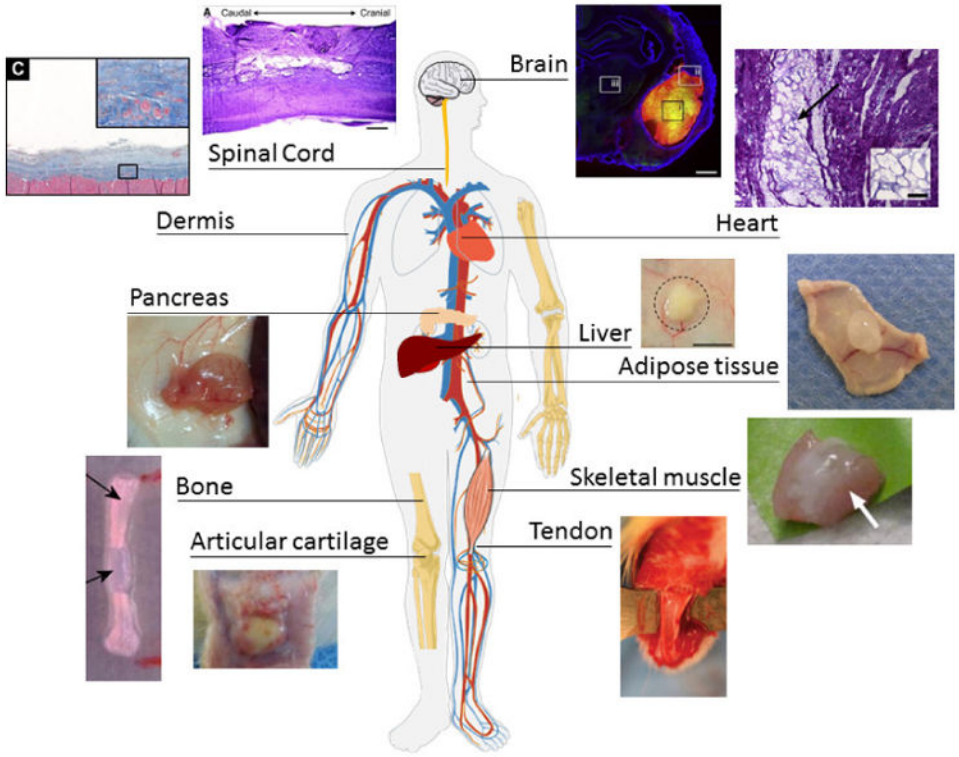

Figure 2.

ECM hydrogels injected for therapeutic applications throughout the body. Images were reprinted with permissions from Elsevier [26, 31, 61, 79, 85, 89, 93, 103], Wolters Kluwer Health, Inc. [82], Mary Ann Liebert, Inc. [97], and the American Chemical Society [22].

Table 1

Progress towards clinical translation. Red indicates an ECM hydrogel that is least translated (in vitro studies only). Orange represents an ECM hydrogel that has been implanted/injected in vivo, but only for biocompatibility/histological assessment. Yellow indicates an ECM hydrogel that has been tested in a functional animal model (described in the last column). Green indicates that an ECM hydrogel has reached clinical trials.

Category	Tissue target	Tissue source	*In vitro* studies	Biocompatibility / histological assessment	Functional studies	Clinical trials	Functional Outcomes
Myocardium	Myocardium	Porcine myocardium	[20, 39, 49, 127, 144-150]	[43, 45, 46, 61, 141, 143]	[44, 48, 52, 58, 62, 63]	Ventrix, Inc. ClinicalTrials.gov Identifier: NCT02305602	Ejection fraction, end diastolic volume, end systolic volume in mouse [48, 52], rat [62, 63], and pig [58, 62] myocardial infarction models and rat full-thickness defect model [44]
		Human myocardium	[67-70]	[141]			
		Rat myocardium					
		Goat myocardium					
		Porcine pericardium		[34, 74]	[35]		Ejection fraction, fractional area change in rat myocardial infarction model [35]
		Human pericardium	[76]	[74]			
		Porcine small intestine submucosa	[151]		[71, 72]		Ejection fraction, end diastolic volume, end systolic volume, contractility in mouse myocardial infarction model [71, 72]
		Human placenta			[73]		Ejection fraction, end diastolic volume, end systolic volume, contractility in rat myocardial infarction model [73]
		Porcine omentum					
		Ovine pericardium					
Musculo-skeletal tissues	Skeletal Muscle	Porcine skeletal muscle	[20, 153, 154]	[77]	[32, 78]		Hindlimb perfusion in rat hindlimb ischemia model [32, 78];

Category	Tissue target	Tissue source	*In vitro* studies	Biocompatibility / histological assessment	Functional studies	Clinical trials	Functional Outcomes
		Human umbilical cord			[78]		Hindlimb perfusion in rat hindlimb ischemia model [78]
		Porcine colon		[80]			
		Porcine dermis		[79]	[37]		Mechanical testing (*ex vivo*) in rat abdominal defect model [37]
		Porcine urinary bladder		[79, 155]			
	Tendon	Porcine tendon					
		Human tendon	[83, 156, 157]	[81, 84]	[25, 82]		Mechanical testing (*ex vivo*) in rat Achilles tendon injury model [25, 82]
		Equine tendon					
	Articular cartilage	Porcine articular cartilage	[40, 49, 87, 158]	[85]			
		Equine articular cartilage					
		Bovine patella tendon					
		Bovine hyaline cartilage					
	Bone	Bovine bone	[26, 36, 88, 161]	[27]			
	Meniscus	Porcine meniscus		[89]			
		Bovine meniscus	[131]	[33]			
		Equine meniscus					
	Nucleus pulposus	Porcine nucleus pulposus	[162]	[163]			
Neural tissues	Brain	Porcine brain	[91, 92, 95, 121, 164, 165]	[28, 90]			
		Porcine urinary bladder	[95, 164, 165]	[28, 93, 94]	[29, 96]		Vestibular motor/ sensorimotor function via foot fault test [29] or beam-walking task [96]; Cognitive function via Morris water maze [29, 96] in rat controlled cortical impact model
		Human umbilical cord		[95]			
		Porcine spinal cord					
	Spinal cord	Porcine spinal cord					

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Category	Tissue target	Tissue source	*In vitro* studies	Biocompatibility / histological assessment	Functional studies	Clinical trials	Functional Outcomes
		Bovine bone					
		Human wisdom teeth					
		Porcine urinary bladder		[97]			
	Optic nerve & Retina	Porcine optic nerve					
		Bovine retina					
Other	Dermis	Rat dermis		[42, 99]			
	Pancreas	Porcine pancreas			[31]		Glucose regulation in diabetic mouse model [31]
	Vocal fold	Porcine small intestine submucosa			[100, 101]		Video-kymographic analysis in rabbit vocal cord injury model [100, 101]
	Adipose tissue	Human adipose	[49, 167, 168]	[47, 103, 104]			
		Porcine adipose		[30]	[106]		Adhesion score in rat laminectomy model [106]
		Rat adipose	[23]	[98]			
		Rat dermis		[105]			
	Colon	Porcine small intestine submucosa		[107]			
	Dental tissue	Porcine urinary bladder	[108]	[109]			
		Bovine bone					
	Esophagus	Porcine esophagus		[129]			
		Porcine urinary bladder		[129]			
		Porcine small intestine submucosa					
	Liver	Porcine liver					
		Human liver					
		Rat liver	[114, 115]	[22]			
		Canine liver					
	Lung	Porcine lung	[150, 170]	[116, 117]			
	Cornea	Porcine cornea					
	Kidney	Porcine kidney					

Category	Tissue target	Tissue source	*In vitro* studies	Biocompatibility / histological assessment	Functional studies	Clinical trials	Functional Outcomes
		Human kidney					