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Cell-derived matrices for tissue engineering and regenerative medicine applications[1]

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Abstract

The development and application of decellularized extracellular matrices (ECM) has grown rapidly in the fields of cell biology, tissue engineering and regenerative medicine in recent years. Similar to decellularized tissues and whole organs, cell-derived matrices (CDMs) represent bioactive, biocompatible materials consisting of a complex assembly of fibrillar proteins, matrix macromolecules and associated growth factors that often recapitulate, at least to some extent, the composition and organization of native ECM microenvironments. The unique ability to engineer CDMs *de novo* based on cell source and culture methods makes them an attractive alternative to conventional allogeneic and xenogeneic tissue-derived matrices that are currently harvested from cadaveric sources, suffer from inherent heterogeneity, and have limited ability for customization. Although CDMs have been investigated for a number of biomedical applications, including adhesive cell culture substrates, synthetic scaffold coatings, and tissue engineered products, such as heart valves and vascular grafts, the state of the field is still at a relatively nascent stage of development. In this review, we provide an overview of the various applications of CDM and discuss successes to date, current limitations and future directions.

Introduction

The extracellular matrix (ECM) of tissues is a complex, highly organized assembly of macromolecules and incorporated signalling factors. In addition to providing tissue structure and a substrate for cell adhesion, the ECM also contributes cues for cell migration, proliferation, differentiation and survival, which are essential processes in development, homeostasis and tissue repair. The primary constituents of the ECM include fibrillar proteins (e.g. collagens, fibronectin, laminin), glycosaminoglycans (GAGs; e.g. heparan sulphate, chondroitin sulphate, hyaluronan), proteoglycans (e.g. decorin, versican, aggrecan) and matricellular proteins (e.g. osteopontin, thrombospondin). As well as contributing directly to the overall structural and mechanical properties of tissue, ECM components are also involved in the binding, sequestration and stabilization of signalling molecules incorporated within the matrix. The composition and distribution of specific matrix components varies with the type of tissue, and can also be altered by the tissue's developmental stage and/or pathological state.

Biomaterials used in tissue engineering and regenerative medicine attempt to recapitulate the multifactorial aspects of ECM function, however synthetic materials and matrices formed from isolated biological materials, such as collagen, fibrin or hyaluronan, fail to achieve the molecular complexity and organization of native tissue matrices. This has motivated the use of native ECM itself as a biomaterial source. ECM derived from allogenic or xenogenic tissues, reviewed in-depth elsewhere [1-3], has emerged as an increasingly common source of natural biomaterials. Decellularized or devitalized skin, small intestinal submucosa, urinary bladder matrix, amniotic membrane, ligaments, blood vessels and heart valves are but a few examples of tissue ECM used in pre-clinical research and clinical therapies. The use of whole organ decellularization is also of interest for tissue engineering applications due to the preserved tissue architecture[4-7]. However, naturally derived matrices are inherently limited in their capacity for manipulation beyond processing procedures and tissue source, and possess uncontrolled variability that may arise from the age, health and gender of individual sources.

ECM derived from cultured mammalian cells provides an alternative to native tissue-derived ECM. Cell-derived matrices (CDM) contain a complex yet organized mixture of macromolecules that can mimic aspects of native tissue microenvironments. In contrast to tissue-derived matrices, CDM have greater ability for customization by selecting the type(s) of cells used to generate the ECM, the culture system (e.g. 2D versus 3D culture; static versus perfusion), the application of external stimuli to modulate ECM production and also the ability to genetically modify the source cells to augment or silence the expression of target molecules. CDM can also be used to confer bioactivity to synthetic scaffolds through the deposition of matrix molecules on the scaffold surface. The trade-off with respect to tissue-derived matrices is that CDM typically have poorer mechanical properties, making them unsuitable for certain applications. However, mechanical preconditioning applied prior to decellularization has been shown to significantly improve the mechanical properties of CDM, particularly for cardiovascular applications[8, 9].

The purpose of this review is to explore the current uses of cell-derived matrices in tissue engineering, stem cell therapies and other biomedical applications, and discuss their current successes, limitations and future directions. CDM have been used in a variety of applications, including as coatings for synthetic scaffolds, biomimetic microenvironments for stem cell differentiation, and decellularized tissue engineered heart valves and vascular grafts. While several results are promising, there remains a fair amount of variability in the reported effects CDM have *in vitro* and *in vivo*, likely due to many differences in the cells, methods and applications examined thus far. Throughout this review we will attempt to identify key factors, such as cell source(s), processing techniques and experimental designs, that may help to explain these inconsistencies and provide insights into the best practices for the derivation and application of CDM in biomedical engineering.

Overview of Cell-derived Matrices Fabrication

Generating cell-derived matrices involves three major considerations: cell source, culture method and processing method. The cell source is the primary determinant of the resulting CDM composition. Cells sourced from different tissues typically yield matrices that mimic the relative composition of the natural tissue matrix. Fibroblasts, a predominant cell type found in connective tissue, are well known for their ability to produce a collage-rich extracellular matrix, a characteristic that has been exploited in a number of different biomedical applications including vascular and dermal tissue engineering. Mesenchymal stem cells (MSCs) are also a common source of CDM due to their ability to deposit ECM that mimics various tissues (e.g. bone, cartilage, adipose) depending on culture conditions, and their prevalent use in tissue engineering applications. Recently, Lu et al. compared the composition of matrices derived from fibroblasts, chondrocytes and MSCs[10]. All matrices stained positive for type I collagen, type III collagen, fibronectin, vitronectin, laminin and decorin. However, versican was only detected in chondrocyte CDM, and aggrecan was detected in both the MSC and chondrocyte matrix, but not the fibroblast CDM[10]. Although this particular study did not quantify the relative amounts of individual matrix components, it does demonstrate the effect of cell source on matrix composition, and the relative complexity of CDM.

Both primary cells and cell lines have been used to generate CDM. Primary cells harvested directly from tissues without passaging are typically considered to be an ideal cell source for tissue engineering and biomedical applications, as they closely resemble their native *in vivo* phenotype and are arguably more capable of generating a matrix that resembles the native microenvironments. An important caveat is that the native matrix can be generated and remodelled by multiple cell types, whereas primary cells are often sorted or isolated into a (quasi)-monoculture. Obtaining sufficient numbers of primary cells for a given application is often impossible or impractical; therefore primary cells are commonly expanded *in vitro* as finite cell lines. However, passaging of cells selects for the most rapidly dividing subpopulations and the *in vitro* environment can alter cell behaviour, resulting in a phenotypic drift from the native cell state. This is commonly

observed for chondrocytes that undergo dedifferentiation and senesce [11], which has been shown to affect the quality of the deposited ECM[12]. Alternatively, continuous (i.e. immortalized) cells lines can be used to generate CDM because they are easily cultured and can yield large numbers of homogeneous cells. However, continuous cell lines are often tumor-derived and can differ significantly from primary cell phenotypes. If mimicking a particular tissue microenvironment is important, a continuous cell line may not be an appropriate source. In contrast, if a particular molecular make-up is not critical, or a cell line produces a matrix with the desired molecular components (i.e. a tumor cell line-derived matrix that promotes angiogenesis), a continuous cell line may prove to be a simple solution. Genetic engineering of cell lines may also enable artificial depletion or enhancement of certain key molecular elements within a CDM

A second important consideration that affects CDM production is the manner in which the cells are cultured. The most common methods for generating CDM are to culture cells for an extended period of time as adherent monolayers (2D) or on scaffold surfaces (coatings), as multicellular aggregates (3D), or within a degradable carrier until sufficient ECM has been deposited (Figure 1). Additional culture considerations may also include mechanical pre-conditioning to improve the mechanical properties of the resulting material[8, 9], or altering the environmental conditions (e.g. hypoxic conditioning[13, 14]) to better model *in vivo* microenvironments (0.5 – 14% O_2, depending on tissue vascularization[15], compared to 21% O_2 in classic culture conditions).

Once a sufficient ECM has been deposited, the cellular component can be disrupted and removed from the ECM through a combination of chemical, physical and/or enzymatic processing methods (Refer to Table 1 for examples of typical decellularization and devitalization protocols). The primary goal of decellularization is the removal of allogenic or xenogenic cellular antigens and other immunogenic components, such as DNA, in order to minimize the risk of adverse immunological responses[1]. This objective must be balanced against preserving the molecular composition, bioactivity and structural integrity of the matrix itself, thus careful consideration and subsequent evaluation must be taken when selecting/developing a decellularization method. Decellularization

techniques and the use of decellularized tissues and organs have been reviewed in depth elsewhere[1, 16-18], therefore it will only be discussed briefly here. Chemical decellularization typically uses alkaline or acidic reagents (ammonium hydroxide, peracetic acid) and/or detergents (Triton X-100, SDS, CHAPS) to solubilize and disrupt cellular components and membranes. Chemical reagents are very efficient in removing cellular components, including DNA, however, chemical processing can also result in the loss of GAGs and disrupt ECM structure. Alternatively, physical methods such as lyophilisation and freeze-thaw cycling devitalize the cells but do not necessarily remove all cellular components from the matrix. Physical methods may enable retention of more matrix components, but can still result in alteration of ECM structure. Often, both chemical and physical methods are combined with DNase treatment to degrade any remnant DNA[16], which can induce sterile inflammatory responses in humans[19]. However, there is some clinical evidence suggesting that in certain cases, CDM generated by allogenic cells do not elicit immune or inflammatory responses when prepared by simple dehydration without DNase treatment[20]. Within the context of this review, the term cell-derived matrices (CDM) is used to describe any non-living cell-generated matrix, regardless of the specific processing method.

Cell-derived matrices in skeletal tissue engineering

Orthopaedic tissue engineering and regenerative strategies aim to improve upon current clinical practices for repairing large bone defects and damaged cartilage. Cell-derived matrices are being investigated (i) as a scaffold coating to improve the biological activity and tissue integration of synthetic scaffolds for regenerative therapies and (ii) as alternative cell culture substrates for maintaining, expanding and differentiating stem cells, and primary chondrocytes and osteoblasts for future use in orthopaedic cell therapies.

The effect of CDM on MSC osteogenic differentiation has been extensively studied in vitro[21-27]. MSCs cultured on titanium (Ti) fibre meshes and poly(e-caprolactone) (PCL) microfibre scaffolds coated with previously deposited osMSC-synthesized matrix

exhibited enhanced osteogenic differentiation, characterized by increased alkaline phosphatase (ALP) activity and calcium deposition, relative to MSCs cultured on unmodified Ti and PCL scaffolds [21-24]. Furthermore, MSCs cultured on Ti/ECM scaffolds upregulated expression of a subset of osteogenic genes (alkaline phosphatase, osteopontin, osteocalcin, osteonectin, osteomodulin and parathyroid hormone receptor) and decreased expression of aggrecan, a protein associated with chondrogenesis[27]. The MSC-derived matrices also supported increased mineralization, even in the absence of dexamethasone, a common osteogenic additive. However, increased calcium deposition appeared to be primarily due to mineralization of the pre-existing matrix as the acellular construct exhibited only slightly less calcium deposition than PCL/ECM construct with MSC[24]. While the ECM/scaffolds did not appear to be able to induce osteogenic differentiation in naïve MSC, they were capable of maintaining osteogenic differentiation of MSC without dexamethasone if MSCs had already been primed towards the osteogenic lineage prior to being seeded on the scaffold[22-24]. The ability to maintain osteogenic differentiation of primed MSC has important implications for promoting osteogenesis *in vivo* in the absence of exogenous soluble factor stimulation.

Decellularized matrices derived from fibroblasts and pre-osteoblasts yielded similar MSC osteogenic differentiation on composite poly(lactic-co-glycolic acid; PLGA)/hydroxyapatite(HA)/β-tricalcium(β-TCP) scaffolds[28]. The CDM-coated composite scaffolds contained fibronectin and collage type I, and stimulated increased ALP activity and mineralization when reseeded with MSC in osteogenic media, compared to uncoated PLGA/HA/β-TCP scaffolds[28]. Decaris et al. demonstrated that osteogenic CDM generated as 2D culture substrates could be coated onto a 3D scaffold, while retaining their capacity to modulate osteogenic differentiation[29, 30]. MSC-derived matrices deposited TCPS culture dishes were decellularized using Triton X-100, NH_4OH and DNAse, then homogenized in acetic acid and deposited as a matrix solution on 3D macroporous poly(lactide- co-glycolide; PLG) scaffolds[29, 30]. The transferred matrix maintained the ability to enhance osteogenic differentiation of MSC in the presence of β-glycerophosphate (β-GP) and dexamethasone, demonstrated by increased expression of osteogenic markers and ALP activity[29, 30]. The retention of bioactivity by the transferred

CDM implies that composition of the matrix, and not necessarily the matrix structure, is sufficient to enhance a cellular response. However, as discussed further below, these positive *in vitro* outcomes did not accurately predict *in vivo* responses.

Cell-derived matrices for bone regeneration and osteogenesis have been evaluated in ectopic and orthotopic sites *in vivo*. Subcutaneous implants of polyesterurethane scaffolds coated with mineralized osMSC-derived ECM induced the ingrowth of host cells and the formation of a mineralized matrix that stained positive for bone sialoprotein (BSP), whereas no mineralization or BSP was found in the uncoated polymer scaffold explants[31]. Similarly, Hong et al. demonstrated enhanced osteogenic differentiation of naïve MSCs implanted on PLGA/HA/β-TCP scaffolds coated with fibroblast-CDM using a subcutaneous ectopic bone formation model[28]. Explanted matrix-coated scaffolds had greater collagen and calcium deposition and ALP expression than uncoated scaffolds[28]. The increased osteogenic differentiation of implanted MSCs was attributed primarily to instructive cues present in the CDM, as the MSC were not treated with soluble exogenous osteogenic factors prior to implantation. Rat femoral segmental defects treated with decellularized MSCs and amniotic fluid stem (AFS) cells matrices on collagen/PCL scaffolds increased the rate of bone bridging, although neither matrix affected the total volume of mineralized matrix in the defect over 12 weeks[32]. Matrix deposited by either calvarial osteoblasts or fibroblasts was found to enhance the osteogenic properties of hydroxyapatite (HA) microparticles in critical-sized calvarial defects in rats. Defects treated with ECM-coated HA microparticles yielded multiple areas of new bone formation throughout the defect, similar to bone formation observed in response to HA microparticles mixed with Tissue Fleece (Baxter), a commercial equine type I collagen scaffold; while defects treated with uncoated HA microparticles had limited new bone formation that was restricted to the defect margins[33]. Despite some of the positive individual results observed in these *in vivo* studies suggesting that CDM can have osteoinductive potential, the current lack of significant improvements over clinical standards, such as Tissue Fleece, indicate that CDM examined to date may only yield modest improvements in skeletal regeneration.

In contrast, several other studies examining the osteogenic properties of CDM failed to observe significant improvements *in vivo*. In an ectopic bone formation model, MSC-derived matrix coating did not improve the mineralization of PLG scaffolds seeded with MSCs, regardless of whether the MSCs had been previously been grown in osteogenic media[29]. However, increased vascular ingrowth was observed in the MSC-containing CDM-coated scaffolds, relative to MSC-containing uncoated scaffolds[29]. At 2 weeks, no human MSC were detected within the coated and uncoated PLG scaffolds, suggesting implanted MSC death due to hypoxia or migration away from the implant site[29]. The lack of cell survival and engraftment may have impaired ectopic bone formation and masked any potential effect of the CDM. Decellularized MSC-derived matrix also failed to induce bone formation on titanium scaffolds implanted intramuscularly for up to 56 days[26], although, again, the matrix did enhance vascularization of the scaffold. The increase in vascular infiltration of ECM-coated scaffolds without significant improvement of bone formation is an interesting observation in both studies, especially considering the increase in vessel density was observed with and without the inclusion of viable MSC in the scaffolds [26, 29]. In both cases, the lack of bone formation may be attributed to reduced or altered osteoinductive properties of the matrices due to processing methods. In the first study, the matrix was transferred from a 2D culture plate and coated onto a scaffold. While previous *in vitro* studies indicated that the bioactivity of the matrix was preserved, it is possible that the homogenization in acetic acid affected the *in vivo* response. In the second study, the matrix was sterilized with ethylene oxide (EtO) following decellularization using freeze-thaw cycles since *in vitro* studies suggested that CDM retained its bioactivity following EtO sterilization[21]. However, EtO sterilization of demineralized bone matrix is still debated due to evidence that the doses required to sterilize the products reduces their osteoinductivity, specifically when high temperatures are used[34].

Cell-derived matrices rich in collagen and GAGs have also been used to enhance the chondrogenic differentiation of MSCs and synovium-derived stem cells (SDSCs) for cartilage regeneration. *In vitro* chondrogenesis of MSC is typically induced by supplementation with transforming growth factor β (TGF-β), dexamethasone and

ascorbate-2-phosphate[35, 36]. Differentiating MSCs on chondrocyte-derived matrices instead of uncoated PCL scaffolds moderately enhanced the GAG deposition and GAG synthetic activity (GAG/DNA per scaffold) in the presence of TGF-β1[25] and decreased the expression of collagen I in the absence of TGF-β1. However, the gene expression of aggrecan, collagen II and collagen I was not affected by the presence of the ECM in TGF-β1 treated cultures. Furthermore, this effect was TGF-β dependent, as the matrix alone was not sufficient to induce chondrogenesis[25]. In the reverse scenario, *in vitro* culture of chondrocytes on MSC-derived matrices had increased collagen type II and aggrecan expression in the presence of chondrogenic media[37], but this improvement was only observed when the matrices were derived from MSCs cultured in a basic growth medium and not chondrogenic medium containing TGF-β3 and dexamethasone. Unfortunately, *in vitro* culture on either MSC-derived matrix did not rescue the phenotype of chondrocytes obtained from an osteoarthritic donor[37].

SDSCs cultured on decellularized SDSC matrix (DSCM) had improved proliferation and attenuated oxidative stress-induced senescence during culture *in vitro*[14, 38]. SDSC expanded on DSCM also had enhanced chondrogenic potential but impaired differentiation along adipogenic and osteogenic lineages, compared to SDSC expanded on tissue culture polystyrene (TCPS) [14, 38]. The benefit of culturing the SDSC on DSCM was retained after the cells were removed from the matrix and cultured as pellets[14, 38] or injected into a cartilage defect *in vivo*[39]. DSCM-expanded SDSC pellets underwent striking chondrogenic differentiation in response to TGF-β1 and TGF-β3 *in vitro*, characterized by increased size, intensified staining for collagen type II, sulphated GAGs and decreased collagen type I staining compared to TCPS-expanded SDSC pellets[39]. Intra-articular injection of DSCM-expanded SDSC improved the repair of partial-thickness cartilage defects in pigs[39]. Upon explant at 3 months, the defect area and newly formed tissue in the group treated with DSCM-expanded cells stained more intensely for GAGs and collagen type II, and less collagen type I compared to defects treated with TCPS-expanded SDSC[39].

In a direct therapeutic application of CDM, Li et al. developed a membrane composed of layered decellularized chondrocyte-derived ECM to improve the outcome of bone marrow stimulation in a canine model[40]. The thin, flexible membrane, composed primarily of collagen and sulphated GAGs, was applied to cover a cartilage defect and protect the bone marrow stem cell-containing blot clot that resulted from bone marrow stimulation. Coverage with the decellularized membrane resulted in more cartilage-like tissue formed within the defect by 18 weeks than uncovered defects[40].

CDM have successfully been applied in *in vitro* osteogenic and chondrogenic differentiation schemes, but thus far they have had limited success in improving regenerative skeletal therapies *in vivo*. For the most part, the CDM in these applications were used as a coating for metallic and polymeric scaffolds. It is possible that the osteogenic or chondrogenic cues within the CDM lack the potency required to drive an *in vivo* response or that the bulk scaffold effects remain dominant in the *in vivo* environment. Another possibility is the lifespan of the CDM, which would likely be remodelled over the span of weeks, could be much shorter than the time scale required for bone or cartilage defect repair, thus limiting the impact of the transiently present CDM. In the case of cell-seeded scaffolds, retention of viable cells within the scaffold continues to be a limitation due to hypoxic cell death or migration of cells away from the scaffold[29] that may interfere with properly evaluating CDM *in vivo*.

Cell-derived matrices in cardiovascular tissue engineering

In contrast to skeletal engineering, CDMs in cardiovascular engineering applications tend to be used as regenerative therapies, where the CDM itself is used to replace damaged or diseased vessels and valves, rather than as a scaffold coating or cell culture substrate. The shortage of human allogenic heart valves and autogenic or allogenic vascular grafts for cardiovascular therapy has motivated the used of decellularized xenogenic and allogenic valves and vascular grafts. However, the limited availability of cadaveric allogenic tissues and potential risk(s) associated with xenogenic tissue sources has prompted

significant research into the development of tissue engineered blood vessels (TEBV) and tissue engineered heart valves (TEHV).

Tissue engineered small diameter vascular grafts have been a critical yet elusive goal of vascular tissue engineering for a long time. Although synthetic grafts function acceptably for large diameter vessels (> 6 mm), there currently is no suitable alternative for small diameter autologous grafts. One method of fabrication for TEBV is culturing fibroblasts[41, 42] or vascular smooth muscle cells[8, 43-45] in sheets, then wrapping the cell sheets around a mandrel to form a tubular structure. The engineered collagen-rich tissue is cultured for months, in some cases conditioned by pulsatile flow, until the tissue is mechanically robust enough for implantation. In some instances, endothelial cells are seeded on the luminal side of the vessel to improve hemocompatibility[8, 41]. While TEBV are considered a success story for the field of tissue engineering with encouraging performances in pre-clinical[44, 46, 47] and clinical studies[42, 48], the long manufacturing times coupled with the regulatory challenges facing the use of living engineered tissues pose potential limitations on widespread clinical use. Such concerns motivated the development of potential "off-the-shelf" products through devitalization of TEBV. Devitalization of vascular grafts has a clinical precedence with the use of cryopreserved allogenic grafts, which retain their mechanical functionality as arterial allografts but are nonviable at the time of implantation[49].

In a 2011 case study, an autologous fibroblast-derived TEBV (Lifeline™, Cytograft) was devitalized by air drying then stored for 5 months at -80°C prior to being thawed, lined with autologous endothelial cells and implanted as an atrioventricular shunt in a 72-year old male patient who required vascular access for hemodialysis[41]. During the first 8 weeks, the graft remained patent with a stable 5 mm diameter, however no reports have been released to date regarding the performance of the graft following puncture for hemodialysis access. With this approach, the patient wait time to prepare the devitalized graft with a viable, autologous endothelium and conduct quality control testing was less then 2 weeks[41]. In a follow-up case study, allogenic devitalized TEBVs prepared without the endothelial layer were implanted as brachial-axillary arteriovenous shunts for

hemodialysis access in three patients[20]. Grafts were used for hemodialysis access as early as 7 weeks post-implantation, and functioned without signs of aneurysms for up to 11 months; however, two of the three patients did require interventions to maintain graft patency. The authors noted that the decellularized graft, similar to synthetic grafts, did bleed slightly into the surrounding tissue at previous puncture sites whereas previously tested living, autologous TEVG did not. An interesting observation was that lymphotoxic cross-reactivity and panel reactive antibody tests indicated that the allogenic grafts were well-tolerated and did not elicit an immune response despite the fact the grafts were simply dehydrated instead of decellularized using detergents and DNase treatments[20].

Using a similar approach, Niklason and collaborators also developed a TEBV using human smooth muscle cells cultured on a tubular biodegradable polyglycolic acid (PGA) scaffold for 8 weeks, at which point the PGA scaffolds had mostly degraded and the constructs were decellularized[8]. *In vitro* mechanical testing indicated that the decellularized grafts possessed similar burst strength and suture retention as a human saphenous vein[8]. Furthermore, the decellularized grafts had an 80% patency rate over six weeks in an arterial graft model in nude rats, despite lacking any endothelial lining prior to implantation[43]. Histological analysis did reveal that host endothelial cells (von Willebrand factor positive) created a confluent endothelium, while host smooth muscle actin (SMA)-positive cells invaded the outer side of the graft. Taken together, these results demonstrate the potential of completely devitalized, non-endothelialized allogenic TEBV to serve as a true “off-the-shelf” tissue engineered product. Furthermore, they demonstrate that CDMs used in this application can be remodelled and repopulated by host cells, without generating a massive inflammatory response.

Similar studies have also been conducted with TEBV developed using ovine dermal fibroblasts seeded in fibrin gels in tubular molds for 2 weeks, then conditioned under pulsatile flow for 3 weeks prior to decellularization[46]. As expected from previous reports, the decellularization process had little effect on the mechanical properties of the TEBV and the grafts remained patent, without dilation, over 24 weeks in an ovine femoral graft model[46]. The grafts were completely re-endothelialized by 24 weeks, and exhibited a high

degree of cellular infiltration with primary α-SMA positive cells. Interestingly, this immune-competent model also showed evidence of immune cell infiltration with $CD45^+CD3^+$ cells (T-lymphocytes) and $CD45^+CD11b^+$ (macrophages) cells present within the graft at 8 weeks, mainly in the acellular region. However, by 24 weeks very few CD45+ cells were observed within the graft, reflecting an acute inflammatory response associated with the remodelling and recellularization of the decellularized TEBV rather than a chronic inflammatory or immunogenic response.

CDMs have also been used to construct tissue engineered heart valves. In this application, human vascular fibroblasts[50], human neonatal dermal fibroblasts[51] and ovine fibroblasts [52-54] have been seeded in fibrin carriers on degradable synthetic templates and cultured 4-5 weeks to allow the deposition of CDM. The matrix derived from fibroblasts generally contained collagen (based upon hydroxyproline content) and GAGs, but not elastin, which is a major component of native valve matrix[50, 52]. Decellularization and long-term storage (18 months) did not significantly alter the mechanical properties or matrix composition of the valves[52]. When implanted in baboons, human vascular fibroblast-derived valves performed well over the 8-week study, similar to decellularized human native heart valve [50]. However, radial shortening of the leaflet valve led to impaired coaptation and regurgitation that persisted throughout the 8 weeks. Contraction of valve leaflets is a well-establish limitation of viable TEHV, and had been attributed to the presence of contractile cells within the scaffold[52, 54, 55]. The contraction of the decellularized leaflets *in vivo* indicate that the remodeling of the valve wall and leaflet matrix by invading host α-SMA positive cells and MAC-387 positive cells (i.e. macrophages), respectively, may be detrimental to the long-term functioning of the engineered tissue. Residual stresses from TEHV being cultured in a closed position have also been suggested as a contributing factor to valve retraction[50]. In contrast, decellularized human native heart valves exhibited limited recellularization and maintained good coaptation without regurgitation over the duration of the study. Culturing the matrix producing fibroblasts with TGF-β1 to stimulate the deposition of elastin may help preserve the leaflet geometry[56].

The use of CDM for tissue engineered heart valve and blood vessel replacement offers a complementary alternative to xenogenic products. The studies discussed in this section have demonstrated that CDM can be repopulated and remodelled by host cells, integrate with the host tissue and maintain mechanical integrity and functionality. While there are still challenges to overcome, "off-the-shelf" tissue engineered vascular grafts and heart valves have the potential to have significant impacts on clinical cardiovascular therapies in the near future.

Cell-derived matrices as models of stem cell niches and *ex vivo* tissue-mimetic microenvironments

A common limitation in expanding sensitive primary cells *ex vivo* is loss of the native phenotype, characterized by loss of function, senescence, dedifferentiation and/or spontaneous differentiation[57-61]. One approach to mitigate culture-induced phenotypic changes is to recapitulate the tissue-specific microenvironment, in addition to the use of soluble factors. Recently, an in-depth evaluation of cell-derived ECM and the ability of MSC-derived ECM to support MSC and hematopoietic stem and progenitor cells (HSPC) expansion *ex vivo* was conducted by Prewitz et al[62]. Matrices generated by mouse embryonic fibroblasts (MEF), human umbilical vein endothelial cells (HUVEC) and human neonatal dermal fibroblasts (nFb) were compared to matrices generated by human MSCs grown in osteogenic factors (osteoECM) or ascorbic acid (aaECM). The matrices were deposited on a fibronectin (FN) layer covalently bonded to the TCPS to prevent the delamination of the matrix, which is a common problem with matrices simply physisorbed onto surfaces. Once decellularized, all the matrices were characterized as having a complex fibrillar structure, with tethered GAG aggregates within the network. The MSC-ECM was thicker than the other matrices, and also had an elasticity that resembled native bone marrow (~0.1 – 0.3 kPa), whereas the other matrices exhibited elasticities of approximately 1 kPa. Proteomic analysis revealed that both MSC-ECM had similar proteomic composition, including proteins associated with the bone marrow matrix such as collagen, decorin, laminins, nidogens, tenascin, thrombospondins, vitronectin and fibronectin. However, the aaECM contained significantly more sulphated

GAG and collagen than the osteoECM. aaECM also released greater amounts of growth factors (hepatocyte growth factor, HGF; fibroblast growth factor, FGF; interleukin-8, IL-8; vascular endothelial growth factor, VEGF) compared to osteoECM. Compared to TCPS and FN-coated TCPS, the MSC matrices supported higher proliferation rates, greater osteogenic and adipogenic differentiation efficiencies and increased secretion of growth factors (angiopoietin 1, Ang-1; stromal cell-derived factor 1,SDF-1; IL-8) in cultured MSCs, with aaECM outperforming osteoECM. The aaECM also supported greater expansion of $CD34^+$ and $CD34^+CD133^+$ cells compared to the other surfaces, without adversely affecting the long-term engraftment of HSPC. Overall, the improved expansion of both the MSC and HSPC populations were attributed to the ability of the aaECM to mimic the native bone marrow niche.

MSCs have the multipotent ability to differentiate into several musculoskeletal cell types, but their low abundance in native tissue is thought to require *ex vivo* expansion to obtain clinically relevant cell numbers. Long-term expansion of MSC typically diminishes their differentiation potential and leads to cellular senescence. Consequently, researchers have attempted to recapitulate the microenvironment of the bone marrow niche using MSC and bone marrow cell (BMC)-derived matrices to prevent the loss of MSC differentiation and proliferative potential[58-60]. BMC-derived matrix contained a composition similar to bone marrow, including the presence of collagen I and III, fibronectin, biglycan, decorine, perlecan and laminin[58]. Expansion of MSCs on BMC-derived matrix promoted proliferation and reduced intracellular levels of reactive oxygen species, relative to expansion on TCPS. Multipotentiality was also retained in MSC expanded on BMC-derived matrix, compared to TCPS. *In vivo*, CDM-expanded MSC retained their potential for osteogenesis, while TCPS-expanded MSCs had reduced osteogenic potential after 7 passages[58]. Similar improvements were reported for adult MSCs grown on fetal MSC-derived ECM and SDSCs grown on SDSC-derived matrix[38, 63].

Human and mouse embryonic stem cells (ESCs) are of great interest for regenerative medicine due to their capacity to differentiate into all somatic cell types[64-67]. Traditionally, they have been cultured with serum on feeder layers of mitotically

inactivated MEFs to maintained the ESCs in an undifferentiated, pluripotent state[64-66]. However, inconsistent and undefined culture conditions produced by the feeder cells and the transmission risk of xenogenic pathogens to human ESCs (hESCs), among other concerns, motivated a move away from feeder-dependent cultures[68]. Undifferentiated mouse ESCs can be grown in feeder-free conditions on gelatin with supplementation of leukemia inhibitory factor (LIF), however, LIF is unable to maintain undifferentiated hESCs on gelatin-coated substrates. Instead, a feeder-free system for undifferentiated hESCs was established by culturing the cells in MEF conditioned media on Matrigel™, a cancer cell derived matrix[69, 70]. Although feeder-free, the Matrigel/MEF-conditioned media culture system does not address the primary concerns of xenogenic contamination and variability of culture conditions[71]. Other serum-free, non-xenogenic culture systems have since been developed for the undifferentiated maintenance of hESCs. Of particular interest for this review, Klimanskaya and colleagues reported using decellularized ECM from mouse embryonic fibroblasts to maintain hESC in a feeder layer-free and serum-free culture[61]. Mitotically inactivated MEFs were grown on gelatin-coated plates for 7 to 21 days, then decellularized with a sodium deoxycholate solution. The MEF-CDM was used to establish and maintain a hESC line (ACT-14), along with six other existing hESC[61]. While the MEFs are of xenogeneic origin, the ability to decellularize and sterilize the matrix layer significantly reduces the risk of animal component contamination and pathogen transmission.

Other examples of *ex vivo* expansion of therapeutic cell populations include the use of CDM to reverse chondrocyte dedifferentiation and prevent loss of phenotype[11]. Cha et al. evaluated matrices derived from fibroblasts, pre-osteoblasts and chondrocytes for their ability to rescue chondrocyte dedifferentiation in culture[57] and each contained collagen type I, collagen type II, fibronectin and laminin. To evaluate the effect of different CDMs on chondrocyte dedifferentiation, rat primary chondrocytes were cultured on TCPS for 4 passages to induce dedifferentiation, marked by loss of collagen type II and aggrecan expression. When transferred to the CDMs and cultured for an additional 2 weeks, the passaged chondrocytes exhibited signs of redifferentiation, including increased GAG and collagen type II deposition and reduced collagen type I gene expression, although the

gene expression levels of collagen type II and aggrecan were similar in all cultures[57]. Pellet cultures of dedifferentiated chondrocytes expanded on CDM for 2 passages also contained more GAGs, stronger peripheral staining of collagen type II and were larger in size compared to pellets formed from TCPS-expanded chondrocytes[57]. Similar studies conducted using SDSC-derived matrix with primary pig chondrocytes[11], and chondrocyte-derived matrix with primary bovine chondrocytes[12, 72] have also demonstrated delayed chondrocyte dedifferentiation when cultured on CDM substrate.

As noted earlier, an important consideration in the use of CDM is that the composition and quality of the matrix is dependent on the source cell population. For example, aging has been shown to have a profound effect on the quality of matrices that MSCs deposit[59]. Matrix derived from BMCs isolated from young mice (3 month old) improved the proliferation and osteogenic potential of MSC from aged mice (18 months old) to a level comparable with young MSC, and reduced intracellular reactive oxygen species[59]. In comparison, both young and aged MSC grown on aged MSC-derived matrix had significant impairment of proliferation and osteogenesis. These results suggest that matrices derived from young MSC can rejuvenate aged MSC populations and contributes to maintaining healthy MSC function. Confocal Raman microscopic analysis of the young and old MSC-derived matrices suggested that the old matrix contained more mineralized phosphate, while the young ECM appeared to contain more collagens, indicating that aging impacts the composition of MSC-derived matrix[59]. This study has far reaching implications for MSC biology and regenerative medicine, as it suggests that culture on matrix derived from young MSCs may improve the quality and quantity of MSC isolated from aged patients for regenerative cell therapies.

Just as disease states are often reflected by cell phenotypes, the native ECM of disease tissues and tumours can also have distinct features that may contribute to or result from a disease state[73]. Studying the composition and properties of matrices derived from diseased cell populations may enable researchers to identify and better understand the relationship between cells and matrix in disease propagation, and enable the development of superior disease models for basic research. Reichert et al. used matrices produced by

human osteoblasts to develop an *in vitro* model of bone metastasis, which recapitulated features of prostate cancer bone metastasis, including loss of epithelial phenotype[74]. Also, the role of ECM in craniosynostosis (CS), the premature fusion of cranial sutures, was investigated using decellularized matrix produced by osteoblasts from patients with CS and healthy individuals[75]. Previous studies had detected decreased expression of TGF- β, a protein involved in regulating bone resorption[76], in MSCs harvested from fused versus unfused sutures in mice[77]. TGF-β had significantly reduced binding affinity for ECM secreted by CS osteoblasts, compared to osteoblasts from healthy individuals, indicating that differences in the ECM of patients with CS likely contribute to the pathogenesis of the disease[75].

Embryonic stem cell-derived matrices

In most cases, cell-derived matrices are used to recapitulate the microenvironment of adult tissues and the cells used to derive the matrix are sourced directly from the tissue of interest. However, there are instances, as noted above, when adult matrices may be limited in their ability to promote a desired behaviour, such as tissue regeneration. For example, scarring and impaired wound healing are significant clinical challenges related to traumatic and surgical wounds, and co-morbidities of diseases like diabetes. The main matrix components in skin are collagens, elastin, GAGs, laminin, nidogen and fibronectin[78], and matrices derived from fibroblasts, the primary cell type within the dermis, recapitulate some the main components in adult skin [10, 57, 79]. Acellular dermal matrix allografts and skin substitutes, such as INTEGRA® Dermal Regeneration Template, are routinely used in the clinic to treat burns and chronic wounds[80-83]. However, adult skin is also known to have reduced regenerative capability compared to fetal skin. At early stages of development, fetal skin can undergo scarless healing and is rich in matrix proteins, GAGs and growth factors that have are known to promote wound repair[84-86]. Therefore, instead of recapitulating adult skin matrix, a matrix reflecting the protein composition of fetal tissue may provide a better therapeutic material for promoting scarless wound healing.

Previous studies from our group have demonstrated that differentiating embryonic stem cells grown in multicellular aggregates, called embryoid bodies (EB), express many of the matrix and matricellular proteins and morphogens that are expressed during early development and are also associated with improved wound healing[87]. Gene expression analysis of spontaneously differentiating mESC EBs revealed expression of many ECM and matricellular proteins, including collagens (*Col1a1, Col2a1, Col3a1, Col4a1-3, Col6a1* etc), laminins (*Lama1, Lamb1, Lamb3* etc) fibronectin (*Fn1*) vitronectin (*Vtn*), osteopontin (*Spp1*), fibulin 1(*Fbln1*), tenascin (*Tnc*) and sparc (*Sparc*)[87]. An interesting trend in the expression profile was that while many matrix proteins had constant or decreased expression initially, the expression for many matrix-associated proteins started to increase expression by day 10. Similarly, the EBs were also shown to express genes of many therapeutically relevant secreted morphogens, including bone morphogenic proteins (BMPs), FGFs, TGFs, platelet-derived growth factors (PDGFs) and VEGFs, some of which would be retained within the EB matrix[87]. The presence of versican and hyaluronan within the EB matrix was independently confirmed in a separate study[88]. The rich and complex nature of the EB matrix, particularly at later time points, led us to postulate that the EB matrix (EBM) and its associated growth factors may provide a therapeutic platform for delivering embryonic-like matrices and paracrine factors to damaged tissues and organs, such as skin and bone. Successful decellularization of EBs has been achieved using both chemical and mechanical methods, matrix components are retained, and the EBM can support exogenous cell attachment and proliferation[89-91].

Similar to the use of MSC-derived matrices to improve somatic stem cell expansion, Goh et al. proposed that ESC-derived ECM be used to recapitulate the embryonic microenvironment to improve the efficiency of current ESC lineage-specific differentiation protocols[92]. Two distinct EBM were generated by culturing mESC EBs with or without retinoic acid (RA), an inducer of neural differentiation. Prior to decellularization, both RA-treated and untreated EBs contained collagen I, collagen IV, fibronectin and laminin, although staining for collagen I, collagen IV and laminin staining appeared to be more intense in the spontaneously differentiating EBs than the RA-EBM and the distribution pattern of fibronectin differed between the two groups.

While the expression of these four proteins was retained following the decellularization process, the total protein content and sulphated GAG content was significantly reduced in both groups. Total collagen and sulphated GAG content remained higher in the EBM generated from spontaneously differentiating EBs (SPT-EBM) than RA-EBM, although the spatial distribution of ECM components was lost. When reseeded with mESC, both SPT-EBM and RA-EBM supported greater cell proliferation compared to the proliferation of ESCs cultured as native EBs. However, SPT-EBM increased expression of early differentiation markers Brachyury, FGF-5 and FGF-8 of mESC, whereas the gene expression of ESC cultured on RA-EBM more closely resembled that of native EB[92]. This study demonstrated that EB-derived matrices can support ESC proliferation and differentiation, and that the differentiation protocol used to obtain the EBM can influence the phenotype of reseeded ESCs. Furthermore, it suggests that the use of an appropriate ESC matrix may improve soluble factor-directed differentiation.

Sart et al. also examined the effect of culture conditions on the composition of ESC-derived ECM and whether any differences in ECM exerted an effect on the proliferation and differentiation of ESCs reseeded on the EBM[93]. Mouse ESCs were cultured as EBs differentiating spontaneously or in the presence of retinoic acid (RA) or BMP-4 to induce ectodermal or mesodermal differentiation, respectively. The matrices derived from these different culture conditions all contained varying amounts of fibronectin, laminin, vitronectin, collagen IV and GAGs, with the highest fibronectin, vitronectin and collagen IV expression occurring in the matrices derived from BMP-4 and RA-treated EBs, compared to the spontaneously differentiating EBs. However, very few differences were observed when fresh ESCs were reseeded and grown on the different EBM for 4 days then replated on gelatin for 7 days, with the exception of ESCs grown on day 10 RA-EBM. However, the decreased proliferation and increased β-tubulin III (ectoderm), α-actinin (mesoderm) and FoxA2 expression (endoderm) observed with the day 10 RA-EBM was thought to be due to residual RA entrapped within the matrix, as the expression of α-actinin and FoxA2 was attenuated by a pan-RA receptor antagonist[93]. Contrary to the results from the previous study, in this experiment the different EBM did not appear to have a direct effect on ESC differentiation. The differing outcomes of the two studies

are not surprising, as there were fundamental experimental differences, including cell type, EB culture time, media formulations, dosing of RA, decellularization and reseeding methods, and culture of reseeded cells[92, 93].

Arguably, one of the strengths of ESC-derived matrices is the rich and complex expression profile of pluripotent cells. While the matrices retained both the matrix proteins and sequestered growth factors and signalling molecules, alternative approaches to deliver stem cell-secreted factors in more concentrated manners may yield greater potency and more significant results. Recently it was reported that hESC-secreted factors significantly improved the viability of human cortical neurons following exposure to a toxic form of Amyloid beta in an *in vitro* model of Alzheimer's disease[94]. Moreover, hESC-conditioned media improved myoblast proliferation and the pro-myogenic proteins secreted by hESCs were found to bind heparin[94]. The heparin-binding capacity of many of the morphogens secreted by ESCs may provide an efficient method for capturing stem cell morphogens for subsequent delivery.

Future directions

The use of cell derived matrices as bulk biomaterials, scaffold coatings and cell culture substrates has permeated the field of regenerative medicine. This review sought to highlight the current status of CDM across various applications and identify some of the successes and major challenges that remain. CDM offer promising alternative biomaterials as decellularization and sterilization techniques are optimized and appropriate cell sources and culture conditions are identified for producing more potent and instructive materials.

As the use of CDM has become more established, one can foresee the potential for engineering matrices with desired properties using genetically engineered cells, small molecules or other soluble factors to induce overexpression of specific matrix components. An example would be inducing overexpression of elastin[95] by fibroblasts generating the TEHV and TEBV matrix in an effort to reduce leaflet retraction or to

improve the elasticity and resilience, respectively. As the cellular components of these transgenic cells would be removed prior to *in vivo* applications, the risk typically associated with transgenic, immortalized or tumorigenic cell lines would be eliminated.

In response to the damage current chemical, enzymatic and physical decellularization and devitalization techniques cause to the ECM, Martin and collaborators have proposed a novel method of decellularization that exploits programmed cell death[96]. The authors propose to induce apoptosis by (i) delivery of a death receptor ligand, such as FasL[97, 98], to activate the extrinsic apoptotic pathway; (ii) induction of mitochondrial apoptosis through lethal environmental conditions, such as hyper- or hypo-thermic temperatures[99, 100], or nitric oxide[101]; or (iii) genetically engineering cells to induce apoptotic pathways in response to a chemical inducer, such as the inducible caspase 9 suicide system[102]. By inducing cell death through apoptotic pathways, the dying cells fragment into small apoptotic bodies, which in theory would be removed by perfusing or washing the ECM[96]. There are a number of limitations and challenges that would need to be addressed to ensure complete decellularization and limit undesirable effects, such as the release of pro-inflammatory factors in response to the stimuli. However, decellularization by intentional activation of programmed cell death is an intriguing proposal that warrants further investigation.

Summary

Cell-derived matrices offer an alternative to tissue-derived matrices and have been used in a number of pre-clinical and clinical applications. Some advantages of CDM are accessibility of human cell sources, increased tunability of matrix properties and conformations (bulk material or scaffold coating) compared to tissue-derived matrices, and the ability to use a variety of somatic and stem cells to generate matrices with desirable properties. CDM have yielded promising results in generating off-the-shelf tissue engineered vascular grafts and heart valves, and novel cell culture substrates mimicking specific niche microenvironments. CDM-scaffold coatings have also shown promise in *in vitro* studies of osteo- and chondrogenesis, however there are apparent

challenges in transferring the technology to *in vivo* orthopaedic applications. The future of CDM in regenerative medicine is exciting, with the potential for using genetically engineered cells and continued optimization of decellularization techniques.

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Abbreviations

Aa	Ascorbic acid
AFS	Amniotic fluid stem cells
ALP	Alkaline phosphatase
ANG-1	Angiopoietin 1
β-GP	β-glycerophosphate
β-TCP	β-tricalcium
BMC	Bone marrow cell
BMP	Bone morphogenic proteins
BSP	Bone sialoprotein
CDM	Cell-derived matrix
CS	Craniosynostosis
DSCM	Decellularized SDSC matrix
EB	Embryoid bodies
EBM	Embryoid body matrix
ECM	Extracellular matrix
ESC	Embryonic stem cells
FGF	Fibroblast growth factor
FN	Fibronectin
GAG	Glycosaminoglycan
HA	Hydroxyapatite
HGF	Hepatic growth facotr
HSPC	Hematopoietic stem and progenitor cells
IL-8	Interleukin 8
LIF	Leukemia inhibitory factor
MEF	Mouse embryonic fibroblasts
MSC	Mesenchymal stem cells
nFb	Neonatal dermal fibroblasts
PCL	Poly(e-caprolactone)
PDGF	Platelet derived growth factor
PGA	Polyglycolic acid
PLG	Poly(lactide- co-glycolide)
PLGA	Poly(lactic-co-glycolic acid)
RA	Retanoic acid
SDF-1	Stromal derived factor 1
SDS	Sodium dodecyl sulfate
SDSC	Synovium-derived stem cells
SMA	Smooth muscle actin
TCPS	Tissue culture polystyrene
TEBV	Tissue engineered blood vessels
TEHV	Tissue engineered heart valve
TGF	Transforming growth factor
Ti	Titanium
VEGF	Vascular endothelial growth factor

References

1. S. F. Badylak, D. O. Freytes and T. W. Gilbert, *Acta Biomater*, 2009, **5**, 1-13.
2. K. E. M. Benders, P. R. van Weeren, S. F. Badylak, D. l. B. F. Saris, W. J. A. Dhert and J. Malda, *Trends Biotechnol*, 2013, **31**, 169-176.
3. J. J. Song and H. C. Ott, *Trends Mol Med*, 2011, **17**, 424-432.
4. H. C. Ott, T. S. Matthiesen, S. K. Goh, L. D. Black, S. M. Kren, T. I. Netoff and D. A. Taylor, *Nat Med*, 2008, **14**, 213-221.
5. T. H. Petersen, E. A. Calle, L. Zhao, E. J. Lee, L. Gui, M. B. Raredon, K. Gavrilov, T. Yi, Z. W. Zhuang, C. Breuer, E. Herzog and L. E. Niklason, *Science*, 2010, **329**, 538-541.
6. H. C. Ott, B. Clippinger, C. Conrad, C. Schuetz, I. Pomerantseva, L. Ikonomou, D. Kotton and J. P. Vacanti, *Nat Med*, 2010, **16**, 927-933.
7. B. E. Uygun, A. Soto-Gutierrez, H. Yagi, M. L. Izamis, M. A. Guzzardi, C. Shulman, J. Milwid, N. Kobayashi, A. Tilles, F. Berthiaume, M. Hertl, Y. Nahmias, M. L. Yarmush and K. Uygun, *Nat Med*, 2010, **16**, 814-820.
8. C. Quint, Y. Kondo, R. J. Manson, J. H. Lawson, A. Dardik and L. E. Niklason, *Proc Natl Acad Sci U S A*, 2011, **108**, 9214-9219.
9. Z. H. Syedain, L. A. Meier, J. W. Bjork, A. Lee and R. T. Tranquillo, *Biomaterials*, 2011, **32**, 714-722.
10. H. Lu, T. Hoshiba, N. Kawazoe and G. Chen, *Biomaterials*, 2011, **32**, 2489-2499.
11. M. Pei and F. He, *J Cell Physiol*, 2012, **227**, 2163-2174.
12. T. Hoshiba, T. Yamada, H. Lu, N. Kawazoe and G. Chen, *J Biomed Mater Res A*, 2012, **100**, 694-702.
13. M. Pei, M. Shoukry, J. Li, S. D. Daffner, J. C. France and S. E. Emery, *Spine (Phila Pa 1976)*, 2012, **37**, 1538-1547.
14. M. Pei, Y. Zhang, J. Li and D. Chen, *Stem Cells Dev*, 2013, **22**, 889-900.
15. Z. Ivanovic, *J Cell Physiol*, 2009, **219**, 271-275.
16. T. W. Gilbert, T. L. Sellaro and S. F. Badylak, *Biomaterials*, 2006, **27**, 3675-3683.
17. T. J. Keane and S. F. Badylak, *J Tissue Eng Regen Med*, 2014.
18. M. L. Wong and L. G. Griffiths, *Acta Biomater*, 2014, **10**, 1806-1816.
19. M. H. Zheng, J. Chen, Y. Kirilak, C. Willers, J. Xu and D. Wood, *J Biomed Mater Res B Appl Biomater*, 2005, **73**, 61-67.
20. W. Wystrychowski, T. N. McAllister, K. Zagalski, N. Dusserre, L. Cierpka and N. L'Heureux, *J Vasc Surg*, 2013.
21. N. Datta, Q. P. Pham, U. Sharma, V. I. Sikavitsas, J. A. Jansen and A. G. Mikos, *Proc Natl Acad Sci U S A*, 2006, **103**, 2488-2493.
22. N. h. Datta, H. L. Holtorf, V. I. Sikavitsas, J. A. Jansen and A. G. Mikos, *Biomaterials*, 2005, **26**, 971-977.
23. J. Liao, X. Guo, D. Nelson, F. K. Kasper and A. G. Mikos, *Acta Biomater*, 2010, **6**, 2386-2393.
24. R. A. Thibault, L. Scott Baggett, A. G. Mikos and F. K. Kasper, *Tissue Eng Part A*, 2010, **16**, 431-440.
25. J. Liao, X. Guo, K. J. Grande-Allen, F. K. Kasper and A. G. Mikos, *Biomaterials*, 2010, **31**, 8911-8920.

26. Q. P. Pham, F. K. Kasper, A. S. Mistry, U. Sharma, A. W. Yasko, J. A. Jansen and A. G. Mikos, *J Biomed Mater Res A*, 2009, **88**, 295-303.
27. Q. P. Pham, F. Kurtis Kasper, L. Scott Baggett, R. M. Raphael, J. A. Jansen and A. G. Mikos, *Biomaterials*, 2008, **29**, 2729-2739.
28. Y. J. Hong, S. E. Bae, S. H. Do, I. H. Kim, D. K. Han and K. Park, *Macromol Res*, 2011, **19**, 1090-1096.
29. M. L. Decaris, B. Y. Binder, M. A. Soicher, A. Bhat and J. K. Leach, *Tissue Eng Part A*, 2012, **18**, 2148-2157.
30. M. L. Decaris, A. Mojadedi, A. Bhat and J. K. Leach, *Acta Biomater*, 2012, **8**, 744-752.
31. N. Sadr, B. E. Pippenger, A. Scherberich, D. Wendt, S. Mantero, I. Martin and A. Papadimitropoulos, *Biomaterials*, 2012, **33**, 5085-5093.
32. E. R. Deutsch and R. E. Guldberg, *J Mater Chem*, 2010, **20**, 8942-8951.
33. G. Tour, M. Wendel and I. Tcacencu, *Tissue Eng Part A*, 2011, **17**, 127-137.
34. J. Glowacki, *Cell Tissue Bank*, 2005, **6**, 3-12.
35. A. M. Mackay, S. C. Beck, J. M. Murphy, F. P. Barry, C. O. Chichester and M. F. Pittenger, *Tissue Eng*, 1998, **4**, 415-428.
36. J. U. Yoo, T. S. Barthel, K. Nishimura, L. Solchaga, A. I. Caplan, V. M. Goldberg and B. Johnstone, *J Bone Joint Surg Am*, 1998, **80**, 1745-1757.
37. S. Thakkar, C. A. Ghebes, M. Ahmed, C. Kelder, C. A. van Blitterswijk, D. Saris, H. A. M. Fernandes and L. Moroni, *Biofabrication*, 2013, **5**, 025003.
38. F. He, X. Chen and M. Pei, *Tissue Eng Part A*, 2009, **15**, 3809-3821.
39. M. Pei, F. He, J. Li, J. E. Tidwell, A. C. Jones and E. B. McDonough, *Tissue Eng Part A*, 2013, **19**, 1144-1154.
40. T. Z. Li, C. Z. Jin, B. H. Choi, M. S. Kim, Y. J. Kim, S. R. Park, J. H. Yoon and B.-H. Min, *Adv Funct Mater*, 2012, **22**, 4292-4300.
41. W. Wystrychowski, L. Cierpka, K. Zagalski, S. Garrido, N. Dusserre, S. Radochonski, T. N. McAllister and N. L'Heureux, *J Vasc Access*, 2011, **12**, 67-70.
42. T. N. McAllister, M. Maruszewski, S. A. Garrido, W. Wystrychowski, N. Dusserre, A. Marini, K. Zagalski, A. Fiorillo, H. Avila, X. Manglano, J. Antonelli, A. Kocher, M. Zembala, L. Cierpka, L. M. de la Fuente and N. L'Heureux, *Lancet*, 2009, **373**, 1440-1446.
43. C. Quint, M. Arief, A. Muto, A. Dardik and L. E. Niklason, *J Vasc Surg*, 2012, **55**, 790-798.
44. S. L. M. Dahl, A. P. Kypson, J. H. Lawson, J. L. Blum, J. T. Strader, Y. Li, R. J. Manson, W. E. Tente, L. DiBernardo, M. T. Hensley, R. Carter, T. P. Williams, H. L. Prichard, M. S. Dey, K. G. Begelman and L. E. Niklason, *Sci Transl Med*, 2011, **3**, 68ra69.
45. S. L. M. Dahl, J. Koh, V. Prabhakar and L. E. Niklason, *Cell Transplant*, 2003, **12**, 659-666.
46. Z. H. Syedain, L. A. Meier, M. T. Lahti, S. L. Johnson and R. T. Tranquillo, *Tissue Eng Part A*, 2014.
47. N. L'Heureux, N. Dusserre, G. Konig, B. Victor, P. Keire, T. N. Wight, N. A. Chronos, A. E. Kyles, C. R. Gregory, G. Hoyt, R. C. Robbins and T. N. McAllister, *Nat Med*, 2006, **12**, 361-365.

48. N. L'Heureux, T. N. McAllister and L. M. de la Fuente, *N Engl J Med*, 2007, **357**, 1451-1453.
49. S. E. Langerak, M. Groenink, E. E. van der Wall, C. Wassenaar, E. Vanbavel, M. C. van Baal and J. A. Spaan, *Transpl Int*, 2001, **14**, 248-255.
50. B. Weber, P. E. Dijkman, J. Scherman, B. Sanders, M. Y. Emmert, J. Grunenfelder, R. Verbeek, M. Bracher, M. Black, T. Franz, J. Kortsmit, P. Modregger, S. Peter, M. Stampanoni, J. Robert, D. Kehl, M. van Doeselaar, M. Schweiger, C. E. Brokopp, T. Walchli, V. Falk, P. Zilla, A. Driessen-Mol, F. P. T. Baaijens and S. P. Hoerstrup, *Biomaterials*, 2013, **34**, 7269-7280.
51. Z. H. Syedain, A. R. Bradee, S. Kren, D. A. Taylor and R. T. Tranquillo, *Tissue Eng Part A*, 2013, **19**, 759-769.
52. P. E. Dijkman, A. Driessen-Mol, L. Frese, S. P. Hoerstrup and F. P. T. Baaijens, *Biomaterials*, 2012, **33**, 4545-4554.
53. D. van Geemen, A. Driessen-Mol, L. G. M. Grootzwagers, R. S. Soekhradj-Soechit, P. W. Riem Vis, F. P. T. Baaijens and C. V. C. Bouten, *Regen Med*, 2012, **7**, 59-70.
54. Z. H. Syedain, L. A. Meier, J. M. Reimer and R. T. Tranquillo, *Ann Biomed Eng*, 2013.
55. T. C. Flanagan, J. S. Sachweh, J. Frese, H. Schnoring, N. Gronloh, S. Koch, R. H. Tolba, T. Schmitz-Rode and S. Jockenhoevel, *Tissue Eng Part A*, 2009, **15**, 2965-2976.
56. Z. H. Syedain and R. T. Tranquillo, *J Biomech*, 2011, **44**, 848-855.
57. M. H. Cha, S. H. Do, G. R. Park, P. Du, K.-C. Han, D. K. Han and K. Park, *Tissue Eng Part A*, 2013, **19**, 978-988.
58. Y. Lai, Y. Sun, C. M. Skinner, E. L. Son, Z. Lu, R. S. Tuan, R. L. Jilka, J. Ling and X.-D. Chen, *Stem Cells Dev*, 2010, **19**, 1095-1107.
59. Y. Sun, W. Li, Z. Lu, R. Chen, J. Ling, Q. Ran, R. L. Jilka and X.-D. Chen, *FASEB J*, 2011, **25**, 1474-1485.
60. X.-D. Chen, V. Dusevich, J. Q. Feng, S. C. Manolagas and R. L. Jilka, *J Bone Miner Res*, 2007, **22**, 1943-1956.
61. I. Klimanskaya, Y. Chung, L. Meisner, J. Johnson, M. D. West and R. Lanza, *Lancet*, 2005, **365**, 1636-1641.
62. M. C. Prewitz, F. P. Seib, M. von Bonin, J. Friedrichs, A. Stissel, C. Niehage, K. Muller, K. Anastassiadis, C. Waskow, B. Hoflack, M. Bornhauser and C. Werner, *Nat Methods*, 2013, **10**, 788-794.
63. C. P. Ng, A. R. Sharif, D. E. Heath, J. W. Chow, C. B. Zhang, M. B. Chan-Park, P. T. Hammond, J. K. Chan and L. G. Griffith, *Biomaterials*, 2014, **35**, 4046-4057.
64. J. A. Thomson, J. Itskovitz-Eldor, S. S. Shapiro, M. A. Waknitz, J. J. Swiergiel, V. S. Marshall and J. M. Jones, *Science*, 1998, **282**, 1145-1147.
65. M. J. Evans and M. H. Kaufman, *Nature*, 1981, **292**, 154-156.
66. G. R. Martin, *Proc Natl Acad Sci U S A*, 1981, **78**, 7634-7638.
67. J. Itskovitz-Eldor, M. Schuldiner, D. Karsenti, A. Eden, O. Yanuka, M. Amit, H. Soreq and N. Benvenisty, *Mol Med*, 2000, **6**, 88-95.
68. T. C. McDevitt and S. P. Palecek, *Curr Opin Biotechnol*, 2008, **19**, 527-533.
69. C. Xu, M. S. Inokuma, J. Denham, K. Golds, P. Kundu, J. D. Gold and M. K. Carpenter, *Nat Biotechnol*, 2001, **19**, 971-974.

70. H. K. Kleinman, M. L. McGarvey, L. A. Liotta, P. G. Robey, K. Tryggvason and G. R. Martin, *Biochemistry*, 1982, **21**, 6188-6193.
71. L. G. Villa-Diaz, A. M. Ross, J. Lahann and P. H. Krebsbach, *Stem Cells*, 2013, **31**, 1-7.
72. T. Hoshiba, H. Lu, N. Kawazoe, T. Yamada and G. Chen, *Biotechnol Prog*, 2013, **29**, 1331-1336.
73. P. Carinci, E. Becchetti and M. Bodo, *Int J Dev Biol*, 2000, **44**, 715-723.
74. J. C. Reichert, V. M. C. Quent, L. J. Burke, S. H. Stansfield, J. A. Clements and D. W. Hutmacher, *Biomaterials*, 2010, **31**, 7928-7936.
75. A. Bhat, S. A. Boyadjiev, C. W. Senders and J. K. Leach, *PLoS One*, 2011, **6**, e25990.
76. Y. Tang, X. Wu, W. Lei, L. Pang, C. Wan, Z. Shi, L. Zhao, T. R. Nagy, X. Peng, J. Hu, X. Feng, W. Van Hul, M. Wan and X. Cao, *Nat Med*, 2009, **15**, 757-765.
77. Y. Xu, P. Malladi, M. Chiou and M. T. Longaker, *Plast Reconstr Surg*, 2007, **119**, 819-829.
78. J. Uitto, D. R. Olsen and M. J. Fazio, *J Invest Dermatol*, 1989, **92**, 61S-77S.
79. P. A. Soucy and L. H. Romer, *Matrix Biol*, 2009, **28**, 273-283.
80. R. S. Kirsner, G. Bohn, V. R. Driver, J. L. Mills, Sr., L. B. Nanney, M. L. Williams and S. C. Wu, *Int Wound J*, 2013.
81. A. Reyzelman, R. T. Crews, J. C. Moore, L. Moore, J. S. Mukker, S. Offutt, A. Tallis, W. B. Turner, D. Vayser, C. Winters and D. G. Armstrong, *Int Wound J*, 2009, **6**, 196-208.
82. N. Moiemen, J. Yarrow, E. Hodgson, J. Constantinides, E. Chipp, H. Oakley, E. Shale and M. Freeth, *Plast Reconstr Surg*, 2011, **127**, 1149-1154.
83. S. A. Rehim, M. Singhal and K. C. Chung, *Hand Clin*, 2014, **30**, 239-252, vii.
84. A. S. Colwell, M. T. Longaker and H. P. Lorenz, *Adv Biochem Eng Biotechnol*, 2005, **93**, 83-100.
85. M. W. Ferguson and S. O'Kane, *Philos Trans R Soc Lond B Biol Sci*, 2004, **359**, 839-850.
86. B. J. Larson, M. T. Longaker and H. P. Lorenz, *Plast Reconstr Surg*, 2010, **126**, 1172-1180.
87. R. Nair, A. V. Ngangan, M. L. Kemp and T. C. McDevitt, *PLoS One*, 2012, **7**, e42580.
88. S. Shukla, R. Nair, M. W. Rolle, K. R. Braun, C. K. Chan, P. Y. Johnson, T. N. Wight and T. C. McDevitt, *J Histochem Cytochem*, 2010, **58**, 345-358.
89. R. Nair, A. V. Ngangan and T. C. McDevitt, *J Biomater Sci Polym Ed*, 2008, **19**, 801-819.
90. R. Nair, S. Shukla and T. C. McDevitt, *J Biomed Mater Res A*, 2008, **87**, 1075-1085.
91. A. V. Ngangan and T. C. McDevitt, *Biomaterials*, 2009, **30**, 1143-1149.
92. S.-K. Goh, P. Olsen and I. Banerjee, *PLoS One*, 2013, **8**, e61856.
93. S. Sart, T. Ma and Y. Li, *Tissue Eng Part A*, 2014, **20**, 54-66.
94. H. Yousef, M. J. Conboy, J. Li, M. Zeiderman, T. Vazin, C. Schlesinger, D. V. Schaffer and I. M. Conboy, *Aging*, 2013, **5**, 357-372.
95. S. H. Li, Z. Sun, L. Guo, M. Han, M. F. Wood, N. Ghosh, I. A. Vitkin, R. D. Weisel and R. K. Li, *J Cell Mol Med*, 2012, **16**, 2429-2439.

96. P. E. Bourgine, B. E. Pippenger, A. Todorov, L. Tchang and I. Martin, *Biomaterials*, 2013, **34**, 6099-6108.
97. M. Rodrigues, H. Blair, L. Stockdale, L. Griffith and A. Wells, *Stem Cells*, 2013, **31**, 104-116.
98. M. Yamaoka, S. Yamaguchi, T. Suzuki, M. Okuyama, J. Nitobe, N. Nakamura, Y. Mitsui and H. Tomoike, *J Mol Cell Cardiol*, 2000, **32**, 881-889.
99. E. H. Nijhuis, A. A. Poot, J. Feijen and I. Vermes, *Int J Hyperthermia*, 2006, **22**, 687-698.
100. U. Rauen, B. Polzar, H. Stephan, H. G. Mannherz and H. de Groot, *Faseb J*, 1999, **13**, 155-168.
101. C. M. Snyder, E. H. Shroff, J. Liu and N. S. Chandel, *PLoS One*, 2009, **4**, e7059.
102. P. Bourgine, C. Le Magnen, S. Pigeot, J. Geurts, A. Scherberich and I. Martin, *Stem Cell Res*, 2014, **12**, 584-598.
103. G. R. Park, J. G. Lee, H. J. Chun, D. K. Han and K. Park, *Macromol Res*, 2012, **20**, 868-874.

Table 2. Examples of common reagents and methods for decellularizing and devitalizing cell-derived ECM.

Culture Method	Application	Examples of Common Processing Methods	DNase Treatment	References
Monolayer	Substrate for cell culture	0.5% Triton X-100 and 20 mM NH_4OH for 5 min at 37°C	Yes	14, 29, 33, 39, 60, 63, 103
			No	38, 59, 62
	Cell sheet layering for tissue engineered blood vessels	Air dried then frozen at -80°C	No	20, 41
Degradable Carrier Scaffold	Tissue engineered blood vessel	8 mM CHAPS, 1M NaCl and 25 mM EDTA for 1 hr at 37°C; followed by 1.8 mM SDS, 1 M NaCl and 25 mM EDTA for 1 hr at 37°C	No	8, 43
	Tissue engineered heart value	1% SDS for 6 - 24 hr at RT; followed by 1% Triton X-100 for 30 min at RT	Yes	46, 51, 54
		0.25% Triton X-100, 0.25% sodium deoxycholate, 0.02% EDTA overnight at 37°C	Yes	50, 52
Aggregate	Embryoid Body Matrices	1% Triton X-100 for 30 min at RT	Yes	89, 90
		1% Triton X-100 for 30 min at RT	No	92
		Lyophilized	No	91, 93
Scaffold Coating	Scaffolds for bone repair and osteogenesis	Free-thaw cycling (3 cycles): liquid nitrogen for 10 min, then 37°C water bath for 10 min	No	21-23, 26, 27, 31, 33

NH_4OH, ammonium hydroxide (alkaline); CHAPS (*zwitterionic detergent*); EDTA, ethylenediaminetetraacetic acid (*chelating agent*); SDS, sodium dodecyl sulphate; Sodium deoxycholate (*ionic detergent*); Triton X-100 (*non-ionic detergent*)

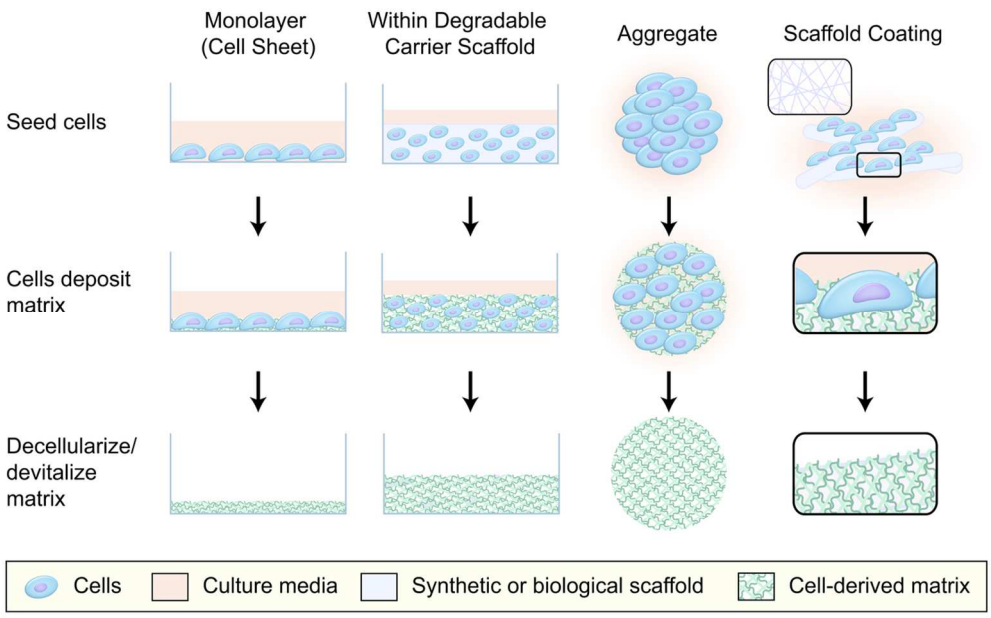

Figure 1. Common culture methods for generating cell-derived matrices (CDM). CDMs can be generated using a variety of culture methods that offer flexibility and tunability to the desired applications. When grown in monolayers, adherent cells deposit a thin layer of matrix molecules upon the underlying surface. For thicker CDM constructs, cells can be embedded in a degradable carrier scaffold. Over time, the degradable scaffold is replaced by ECM deposited by the embedded cells, which yields a three-dimensional ECM construct following decellularization. Culturing cells as multicellular aggregates also produces three-dimensional matrices, without the need for a carrier scaffold. However, individual aggregates tend to be much smaller than the thick constructs obtained when using a carrier scaffold. Similar to monolayer cultures, cells can also be cultured on the surface of synthetic scaffolds. The deposited matrix molecules remain on the scaffold surface following decellularization, and can improve the bioactivity and biocompatibility of the synthetic material.
120x74mm (300 x 300 DPI)