Review

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Extracellular matrix scaffolds for cartilage and bone regeneration

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Regenerative medicine approaches based on decellularized extracellular matrix (ECM) scaffolds and tissues are rapidly expanding. The rationale for using ECM as a natural biomaterial is the presence of bioactive molecules that drive tissue homeostasis and regeneration. Moreover, appropriately prepared ECM is biodegradable and does not elicit adverse immune responses. Successful clinical application of decellularized tissues has been reported in cardiovascular, gastrointestinal, and breast reconstructive surgery. At present, the use of ECM for osteochondral tissue engineering is attracting interest. Recent data underscore the great promise for future application of decellularized ECM for osteochondral repair. This review describes the rationale for using ECM-based approaches for different regenerative purposes and details the application of ECM for cartilage or osteochondral repair.

The need for improved repair of osteochondral defects

Joint injuries are common in the young and active population and often result in cartilage or osteochondral lesions. If untreated, these defects lead to joint swelling, pain, and serious restrictions in daily activities and can eventually progress towards osteoarthritis (OA), of which the only end-stage, salvaging therapy is artificial joint replacement. Over 151 million people suffer from OA worldwide [1], representing a huge clinical and socioeconomic burden. Established OA is notoriously difficult to treat, but prevention through successful treatment of cartilage lesions will significantly reduce this socioeconomic impact.

Natural wound healing in full-thickness cartilage defects leads to the formation of so-called fibrocartilage, which is functionally and biomechanically inferior to the original hyaline cartilage. This makes the tissue more prone to further deterioration, and thus initiates a vicious cycle.

Currently, many different cartilage repair-enhancing treatments are applied in patients with (osteo)chondral defects. These techniques are either based on cell therapy, such as autologous chondrocyte implantation (ACI) [2] and matrix-induced chondrocyte implantation (MACI) [3], on replacement of the damaged tissue within the joint, for example, by mosaicplasty [4] and osteochondral allografting (see Glossary) [5,6], or on the recruitment of mesenchymal stromal cells (MSCs) through, for example, microfracture [7]. All of these techniques provide fairly acceptable clinical results, but none results in restoration of fully functional hyaline cartilage, making long-term prognosis uncertain.

In an attempt to optimize the functional restoration of cartilage, tissue engineering has been suggested as a good basis for new regenerative therapies. The key to successful engineering of cartilage with optimal restoration of function lies in finding the optimal combination of biomaterials, biofactors, and cells [8]. Biomaterials currently used in the field of cartilage tissue engineering can be grossly divided into two groups: (i) natural biomaterials such as collagen [9], gelatin [10], and fibrin [11], and (ii) synthetic biomaterials such as polycaprolactone (PCL) [12], and polylactic acid (PLA) [13]. The synthetic materials often have good biomechanical strength and their specific properties can be tailored by changing the polymer composition. However, the major challenge for these materials, which are foreign to the body, is to achieve satisfactory tissue integration and differentiation. Natural biomaterials may overcome this challenge because they are biocompatible and biodegradable.

Despite the great advances that have been made in the field of material sciences in mimicking the natural tissue

Glossary

Allograft: graft obtained from a donor of the same species.
bFGF: basic fibroblast growth factor; involved in angiogenesis, wound healing, and embryonic development.
EGF: epidermal growth factor; stimulates proliferation and differentiation.
IGF: insulin-like growth factor; regulates cellular proliferation and apoptosis.
Mosaicplasty: surgical procedure during which a defect is filled with osteochondral plugs taken from a non-load-bearing region of the joint.
Osseous phase: bone compartment.
Proteoglycans: glycoproteins of high molecular weight present in connective tissue and cartilage.
TGFβ: transforming growth factor beta; regulates many cellular functions including proliferation, differentiation, and apoptosis.
VEGF: vascular endothelial growth factor; stimulates vasculogenesis and angiogenesis.

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environment to drive cell proliferation and differentiation, oversimplified biomaterials for (cartilage) tissue regeneration are still being used. In fact, all tissues in the body are composed of a complex mixture of different biomaterials and this situation is not different for cartilage, notwithstanding its seemingly homogeneous and straightforward appearance. In reality, the extracellular matrix (ECM) of cartilage is a structurally complex 3D environment composed of various types of collagens and proteoglycans in which multiple bioactive factors, such as growth factors, integrins, and functional peptides, are incorporated. Even highly sophisticated, newly developed biomaterials will probably never reach this complexity.

The abovementioned circumstances and considerations have driven the tissue engineering field towards increased use of biomaterials or scaffolds based on (processed) natural ECMs, an approach that might be a very valid option for cartilage repair as well.

ECM-based regenerative medicine

All tissues are composed of cells surrounded by ECM that consists of a unique and tissue-specific 3D environment of structural and functional molecules secreted by the resident cells [14]. There is reciprocal interaction between cells and ECM; cellular products, including proteinases, modify the ECM, and ECM-incorporated growth factors and cytokines act as functional cues, steering the metabolic and secretory activity of cells. This situation becomes even more complex because the intricate interplay of cells and ECM in a given microenvironment is not static but is rather a dynamic event that responds to external influences, such as biomechanical triggers and hormonal actions [15]. It is the eventual outcome of these dynamic processes that determines tissue homeostasis and possible aberrations thereof. Given the high complexity of these processes and the multiple roles of the ECM, constructs based on natural ECM sources are likely better prepared to produce a tissue with optimal functionality than those built from artificial compounds.

ECM-based tissue engineering strategies are already successfully being used clinically for the regeneration of a range of different tissues, including heart valves [16], trachea [17], muscle [18], tendon [19], and abdominal walls [20], with matrices derived from bladder and small intestinal submucosa [21] the most widely used implants. The main advantage of ECM as a scaffolding material is that it allows for so-called constructive remodeling [22], that is, it supports and encourages specific tissue formation at the implantation site rather than forming inferior and less functional scar tissue. However, the functional outcome of ECM-derived scaffolds depends on several factors, including retention of growth factors within the ECM, its surface topology, modulation of the immune response (Box 1), and the microenvironmental cues exerted on the cells, such as biomechanical loading (Box 2) [23].

The underlying mechanisms are still not fully understood, but several potential explanations are possible for the positive outcomes obtained with ECM-derived scaffolds. First, the process of dynamic reciprocity explained above [24], which is vital for proper functioning of tissues, is more likely to be effective in a natural tissue that contains bioactive cues, such as growth factors, polysaccharides, and functional peptides, than in an artificial

Box 1. The immune response to decellularized matrix

Several decellularized products for different regenerative purposes are available for clinical use. However, the amount of cellular material that remains after decellularization is variable [39]. There are no clear-cut guidelines for the degree of decellularization required, because cell remnants in devitalized tissue do not always hinder tissue regeneration [44,69].

The immune response that may occur in response to implantation of foreign cellular material is partly macrophage-mediated [39]. A macrophage response to implantation of a scaffold is a necessary event, because macrophages are involved in scaffold degradation. However, macrophages release several soluble factors on activation that can be both beneficial and detrimental to neotissue formation, depending on macrophage phenotype. Activation of M1 macrophages leads to adverse remodeling through the release of catabolic cytokines, whereas activation of M2 macrophages leads to constructive remodeling through anabolic cytokines [39]. For example, M1 macrophages release IL-1β and IL-6, which are upregulated in patients with damaged knee cartilage. The balance between M1 and M2 macrophages after implantation tends to shift to M2 macrophages if decellularization is more successful [39].

The avascular nature of cartilage is one of the major challenges in initiating intrinsic repair but may also be advantageous, because the tissue is immunopriviliged to a large extent, which opens up many more options in choosing the ECM source, including allogeneic and xenogeneic sources, without rejection issues [70]. In addition, the dense nature of cartilage ECM may further contribute to the weakly immunogenic, or even non-immunogenic, status, because it physically protects chondrocytes from T and NK cells that are released in graft rejection [70]. The application of xenogeneic products for cartilage repair is still in its infancy but should be explored further, because it overcomes the limited availability of human tissue or cells. The question remains, however, which tissue components may lead to an inappropriate immune response, the cells or the ECM.

Box 2. Biomechanical properties of decellularized matrix

The biomechanical characteristics of articular cartilage in terms of resilience and stiffness are crucial to proper functioning of the tissue in a strictly mechanical sense, but also with respect to tissue homeostasis, because biomechanical cues steer chondrocyte behavior to a large extent via mechanotransduction pathways [71]. In this context, biomechanical properties influence the growth factor reservoir within the ECM and matrix stiffness may, for instance, mediate TGFβ-driven processes through which this reservoir is continuously replenished and depleted [27].

The processes of harvesting, decellularization, and sterilization of ECM scaffolds affect the hydration status and 3D configuration and hence strongly influence biomechanical behavior. Washing steps using SDS or other processes that lead to removal of GAGs entail loss of water and produce a more loosely packed collagen network and hence loss of viscoelastic properties [22,72]. Freeze–thaw cycles may result in disruption of the collagen network through crystal formation.

The biomechanical behavior of ECM scaffolds *in vivo* will depend on the way the scaffold was processed, on the properties and geometry of the surrounding tissue, the pattern and magnitude of forces exerted on the scaffold, its degradation rate, and the extent to which new ECM is formed [73]. The biomechanical properties of any ECM-based scaffold will almost invariably be inferior to those of the original tissue. The extent and rate at which neotissue is formed and takes on more physiologic biomechanical characteristics depend mainly on the capacity of the scaffold (and/or the cells seeded therein, if any) to properly respond in an anabolic way to the cues elicited by joint loading and motion.

tissue that does not. Along the same line, incorporation of a certain cell type in a scaffold made from the target ECM will more easily drive the cell towards the appropriate terminal differentiation [25–27]. Second, naturally occurring ECM is the product of the resident cells and has a 3D structure that may guide cell behavior, attachment, and migration [28], but incorporated growth factors or other functional proteins are often also associated with alignment of the collagen fibers that mostly make up the 3D structure of a tissue and that give a tissue its biomechanical strength and resilience [29]. The biomechanical environment of the cell, which is largely dictated by the biomaterial, can have a great influence on cell differentiation. For example, MSCs commit to the osteogenic lineage in stiff biomaterials, but to the neuronal lineage in more flexible biomaterials [30].

The mechanism behind the successful use of ECM-based scaffolds seems to be generic to a certain extent and not exclusively tissue-specific, because ECM scaffolds originating from tissues other than the target tissue have been used with success. For example, small intestinal submucosa (SIS) ECM has been used as a scaffold for repair of the musculotendinous junction between the gastrocnemicus muscle and the Achilles tendon in dogs [31,32]. The scaffold was recellularized by progenitor cells from its surroundings and was ultimately completely replaced by functional contractile muscle and tendon, including one of the most challenging types of tissue to regenerate, the neurovascular bed [31]. ECM-based scaffolds can even be of xenogeneic origin [31,33–35] after successful decellularization to remove cellular antigens.

Decellularization of tissues can be accomplished using various methods or combinations thereof (Table 1). Physical treatments such as thermal shock, freeze–thaw cycles, and mechanical crushing of the tissue will lead to cell lysis and tissue breakdown, allowing for easier infiltration of the chemical and enzymatic treatments that often follow [24]. Treatments with detergents or other chemicals, including SDS and Triton X-100, are used to break down cellular and nuclear membranes [36], which can then be removed in subsequent washing steps. Enzymatic treatments depend on the tissue type, but often trypsin and nuclease solutions are used to break down peptides, DNA, and RNA [36].

Decellularization should ideally remove all cells and cellular antigens while retaining the bioactive cues that reside in the ECM. Decellularization of bladder submucosa matrix using several washing steps with enzymatic agents and detergents led to full decellularization but also ensured that important growth factors, such as VEGF, TGFβ1, bFGF, and EGF, typically remained present within the decellularized tissue [37]. In the case of cartilage, preservation of proteoglycans, one of the main ECM components, may be important. Proteoglycans not only contribute to the mechanical characteristics of the tissue through attraction of water by variations in fixed charge density [38] but are also thought to be a reservoir of several growth factors at times when these are not readily produced and released by the resident cells [37].

Both single tissues and whole organs can be decellularized, providing a biological scaffold of resident ECM with the complex geometry of an organ and an intact vascular network that will enhance nutrient supply, benefitting regeneration and recellularization [24]. In the case of organ decellularization, it is imperative that the process does not disrupt the natural integrity of the tissue; in the case of tissue decellularization, the process can be more rigorous.

Certain criteria have been proposed for successful decellularization, or perhaps better denuclearization: (i) the absence of nuclei on histological evaluation [hematoxylin–eosin or 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI)], (ii) DNA quantification <50 ng/mg dry tissue, and (iii) DNA fragments <200 bp [24]. However, these criteria were based on the decellularization of loosely organized tissues (SIS and urinary bladder matrix (UBM)) and may not apply to more dense tissues such as cartilage. Rigorous decellularization enhances loss of structural integrity of the ECM and of certain ECM compounds. However, whether absolute decellularization is necessary is still under discussion because ineffectively decellularized ECM still induced similar host remodeling to that induced by effectively decellularized material [39].

In addition to decellularization, artificial crosslinking of ECM scaffolds is often applied to enhance the biomechanical strength of the scaffold in the initial stages after implantation. However, this practice unequivocally affects ECM properties. Artificial, and more specifically, chemical crosslinking will ultimately decrease the degradation rates and thus the controlled release of bioactive factors [40]; chemical crosslinking may also physically hamper tissue remodeling because it elicits an adverse recipient immune response [41].

The successful application and encouraging results for *in vitro* and *in vivo* work using ECM scaffolds in several different fields hold great promise for this approach in attempts to regenerate (osteo)chondral tissue.

Application of ECM-based scaffolds to treat osteochondral defects

Treatments during which osteochondral plugs, taken either from a non-load-bearing region of the joint (mosaicplasty) or from a donor (allogeneic osteochondral grafting), are used to fill the defect can theoretically be considered ECM-based strategies because they imply the direct implantation of cartilage and bone matrix (Figure 1). However, the use of seeded or unseeded ECM-based scaffolds is a new and emerging approach within the field of cartilage tissue engineering, supported by a slowly increasing body of evidence of success.

One of the major advantages of using the ECM as a scaffolding material is its potential to retain the growth factors that the tissue is naturally inclined to respond to. For cartilage, some of the most important growth factors are TGFβ, FGF, and IGF [8]. The retention of bioactive molecules will be especially beneficial in regenerating cartilage, because this tissue naturally lacks a supply of appropriate growth factors and nutrients owing to its avascular nature.

Bioactive ECM for (osteo)chondral repair can be applied in many different ways that fall in three general categories (Figure 1). First, non-decellularized cartilage particles [42] combined with a degradable biomaterial led to initial clinical results that at least matched the outcomes for microfracture. Even ECM particles from osteoarthritic

Table 1. Possible decellularization techniques for (osteo)chondral repair

Decellularization method	Tissue type	Refs
Cartilage tissue		
1. Rinsing in PBS 2. Lyophilization 3. Tissue grinding 4. Trypsin treatment 5. Rinsing in PBS 6. Nuclease treatment 7. Hypotonic Tris–HCl treatment 8. Incubation in Triton X-100 9. Rinsing in PBS 10. Lyophilization 11. Crosslinking with UV 12. Sterilization by ethylene oxide	Bovine cartilage	[49]
1. Rinsing in PBS 2. Shattering of the tissue in PBS 3. Differential centrifugation 4. Incubation in Triton X-100 5. Hypotonic Tris–HCl treatment 6. Nuclease treatment 7. Rinsing in PBS 8. Tris–HCl treatment 9. Rinsing in PBS 10. Lyophilization 11. Dehydrothermal treatment 12. Crosslinking with carbodiimide 13. Rinsing in PBS 14. Sterilization by cobalt γ-irradiation	Human cartilage	[48]
1. Rinsing in distilled water 2. NaOH treatment 3. Rinsing step 4. Defatting in ethanol 5. GndHCl and NaOAc treatment 6. Rinsing step 7. H_2O_2 treatment 8. Rinsing step (0.9% NaCl)	Human nasal cartilage Porcine nasal cartilage Porcine meniscus	[35,47]
1. Rinsing in PBS 2. Freeze and thaw cycles 3. Hypotonic Tris–HCl treatment 4. SDS–EDTA treatment 5. Rinsing in PBS 6. Nuclease treatment 7. Rinsing in PBS 8. Peracetic acid treatment 9. Rinsing in PBS	Porcine cartilage	[72]
1. SDS treatment 2. Rinsing in water 3. Lyophilization	Cartilage ECM sheets of 10 μm	[52]
Bone Tissue		
1. Rinsing in demiwater 2. NaN_3 treatment 3. Chloroform and methanol treatment 4. Incubation in Triton X-100 5. SDS treatment 6. Rinse in PBS	Human cancellous bone	[57]
1. Defatting in acetone 2. Rinsing in saline 3. Trypsin treatment 4. Rinsing in saline 5. Rinsing in acetone 6. Crosslinking with hexamethyl diisocyanate 7. Rinsing in acetone 8. Rinsing in saline 9. Sterilization by γ-irradiation	Porcine trabecular bone	[56]
Cultured cell matrices		
1. Incubation in Triton X-100 with NH_4OH	Human MSC matrix	[27]
2. Rinsing in PBS 3. Rinsing in double distilled water 4. Freeze and thaw cycles 5. NH_4OH treatment 6. Rinsing in double distilled water 7. Na_3PO_4 treatment 8. Rinsing in double distilled water	Human MSC matrix, normal human articular chondrocyte matrix, and normal human dermal fibroblast matrix cultured on PLGA meshes	[51]
1. SDS with nuclease and EDTA treatment 2. Rinsing in PBS 3. Culturing for 4 weeks 4. SDS with nuclease and EDTA treatment 5. PBS rinsing	Immature bovine chondrocyte matrix cultured in agarose wells	[46]
1. Freeze and thaw cycles 2. Rinsing in PBS 3. Rinsing in double distilled water 4. Perfusion based washing in bioreactor	Human MSC bone matrix cultured on polyesterurethane	[58]
1. Freeze and thaw cycles 2. Rinsing in distilled water 3. Lyophilization	Human MSC bone matrix cultured on tissue plastic	[59]

patients can be used for this purpose [43]. A combination of OA cartilage particles that had undergone freeze–thaw cycles (devitalization) with MSCs in fibrin glue for implantation in subcutaneous pockets in mice led to better shape fidelity; glycosaminoglycan (GAG) content and chondrogenic gene expression were also enhanced compared to non-supplemented glue [43]. Cartilage tissue can also be processed into cartilage microparticles [44] that may be used as an additive to enhance current cell-centered techniques (ACI or MACI) by mixing it with the cell suspension or biomaterial that fills the defect. The addition of microparticles to pellet cultures leads to upregulation of chondrogenic gene profiles and moderately decreases hypertrophic gene expression [44].

Second, cartilage matrix can be harvested from allogeneic or even xenogeneic sources and then used in a scaffold form. Preclinical results underscore the benefits of devitalized or decellularized tissue over implantation of living cartilage, because the formation of neocartilage of the latter tends to lag behind [45]. Decellularized cartilage matrix can be obtained from different sources and through different decellularization processes. Owing to the dense nature of cartilage ECM in which the cells are embedded, more vigorous protocols are required to decellularize cartilage than for many other tissues. This inevitably leads to greater destruction of the ECM components; GAGs will be especially affected [46]. Moreover, cartilage thickness decreases and the tissue loses some of its biomechanical resilience [46]. The effect of GAG loss on the final concentrations of bioactive cues such as growth factors still needs to be evaluated for different decellularization protocols. Decellularized cartilage ECM can also be rebuilt into a

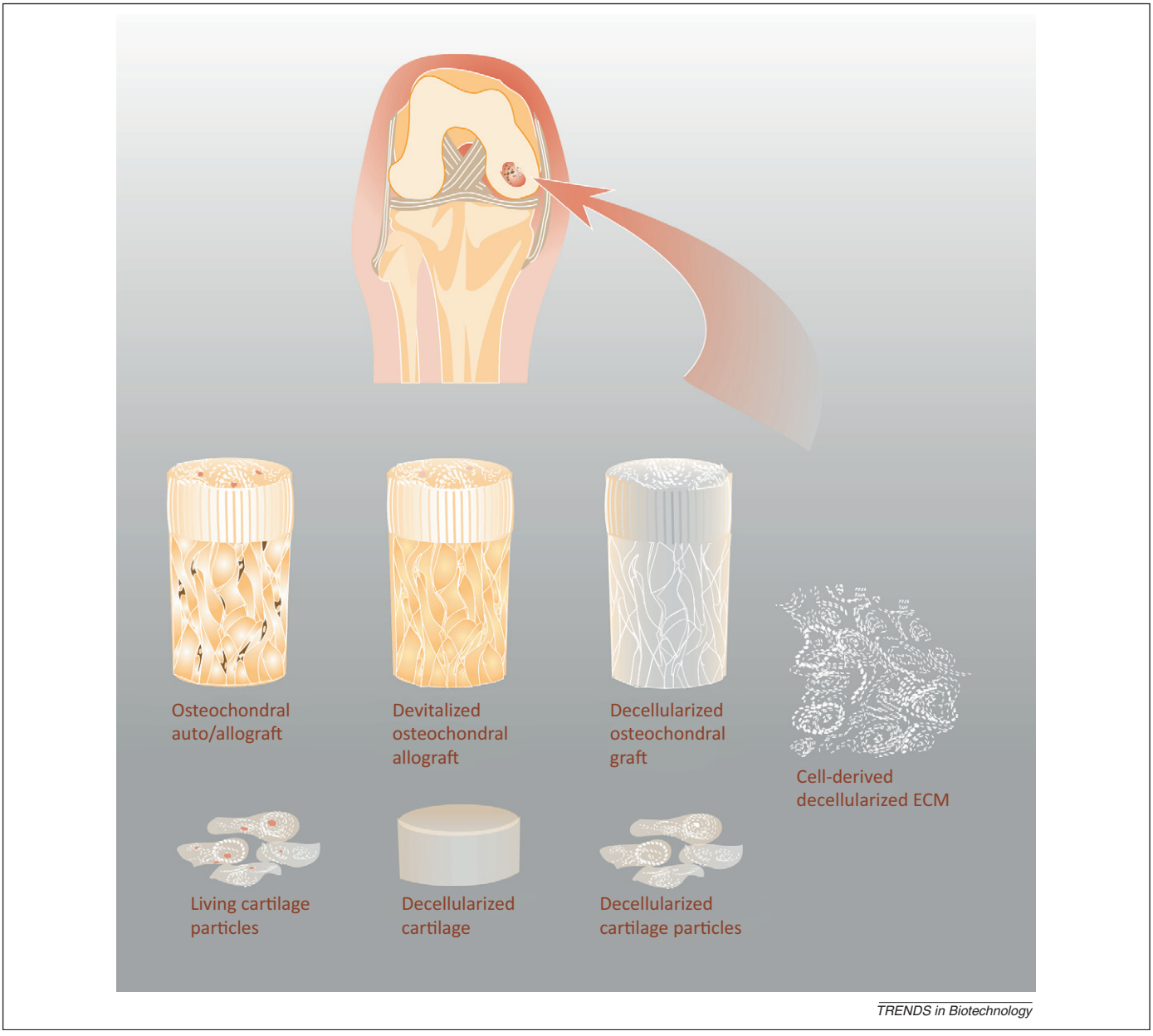

Figure 1. Various possibilities for matrix-based approaches to (osteo)chondral repair: osteochondral defects can be filled with fresh, devitalized, or decellularized osteochondral grafts, which can be from autologous or allogeneic origin. Defects can be treated with allogeneic living cartilage particles [42], a decellularized cartilage graft, or decellularized cartilage particles [45]. In addition, use of *in vitro* produced cell-derived decellularized matrix is also being actively explored [27,28,50,51].

scaffold through lyophilization [47–49]. In rabbits, this type of scaffold resulted in the regeneration of hyaline cartilage when combined with rabbit MSCs [49].

Finally, the ECM to produce a scaffold for cartilage repair can be harvested from cultured cells to create so-called cell-derived ECM scaffolds [27,28,50,51]. Cell-derived ECM overcomes the issues of possible exogenous pathogen transfer and allows ECM produced by the patient's own cells to be used. Moreover, different cell types can be mixed to create the appropriate ECM for more complex tissues, and use of thin ECM sheets allows much easier decellularization and recellularization [50]. ECM sheets seeded with MSCs or chondrocytes show superior chondrogenesis compared to pellet cultures [50,52]. The main challenge in using cell-derived ECM is finding a way to upscale the process in such a way that it can be clinically applied for human regenerative therapies. One way to accomplish this is to stack several different decellularized cartilage sheets to create a layered construct [52,53].

The process of decellularization paves the way for the use of xenogeneic material, the major advantages of which are cost-effectiveness and the relatively limitless availability of ECM. With a xenogeneic matrix, the age of the source animal should be taken into account. Young individuals heal better than adults and the tissues may be morphologically different. SIS ECM, for example, is thinner in older animals and has lost its elastic properties, as well as some proteoglycans and growth factors [23]. Therefore, use of tissue from younger donors may be advantageous [23]. For a tissue such as cartilage that is metabolically stable in mature individuals, the age up to which this is true is a relevant question. Products of non-enzymatic glycation

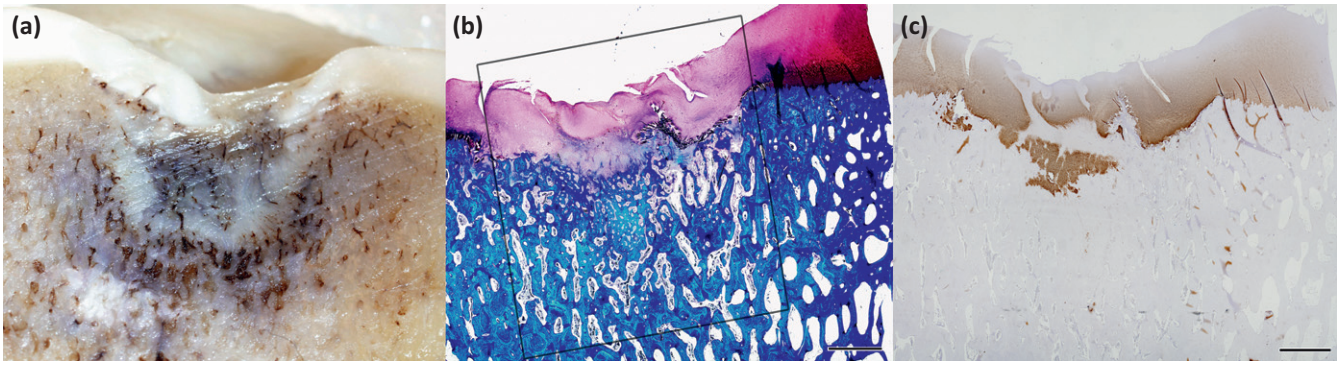

Figure 2. Osteochondral repair in a horse using decellularized cartilage. **(a)** Macroscopic overview of osteochondral repair tissue after 8 weeks of implantation. Both **(b)** glycosaminoglycan-rich (Safranin-O, Fast Green) and **(c)** collagen type II-rich neotissue was found after 8 weeks, with clear distinction between the cartilage and bone phase. Scale bars represent 2 mm; the box approximates the osteochondral defect created.

such as pentosidine crosslinks start to accumulate linearly in cartilage from approximately the age of 15 onwards [54]. This might be an indication of the cutoff age after which ECM from young individuals can be supposed to have acquired a mature metabolic rate. Xenogeneic use of cartilage has already been successful when implanting human cartilage-derived scaffolds seeded with canine MSCs in nude mice [48]. Cells showed good viability and the neocartilage contained both GAGs and collagen type II [48].

An important feature of the ultimate ECM scaffold is its biomechanical behavior. This is an especially challenging topic when considering the mechanical forces that are exerted daily on the cartilage and underlying bone in a human joint. Combining ECM with a stronger synthetic or ceramic material could potentially enhance the biomechanical properties of an ECM scaffold, an approach that may be especially attractive for the repair of osteochondral defects. Alternatively, a novel lyophilization method has been used to control the orientation of collagen fibers within a fabricated scaffold. This approach ultimately led to a Young's modulus that was almost three times higher than that in non-oriented scaffolds [55]. Moreover, the chondrocytes that were seeded on these scaffolds tended to align along these fibers, proliferated more rapidly, and produced similar amounts of GAG- and collagen-rich neotissue compared to scaffolds without collagen fiber alignment [55].

The repair of cartilage defects penetrating into the subchondral bone (osteochondral defects) poses additional challenges. First, bone regeneration should not extend beyond the osseous phase of the defect, so there may be a need for different biomaterials for the cartilaginous and osseous phases. Second, integration between cartilage and bone is challenging and depends on simultaneous maturation of both tissues, which is influenced by the biomaterials chosen for both tissue types. Similar to decellularized cartilage, decellularized bone promotes tissue growth on subcutaneous implantation, even outperforming the bioactivity of established biomaterials such as bioactive glass [56]. Attempts have been made to combine decellularized cartilage and decellularized bone to create biphasic constructs for osteochondral defect repair [57]. Preculture of a biphasic construct with MSCs for 4 days before implantation into an osteochondral defect in canine knees led to full regeneration after 6 months with near-hyaline cartilage repair [57]. In the case of bone regeneration, decellularized tissue-engineered ECM can also be used to enhance the biological interaction of synthetic or ceramic biomaterials with cells [58–60], and may even aid in the controlled release of incorporated and normally rapidly released growth factors such as bone morphogenetic protein 2 (BMP2) [59].

Current work by our group is focused on the use of decellularized equine cartilage matrix for osteochondral repair. We performed an equine pilot study in which an osteochondral defect of critical size (11 mm ∅ × 10 mm) was created in the stifle (knee) joint of a horse. This defect was treated using a decellularized cartilage matrix scaffold and clear regeneration of both the bone and cartilage phase was present after 8 weeks (Figure 2). The two tissues could clearly be distinguished and the integration between the two was satisfactory (Benders *et al.*, manuscript under review). This indicates that a biphasic construct might not be a biological necessity for osteochondral regeneration, but may only serve as a biomechanical stabilizer during the initial phases of tissue repair in a challenging environment such as the joint. An issue that needs attention is assessment of the possible long-term ossification of neocartilage tissue *in vivo* in long-term studies.

Future perspectives for ECM-based scaffolds for osteochondral repair

The use of decellularized ECM is gaining ground within the field of cartilage tissue engineering and may prove to be of great potential because it allows for multifactorial mimicry that has not yet been achieved by man-made biomaterials. The approach is still relatively underexplored and extensive research is required to understand the biologic responses to ECM scaffolds within the joint environment and to optimize the decellularization techniques and ultimately the final repair tissue. There are several issues that need to be addressed.

First, cartilage naturally consists of different zonal layers that exert different functions due to differences in matrix composition and chondrocyte phenotype [29,61,62]. The use of ECM sheets may offer a possibility to represent this natural microenvironment by stacking ECM sheets

produced by the different zonal cell types. Recreation of the zonal structure can be further stimulated through the use of bioprinted 3D porous constructs to deposit zone-specific matrices [63], combining hydrogels and strong synthetic polymers, so that the mechanical properties can be tailored [64]. The synthetic materials or hydrogels that are ideal for bioprinting are often suboptimal in stimulating cell differentiation [65] and could be functionalized using ECM particles to optimize cell responses to the biomaterial.

Second, current decellularization approaches have focused on decellularization of cartilage tissue [49,57] or ECM produced by either stem cells or chondrocytes [50,52,53]. However, the need to use a cartilage-specific matrix may be questioned, and more readily available tissues such as SIS and bladder matrices may have similar effects. For example, SIS ECM has been successfully used to regenerate other tissue types, such as cardiac and vaginal tissues [66,67]. The use of non-cartilage-specific matrix would have many advantages, because the scaffolds can be produced via standardized protocols that have already been established, the tissue is more easily accessible and available in larger volumes, and the use of SIS ECM, for example, has already been evaluated both *in vitro* and *in vivo* and is currently applied clinically.

Finally, repair of osteochondral defects remains a huge orthopedic challenge owing to the complex combination of cartilage and bone, which frequently leads to overgrowth of bone. Osteoinductive materials such as tricalcium phosphate (TCP) and biphasic calcium phosphate (BCP) are already available and successful in the regeneration of critical-size bone defects [68]. Therefore, it seems logical to create a biphasic construct of such a successful ceramic and combine it with bioactive decellularized cartilage, which on its own seems to drive tissue regeneration *in vivo*. The use of ECM scaffolds may even allow for a non-cell-laden approach to osteochondral repair because they can attract cells from the implant site that will then differentiate into the appropriate cell type and elicit endogenous repair. Eventually, this may lead to natural off-the-shelf products that can be applied for a wide range of cartilage and osteochondral defects.

Concluding remarks

ECM scaffolds have shown great promise within the field of tissue engineering and are now being developed specifically for cartilage repair. Decellularized ECM-based scaffolds may solve many problems associated with the matrix-based approaches currently used for the repair of cartilage or osteochondral defects, such as osteochondral allografting and mosaicplasty. This approach may lead to the development of the ideal cartilage or osteochondral scaffold, providing the injured site with the right bioactive cues that stimulate the regeneration of functional tissue that resembles the healthy situation.

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