

The rationale for using microscopic units of a donor matrix in cartilage defect repair

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Abstract The efficacy of existing articular cartilage defect repair strategies are limited. Native cartilage tissue forms via a series of exquisitely orchestrated morphogenic events spanning through gestation into early childhood. However, defect repair must be achieved in a non-ideal microenvironment over an accelerated time-frame compatible with the normal life of an adult patient. Scaffolds formed from decellularized tissues are commonly utilized to enable the rapid and accurate repair of tissues such as skin, bladder and heart valves. The intact extracellular matrix remaining following the decellularization of these relatively low-matrix-density tissues is able to rapidly and accurately guide host cell repopulation. By contrast, the extraordinary density of cartilage matrix limits both the initial decellularization of donor material as well as its subsequent repopulation. Repopulation of donor cartilage matrix is generally limited to the periphery, with repopulation of lacunae deeper within the matrix mass being highly inefficient. Herein, we review the relevant literature and discuss the trend toward the use of decellularized donor cartilage matrix of microscopic dimensions. We show that

2- μ m microparticles of donor matrix are rapidly integrate with articular chondrocytes, forming a robust cartilage-like composites with enhanced chondrogenic gene expression. Strategies for the clinical application of donor matrix microparticles in cartilage defect repair are discussed.

Keywords Cartilage · Tissue engineering · Chondrocytes · Extracellular matrix · Decellularized tissues · Autologous chondrocyte implantation · Matrix-induced autologous chondrocyte implantation

Introduction

Scaffolds derived from decellularized tissues have enabled advancements in tissue engineering that, in many cases, exceed achievements achieved using synthetic approaches. Much of the value of these scaffolds is associated with the fact that the composition and organization of the extracellular matrix (ECM) remain largely intact following the decellularization process. The remaining high-resolution chemical and physical architecture guides the accurate repopulation of the decellularized donor tissue with host cells. Successful tissue repair in the clinic, and even partial restoration of organ function in vitro, has motivated attempts to regenerate virtually all tissues of the body via these same strategies. Cartilage is an ECM-rich tissue that, in theory, should be ideally suited for regeneration via the decellularization approach. However, this very feature makes classical decellularization and repopulation strategies non-viable. Here, we briefly review the literature in this area and describe what we believe is an evolutionary route toward the use of microscopic units of donor cartilage matrix in articular cartilage tissue engineering.

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Use of ECM in tissue repair

ECM is a product secreted by the cells that populate a given tissue or organ (Badylak 2004). The functions of ECM are many and its precise role in specific tissues varies. However, in general, the ECM plays the role of nature's ultimate scaffold providing structural support, facilitating cell-cell communication, functioning as a reservoir for the controlled release of growth factors and being fully biocompatible and biodegradable. The complex architecture of the ECM is designed and manufactured by the resident cell population, which, in turn can accurately facilitate its own repopulation by new cell populations, thus playing a critical role in tissue regeneration. This is a complex and circular relationship that is initially driven by morphological processes staged during embryonic and fetal development. Replication of these morphological events, or the resulting tissue ECM architecture, remains beyond existing scientific technologies. Indeed, we can accurately say that even the full composition and organization of tissue-specific ECM remains unknown. For this reason, some of the most successful tissue engineering outcomes have so far utilized portions of decellularized tissues, rather than synthetic mimics. The clinical application of this concept is rapidly becoming routine, with decellularized donor tissue being utilized in skin repair, heart valve replacement, bladder repair/replacement, hernia repair and many other reconstructive procedures (Crapo et al. 2011). Experimentally, whole organ decellularization of a rat heart, followed by its semi-functional repopulation has been reported (Ott et al. 2008).

Cartilage is an ECM-rich tissue

The volume ratio of ECM in cartilage is in excess of 95%, making it a uniquely ECM-rich tissue. The primary ECM components are collagen and the highly negatively charged proteoglycan aggrecan, which account for 20–30% and 10% of cartilage mass (w/w), respectively (Han et al. 2011). Aggrecan, in conjunction with chondroitin sulfate glycosaminoglycan (CS-GAG), keratan sulfate glycosaminoglycan (KS-GAG) and hyaluronan (HA) attract water forming a super-hydrated gel that endows cartilage with its ability to absorb loads of up to 20 MPa and withstand compressive strains of 10–40%. A highly structured (anisotropic) network of collagen fibrils functions to provide this hydrogel shock absorber with tensile strength. As the stiffness of the resident chondrocytes is ~1000-fold less than the ECM, they essentially do not contribute directly to the bulk mechanical properties of the tissue. Further, the cartilage ECM fraction appears to be mechanically stable and largely unaffected by freeze-thaw cycles (Szarko et al. 2010), implying that this

tissue can realistically be expected to be mechanically functional post-decellularization.

Previous application of decellularized cartilage ECM in cartilage tissue engineering

A handful of studies are now available outlining the manufacture of scaffolds from either pieces or sheets of decellularized cartilage tissue (Gong et al. 2011; Peretti et al. 1998, 2000) and some patents even describe the decellularization of the entire articular surface (Chen et al. 2009). Pivotal contributions to the development of these concepts began in 1998 with Peretti et al. demonstrating that devitalized donor cartilage pieces could be effectively bonded together by chondrocytes seeded between the pieces (Peretti et al. 1998). Specifically, 1 mm (by 3×5 mm) thick slices of ovine donor cartilage were prepared via 5 cycles of freeze/thawing to destroy the resident chondrocytes. Three pieces of devitalized tissue were mixed with 10^6 ovine chondrocytes, with the layered structure initially being held together with fibrin glue. Bonding of the donor cartilage continued to improve *in vivo*, with a plateau at 28 days. At this time, the fibrin glue had been replaced by new cartilage tissue. Qualitative organization of the new tissue continued to improve until day 42. When the experiment was terminated some recolonization of the donor cartilage slices was observed, although only near the exposed surfaces rather than throughout the construct. One of the challenges frequently encountered in cartilage repair is failure of the graft or of the *de novo* cartilage to bond effectively with the adjacent native tissue. Thus, the observations made by this team, suggesting that the donor matrix and presumably native matrix can be joined in a continuous manner by chondrocyte populations seeded between the two surfaces, were particularly promising.

In the previous study (Peretti et al. 1998) the repopulation of donor matrix was limited, resulting in large regions devoid of functional cell populations. Typically, chondrocyte migration through dense cartilage matrix is poor and this is a feature partially responsible for the tissue's limited intrinsic repair capacity. Indeed, migration is so poor that in repair procedures such as mosaicplasty a border of "dead tissue" commonly occurs at the donor-host tissue interface, where the lacunae are simply never repopulated (Redman et al. 2005). Figure 1 shows the relative cell:matrix volume ratio in cartilage versus dermal tissues commonly utilized in acellular repair applications. The decellularized products are markedly different and consequently, so is their repopulation potential. The high matrix content in the cartilage tissue makes decellularization challenging and compromises subsequent repopulation (Kheir et al. 2011).

Given the limited repopulation potential of decellularized or devitalized cartilage donor tissue, more recent efforts that

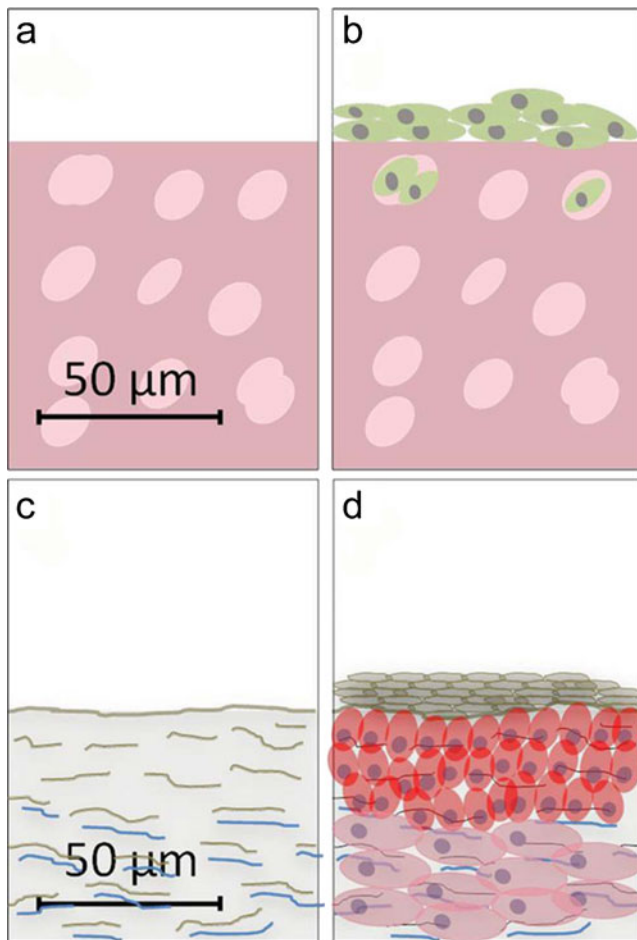


Fig. 1 Decellularized cartilage and dermis and their subsequent repopulation with host cells. **a** Decellularized cartilage with empty lacunae (pink) and dense surrounding matrix (purple). **b** Chondrocytes (green) attempting to repopulate decellularized cartilage, with minimal penetration into the donor matrix and repopulation of lacunae only occurring at the periphery (based on Kheir et al. 2011). **c** Decellularized dermis with sparse matrix and significant void space. **d** Repopulation of decellularized dermis with organized zones of fibroblasts (bottom, pink), keratinocytes (middle, red) and differentiated keratinocytes (top, brown; based on Nunez et al. 2009)

have focused on exploiting the donor matrix in tissue engineering applications have had the greatest success when the thickness of the donor tissue(s) were reduced. For example, in the study reported by Gong et al. (2011) donor tissue dimensions were reduced to 10–30 μm in thickness to enhance cell infiltration and repopulation of the matrix. Specifically, discs of porcine ear cartilage matrix were prepared by decellularization in sodium dodecyl sulfate followed by lyophilization. Suspensions of ovine chondrocyte populations, derived from the ears of new-born pigs, were seeded between the discs. This strategy enabled the authors to generate cartilage constructs, reported to have recovered 87% of the native (ear) cartilage tissue's mechanical properties, following a 12-week in vivo incubation subcutaneously in

nude mice. The mechanical properties of this porcine ear-based cartilage construct were only ~ 0.015 MPa following 4 weeks of in vitro culture and ~ 4 MPa following an additional 12 weeks of in vivo incubation. By contrast, mature articular cartilage, measured using the same methodology, has a Young's modulus of approximately 40 MPa (Yan et al. 2009). Whereas more rigorous mechanical evaluations could have provided a more precise mechanical characterization (Little et al. 2011), the presented relative comparison provide an appreciation of the tissue's potential. The fragile modulus in the final in vivo incubated tissues and particularly in the in vitro cultured tissues, suggest that such constructs are not suitable for use in the repair of articular cartilage defects. However, this study provides valuable insight into the utilization of decellularized cartilage tissue. Specifically, this study further demonstrated that the repopulation of the donor tissue was a limiting factor. In the thinner 10- μm sheets, $\sim 60\%$ of the donor tissue lacunae were repopulated, whereas less than 10% of donor tissue lacunae were repopulated in the thicker 30- μm sheets.

The obvious trend toward better outcomes with reduced donor tissue dimensions suggests that additional reductions in donor tissue dimensions should enhance de novo tissue quality. An important consideration in the development of cartilage repair technologies is whether the objective is to regenerate fully functional tissue in vitro for ultimate use in joint resurfacing, or to attempt to enhance more modest in situ repair procedures such as autologous chondrocyte implantation (ACI) or matrix-induced autologous chondrocyte implantation (MACI). In both the ACI and MACI procedures, a biopsy of cartilage tissue is harvested from a non-weight-bearing region of the joint in preliminary surgery (Haddo et al. 2004). Chondrocytes are isolated from this biopsy and expanded in culture for 3–5 weeks. In a second surgical intervention, the expanded cell population is transplanted into the defect site, either beneath a periosteal membrane (ACI) or on a collagen gel membrane (MACI). However, the initial repair tissue obtained following ACI or MACI procedures is essentially only a cell suspension or a population of cells on a collagen gel, respectively. Actual cartilage tissue is regenerated in situ by the transplanted cell population over a period of months to years (Reinold et al. 2006).

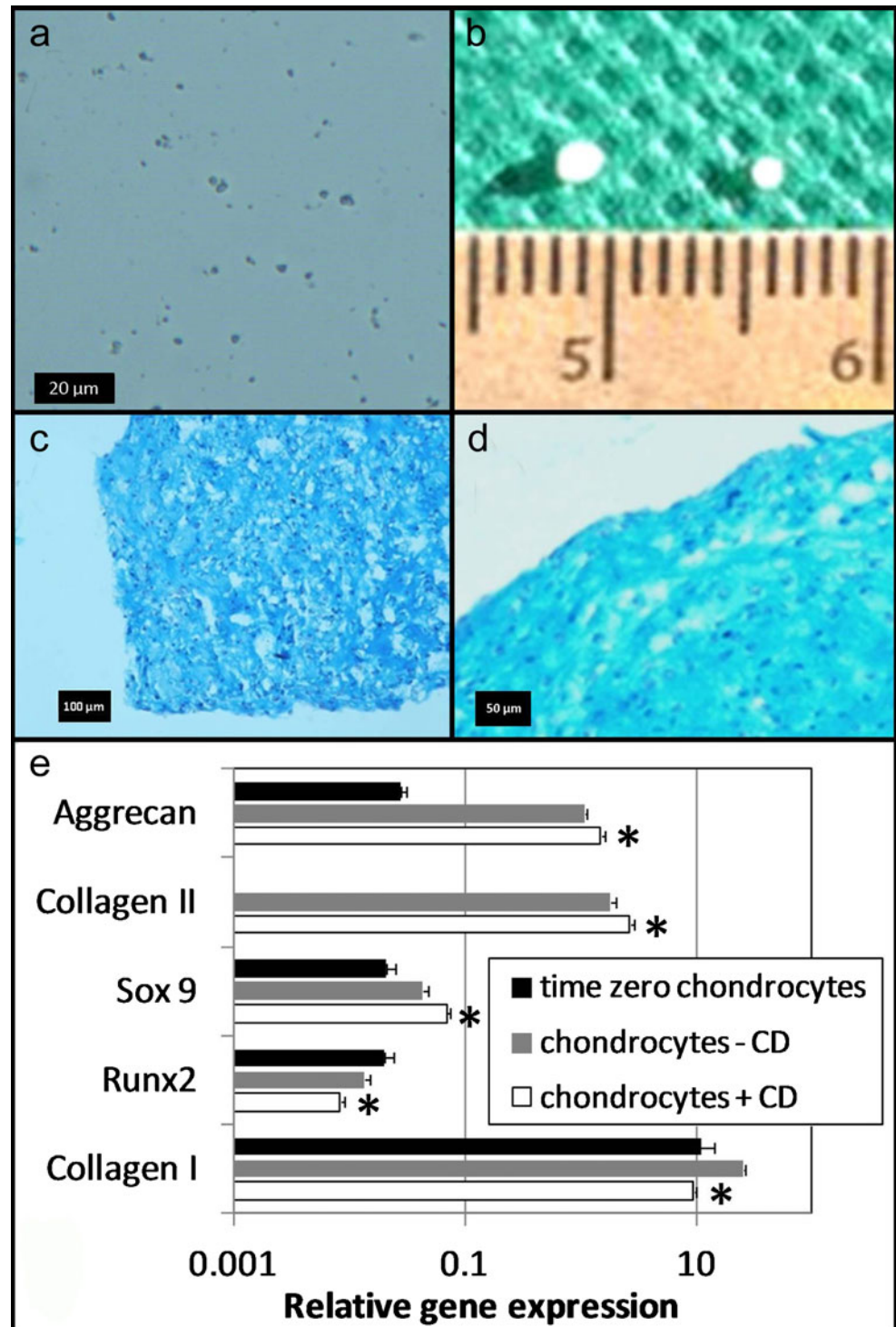
We would argue that, in the near-term, enhanced in situ repair strategies are more likely to be clinically achievable than strategies aiming to generate mature cartilage tissue in vitro for use in subsequent joint resurfacing. In such cases, in situ application of the donor matrix concept will probably require approaches in which the donor matrix and donor cells self-assemble into an organized tissue. More sophisticated manufacturing methods, like those reported previously (Gong et al. 2011; Peretti et al. 1998, 2000; Yang et al.

2008), will probably be more suited to in vitro cartilage construct assembly and cultivation.

Our team has recently undertaken in vitro and in vivo studies on the self-assembly of cartilage constructs generated from either mesenchymal stem/stromal cells (MSC) or articular chondrocytes in combination with lyophilized donor cartilage matrix that has been pulverized into ~2- μ m

microparticles (so-called cartilage dust; CD). Herein, we briefly describe a part of our in vitro studies demonstrating that CD is well integrated into cartilage constructs after only 14 days of in vitro culture and that the CD supplement results in larger tissues having enhanced chondrogenic gene expression. In these studies, 200,000 passage-2 articular chondrocytes were pelleted in 15-ml Falcon tubes (Becton

Fig. 2 Cartilage dust (CD) enhances the quality of cartilage constructs formed from articular chondrocytes. **a** CD particles were approximately 2 μ m in diameter. Bar 20 μ m. **b** CD-containing pellets (*left*) were approximately eight-fold greater in volume than cell-only pellets (*right*). **c, d** Alcian Blue GAG staining and hematoxylin nuclear staining (*dark blue*) of pellet cultures. **c** CD-containing pellet cultures. CD was evenly distributed within pellets. Bar 100 μ m. **d** Cell-only pellets. Bar 50 μ m. **e** Chondrogenic and osteogenic gene expression (\pm Standard deviation) in pellet cultures. CD-containing cultures had enhanced chondrogenic gene expression and reduced osteogenic gene expression. *Significant difference relative to the *chondrocytes-CD* group ($P<0.05$, $n=4$)



Dickenson) in medium composed of high-glucose Dulbecco's modified Eagle's medium (Gibco), 10 ng/ml recombinant human transforming growth factor- β 1 (TGF- β 1; Gibco), 10^{-7} M dexamethasone (Sigma), 200 μ M ascorbic acid 2-phosphate (Sigma), 100 μ g/ml sodium pyruvate (Sigma), 40 μ g/ml proline (Sigma), 1% insulin-transferin-selenium (ITS-X, Gibco) and 1% penicillin/streptomycin. Pellet cultures supplemented with 500 μ g CD were compared with controls without CD. All cultures were maintained under a 5% CO₂, 2% O₂ atmosphere at 37°C for 14 days. Medium was exchanged twice per week.

Figure 2a shows a phase contrast image of CD for size characterization. CD particles had an average diameter of \sim 2 μ m. We found that the CD particulate supplement added to each pellet culture retained approximately 50 μ g GAG after being lyophilized, pulverized and washed. The addition of the CD supplement resulted in an approximate two-fold increase in pellet diameter, corresponding to an approximate eight-fold increase in pellet volume relative to cell only controls (Fig. 2b). Figure 2c and d show Alcian Blue GAG staining and hematoxylin nuclear staining. CD-containing pellets appeared to have a greater GAG content and this qualitative assessment was supported by GAG quantification (data not shown). Critically, a reasonably uniform distribution of cells and CD could be seen and as the CD particle diameter was less than the diameter of a single cell, all donor tissue lacunae were exposed and could potentially be repopulated by the supplied chondrocyte population. Supplementation of pellet cultures with CD resulted in a significant enhancement of chondrogenic gene expression (Aggrecan, Collagen II and Sox 9) and even a moderate reduction in the expression of genes commonly associated with hypertrophy (Runx2 and Collagen I; Fig. 2e). This shift in gene expression is perhaps not surprising as similarly prepared cartilage matrix (Gong et al. 2011) has been previously reported to contain growth factors, including insulin-like growth factor (IGF) and TGF β , known to support cell expansion (Doucet et al. 2005) and chondrogenic differentiation (Johnstone et al. 1998).

We reason that the microscopic nature of the CD, and the observed self-assembly with MSC or chondrocytes, will make this strategy suitable for in situ repair strategies in which the repair components will probably need to be injected into defect sites (see Fig. 3). We envision that CD can be combined with expanded chondrocytes or MSC shortly before transplantation via the ACI or MACI protocol(s). The microscopic nature of CD should enable the self-assembled composite to mold to fit complex defect geometries, unlike prefabricated scaffolds. Importantly, the GAG fraction in the CD should enhance the mechanical properties of the initial repair tissue, relative to current ACI or MACI approaches, which initially lack any functional cartilage matrix. This alone could revolutionize existing therapies,

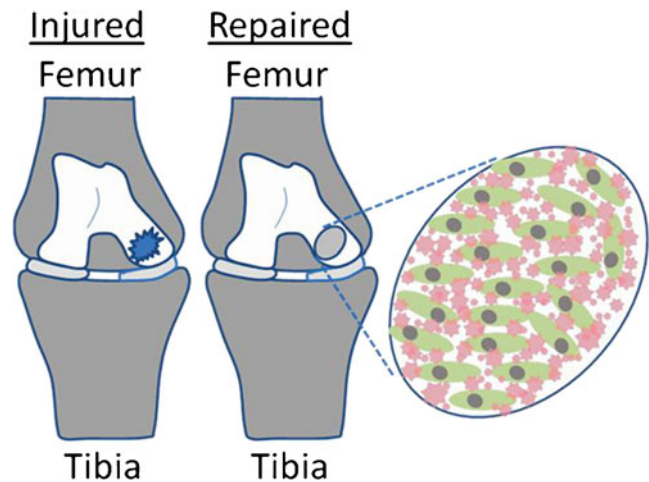


Fig. 3 Use of a slurry of CD (pink) and ex-vivo-expanded autologous chondrocytes (green) might enable a defect site to be filled, thereby generating a more stable initial repair tissue and ultimately achieving enhanced clinical outcomes (adapted from Redman et al. 2005)

as these strategies are plagued by the fragile nature of the initial repair tissue and its extraordinary sensitivity to unintended microtrauma (Van Assche et al. 2010).

The CD concept can probably be extrapolated to include the use of a cadaveric or xenogeneic matrix recovered from bovine or porcine cartilage, thus providing an essentially unlimited supply of donor matrix for human joint repair. Numerous reports are available describing the clinical use of other decellularized xenogeneic tissues (Badylak et al. 2011) and the preliminary development of protocols to eliminate immunogenic antigens from xenogeneic cartilage matrix (Elder et al. 2009). Whereas the processing of donor tissue should be possible such that pathogen transfer or immunological concerns are minimized, a risk exists that “over-processing” will reduce both CD matrix content and its ability to support enhanced chondrogenesis. Studies comparing donor matrix sources and processing methodologies will guide future clinical decisions.

Further innovations might enable CD to be used in conjunction with newer single-step repair strategies such as autologous matrix-induced chondrogenesis (AMIC; Benthien and Behrens 2011). In these procedures the surgeon penetrates the subchondral bone (microfracture) liberating bone marrow MSC into the defect site, which is then sealed with a MACI collagen membrane. No preliminary surgery or cell culture is required for the AMIC procedure, making this a potentially versatile and cost-effective alternative to ACI and MACI. The addition of CD could enhance the in situ chondrogenesis of the liberated MSC in the AMIC process and support the formation of a more robust initial repair tissue.

In conclusion, we hypothesize that just as the donor matrix has proven to be exceedingly valuable in the regeneration of other tissue types, donor cartilage matrix will enable

similar outcomes in articular cartilage repair providing that the latter matrix is supplied in the appropriate microscopic dimensions.

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