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Cartilage regeneration using zonal chondrocyte subpopulations: a promising approach or an overcomplicated strategy?

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Abstract

Cartilage defects heal imperfectly and osteoarthritic changes develop frequently as a result. Although the existence of specific behavior of chondrocytes derived from various depth-related zones *in vitro* has been known for over 20 years and the zones are known to differ in biochemical and biomechanical characteristics, only a relatively small body of *in vitro* work has been performed with zonal chondrocytes and current clinical treatment strategies do not reflect these native depth-dependent (zonal) differences. This is surprising, as it lies at hand that mimicking the zonal organization of articular cartilage in neo-tissue by the use of zonal chondrocyte subpopulations may enhance the functionality of the graft.

Although some research groups have made considerable progress in tailoring culture conditions using specific growth factors and biomechanical loading protocols, it is concluded that an optimal regime for the acquisition of zonal subpopulations has not been determined yet. Other yet unmet challenges include the lack of specific zonal cell sorting protocols and limited amounts of cells harvested per zone. As a result, the engineering of functional tissue has not yet been realized, and no long-term *in vivo* studies utilizing zonal chondrocytes have been described.

This paper critically reviews the research performed so far and outlines our view of the potential future significance of zonal chondrocyte populations in regenerative approaches for the treatment of cartilage defects. Secondly, we discuss briefly the capabilities of additive manufacturing technologies which can not only create patient-specific grafts directly from medical imaging data sets, but may also more accurately reproduce the complex 3D zonal extracellular matrix architecture by using techniques such as hydrogel-based cell printing.

1. Introduction

Cartilage impresses as a relatively simply structured tissue: it is aneural, avascular, and it contains few cells of supposedly only one cell type, the chondrocyte. Mature articular cartilage has limited healing capacity, and when a cartilage defect occurs, this eventually leads to the development of osteoarthritic changes (Prakash *et al.* 2002). Current clinically applied strategies include autologous chondrocyte implantation (ACI) (Peterson *et al.* 2002), microfracture (Lane *et al.* 2010), and mosaicplasty (Hangody *et al.* 2008). Although short-term results of these treatment modalities are satisfactory, all of these approaches have drawbacks. For example, long-term outcome is less favourable for microfracture (Saris *et al.* 2009) and mosaicplasty (Bentley *et al.* 2003). Moreover, the latter approach is also associated with donor site morbidity (Hangody *et al.* 2008). ACI on the other hand requires a two-stage procedure and comes with high expenses (Gerlier *et al.* 2010). Hence, notwithstanding reasonable clinical success, all current therapies for cartilage defects have major drawbacks and often result in the formation of a fibrous repair tissue rather than regeneration of hyaline cartilage with its specific layered organisation. This means that there is room for improvement of current clinical practice and use of cartilage constructs with a zonal organisation may be a viable alternative.

Despite its simple appearance, cartilage is in fact a heterogeneous tissue with a composition that varies greatly with depth (Figure 1). Articular cartilage can be divided into three zones: the superficial (the top 10–20% of the cartilage), middle (the next 40–60%), and deep (the bottom 30-40%) zone. The superficial zone (SZ) has the highest cell density, the lowest amount of glycosaminoglycans (GAGs) (Buckwalter *et al.* 1998), and the lowest biosynthetic activity (Wong *et al.* 1996). With increasing depth,

the cell density decreases and the amount of GAGs increases (Buckwalter *et al.* 1998), resulting in the highest amount of GAGs and the lowest cell density in the deep zone (DZ). With increasing GAG amount, the compressive modulus of the tissue also increases (Schinagl *et al.* 1997). The cells in the different zones differ in morphology and size. Cells in the SZ are small and flattened, while in the DZ cells are larger and round (Siczkowski *et al.* 1990). Further, the collagen fiber alignment differs between zones. The collagen fibers have an arcade-like structure (Benninghoff 1925). They originate from the calcified cartilage and continue perpendicular to the surface, through the DZ to make a transition in the middle zone (MZ) towards an orientation parallel to the surface in the superficial layer. The specific orientation of the collagen fibers, together with the proteoglycan aggregates that are interspersed between these fibrils, provide the tissue with its unique biomechanical characteristics, combining compressive stiffness, resilience and shear resistance. Additionally, zonal variations in the collagen network and proteoglycans result in vast differences in compressive, shear, and tensile properties with depth. These matrix biomechanical differences in-turn lead to significant variations in strains and stresses being experienced by the cells in different zones during joint loading.

Various proteins are preferentially secreted among zones: in the superficial zone, clusterin (Khan *et al.* 2001; Malda *et al.* 2010), proteoglycan-4 (PRG4) also known as superficial zone protein (SZP) (Flannery *et al.* 1999) and Del-1 (Pfister *et al.* 2001) are more prominent, while in the MZ the levels of cartilage intermediate layer protein (CILP) (Bernardo *et al.* 2011; Lorenzo *et al.* 1998) are highest. Cartilage oligomeric matrix protein (COMP) is mainly seen in the MZ and DZ (DiCesare *et al.* 1995; Murray

et al. 2001). These proteins most probably contribute to the zone-specific functionality of the cartilage. The exact specific functions of many of the zone-related proteins remain unclear, but some information about their function is known. Del-1, for example, acts as a regulator of vascularization (Penta *et al.* 1999), clusterin plays a role in cellular stress protection (Rosenberg *et al.* 1995), and COMP is a mediator in chondrocyte attachment and matrix assembly (Blumbach *et al.* 2009; Di Cesare *et al.* 1996). The presence of PRG4 in the SZ of cartilage ensures boundary lubrication and low-friction articulation (Jay *et al.* 1998), which has led to the hypothesis that transplanting or implanting cells or tissues that can secrete SZP may aid functional lubrication (Klein *et al.* 2003; Schmidt *et al.* 2004).

The heterogeneity of articular cartilage is not explicitly addressed in current therapies for cartilage defects. For this reason, under the hypothesis that replicating the zonal hierarchy will lead to better long-term function of cartilage constructs, attempts have been made to mimic the zonal architecture of the tissue using zonal chondrocyte subpopulations. It has become clear, however, that this approach is fraught with difficulties and that there are many obstacles on the road. While still going from the premise that the creation of zonal constructs leads to superior results, this has raised the question whether the approach using zonal chondrocyte subpopulations is not overcomplicated and whether possibly zonal characteristics could be obtained as well using less complicated approaches (*e.g.* through manipulation of the biochemical or biomechanical environment).

The goals of this discussion paper are to 1) give a critical appraisal of tissue engineering approaches that have used zonal chondrocytes to mimic native cartilage, 2) to present

our view of the zonal concept's role in optimizing constructs for cartilage regeneration, and 3) to suggest the most effective and reliable approaches to create zonal differences.

2. Current status of research

2.1 Isolation and culture of zonal chondrocytes

Experiments have been performed to elucidate the role of zonal cell populations in cartilage tissue formation *in vitro*. It has been demonstrated that cartilage tissue from the different zones can be harvested under sterile conditions. The zonal tissue can then be separated directly using dissection (Coates *et al.* 2011; Hwang *et al.* 2007; Sharma *et al.* 2007; Siczkowski *et al.* 1990; Waldman *et al.* 2003) or abrasion (Darling *et al.* 2005) techniques by using a surgical blade. Alternatively, (osteo)chondral cores can be obtained from which the cartilage is dissected using a microtome (Schmidt *et al.* 2004) or manually (Kim *et al.* 2003). SZ chondrocytes have also been isolated from mouse cartilage using sequential brief treatment with trypsin and collagenase (Yasuhara *et al.* 2011). Although these methods do not yield "pure" zonal cell populations, the enriched populations of zonal chondrocytes harvested this way show clear differences both directly after isolation and in expansion culture (Aydelotte *et al.* 1988; Kim *et al.* 2003; Klein *et al.* 2003; Ng *et al.* 2009; Schrobback *et al.* 2011; Schuurman *et al.* 2009; Sharma *et al.* 2007). It is important to note that in the research focusing on the (stratified) combination of zonal chondrocytes, almost exclusively unexpanded, articular cartilage chondrocytes from skeletally immature donors have been used thus far (Kim *et al.* 2003; Klein *et al.* 2003; Ng *et al.* 2009; Sharma *et al.* 2007) (Table 1). An important characteristic of immature chondrocyte subpopulations is their high metabolic activity and chondrogenic potential (Hidaka *et al.* 2006; Pestka *et al.* 2011), which makes them

well-suited for this type of research. However, it should be realized that immature DZ cells significantly differ from mature DZ zone cells, because the former originate from a zone that is vascularized and later becomes bone through the process of endochondral ossification (Karaplis 2008). It is known that already early in fetal life discrimination can be made between chondrocytes that will undergo the process of endochondral ossification and those destined to retain their chondrocyte phenotype in the mature articular cartilage layer (Lecocq *et al.* 2008). This makes the use of immature chondrocytes for this type of research highly questionable.

In this same line of argument, it was shown in a direct comparison that SZ cells have a more stable phenotype during growth than DZ cells do (Hidaka *et al.* 2006). As differences between zonal populations are more prominent in immature cells, immature cells should be solely seen as a source for models and caution should be taken when translating outcomes to the human situation because of the limited clinical relevance.

Under clinical circumstances, only a limited number of chondrocytes is available, and in order to yield clinically relevant numbers, expansion of cells is necessary, which entails dedifferentiation. The general signs of dedifferentiation in cultured chondrocytes are the expression and secretion of collagen type I instead of collagen type II, and a lower synthesis of GAGs (Benya *et al.* 1982). This process can be reversed by using growth factors (Jakob *et al.* 2001) and 3-dimensional culture methods (Benya *et al.* 1982; Martin *et al.* 1999; Stewart *et al.* 2000). Zonal populations will also undergo general dedifferentiation and thus lose their chondrogenic phenotype during expansion. Additionally, they will lose their specific zonal phenotype characteristics as well (Darling *et al.* 2005). Zonal cells will entirely or partly lose their size differences

(Siczkowski *et al.* 1990) and the expression and secretion of specific zonal proteins, such as PRG4 (Darling *et al.* 2005), clusterin (Malda *et al.* 2010), COMP (Schuurman *et al.* 2009), and CILP (Schrobback *et al.* 2012). Using the growth factors basic fibroblast growth factor (basic FGF) and transforming growth factor-β (TGF-β) during expansion and redifferentiation (Darling *et al.* 2005), or culturing under low oxygen conditions (Schrobback *et al.* 2011), chondrocytes can reacquire at least part of their specific zonal chondrogenic characteristics. Culturing cells in a 3-dimensional (3D) environment, either scaffold-free or in a hydrogel (Darling *et al.* 2005; Schuurman *et al.* 2009), favors redifferentiation, although even this does not completely reverse the dedifferentiation process (Darling *et al.* 2005). Moreover, it has been suggested that growth factor use for re-inducing specific features of SZ and DZ cells should be differential, since growth factors affect zonal cells differently (Darling *et al.* 2005).

Separately cultured chondrocytes from the DZ produce more GAGs than those from the SZ (Aydelotte *et al.* 1988; Cheng *et al.* 2007; Hwang *et al.* 2007; Lee *et al.* 1998; Schuurman *et al.* 2009; Siczkowski *et al.* 1990) and during redifferentiation zonal cells will start producing (part of) their specific proteins (Darling *et al.* 2005; Klein *et al.* 2003; Schuurman *et al.* 2009). Altogether, it is possible to harvest enriched subpopulations of zonal chondrocytes and at least partially re-induce the zonal phenotype of these cells using 3D culture, growth factors and low oxygen tension. This makes the use of zonal chondrocytes for the fabrication of stratified cartilage constructs in principle feasible, but there are several potential hurdles in the way.

An important challenge lies in isolating pure zonal chondrocyte populations. Whilst separation of zonal cartilage tissue is possible, as mentioned previously, there are no methods described to separate pure zonal chondrocyte subpopulations from a mixed population isolated from a cartilage biopsy. Chondrocyte subpopulation sorting using a fluorescence-activated cell sorter (FACS) based on cell surface molecules may be feasible, as SZ chondrocytes express greater levels of $\alpha_V\beta_3$ integrins {Woods, 1994 #959}, and DZ chondrocytes have higher levels of cell-surface keratan sulfate *in situ* {Zanetti, 1985 #299}. Alternatively, various candidate markers based on proteins present in the ECM of native cartilage have also been proposed to distinguish between zonal cells (Bernardo *et al.* 2011; DiCesare *et al.* 1995; Flannery *et al.* 1999; Khan *et al.* 2001; Malda *et al.* 2010; Pfister *et al.* 2001). However, though preferentially secreted in native cartilage zones, they are not specific for a single chondrocyte subpopulation. Additionally, sorting cells based on secreted molecules is problematic since both the secreted molecule must be retained in the cell and the antibody must be transported into the cell. Theoretically, zonal chondrocytes could instead be sorted based on their size using a fluorescence-activated cell sorter. Sorting cells based on volume may give better outcomes than cell size, since the cell volume scales with the cube of the diameter; the average difference of 32% between SZ and DZ in cell diameter would translate to a difference of 124% in volume (Hunziker *et al.* 2002). However, although the original method for electronic cell sorting was based on Coulter volume {Fulwyler, 1965 #2092}, modern cell sorters all focus on optical properties of cells, thus such separation of zonal chondrocytes has not been shown. Surrogate optical measures of volume, such as forward scatter width (FSC-W), side scatter area (SSC-A), or autofluorescence may be applicable, but need to be optimized for each cell type and cell sorter {Tzur, 2012

#2093}. Despite improvements in separating cell subpopulations based on volume, there remains a significant overlap in cell volume even between the most divergent chondrocyte populations. Therefore, size-based sorting is unlikely to result in pure populations from the different zones.

Another problem regarding the creation of zonal constructs based on the use of zone-associated chondrocyte populations is the current lack of a gold standard for the determination of the zonal phenotype of newly formed cartilage. The International Cartilage Repair Society histological grading scheme includes depth –dependent characteristics (Mainil-Varlet *et al.* 2010), however, this assessment and other assessments of tissue are only based on the resemblance of the neo-tissue to the native tissue with respect to GAG content, cell morphology, and a number of – not completely understood - native cartilage proteins. These observations raise the somewhat philosophical but very relevant question to what extent the division of articular cartilage in three different zones reflects reality or represents just the use of a simplified categorical scale to describe an underlying continuous phenotypic transition in cartilage characteristics from the surface to the calcified layer.

2.2 Mechanical loading to enhance zonal differences *in vitro*

Biomechanical loading experienced by native tissue *in vivo* influences ECM synthesis and remodeling, and is thus critical for the zonal structure of articular cartilage. It has previously been shown in young, growing animals, that mechanical loading directs the formation of "horizontal" topographical heterogeneity over the surface of a given joint and can affect the development of collagen orientation (Brama *et al.* 2001, 2002, 2009),

and that also in vertical direction, *i.e.* depth-related, changes in the distribution of extracellular matrix components is influenced by exercise (van Weeren *et al.* 2008). Experimental evidence from *in vitro* research on the effect of force direction on collagen alignment supports these *in vivo* data (Kock *et al.* 2011). Further numerical models predict that a combination of compression and shear stress holds promise to result in a physiological collagen structure (Khoshgoftar *et al.* 2011). It is therefore likely that mechanical stimulation will be necessary to truly recapitulate the zonal structure of cartilage in an engineered tissue.

Mechanical loading can clearly promote *in vitro* ECM formation, as reviewed in (Grad *et al.* 2011), and can also be used to specifically influence the behavior of zonal chondrocytes (Table 2). However, chondrocytes vary dramatically in their response to different loading types (compression, tension, shear, hydrostatic pressure), and specific loading parameters (frequency, amplitude, duration, duty cycle), and therefore selection of an appropriate loading regime is a complicated and challenging task, especially if multiple cell types and biomaterials are to be used in the construct. SZ and DZ bovine chondrocytes in agarose hydrogels responded differently to dynamic compression (0-15% compression, 0-3 Hz, 24 hrs/day); GAG was generally inhibited and protein synthesis improved for SZ at all frequencies, whereas GAG was increased at 1 Hz protein synthesis virtually unchanged for DZ at all frequencies {Lee, 1998 #100}. Nonetheless, these effects are not consistently reported over a range of studies using bovine cells of different ages or human chondrocytes, different construct specifications and various compression protocols. Surprisingly, when optimising dynamic compression loading parameters for SZ and DZ human OA chondrocytes in alginate

constructs, the same high strain protocol (0-50% compression, 1 Hz, 3 hrs/day) was best for both cell types {Jeon, 2012 #2094}. In this study, as well as others (Raizman *et al.* 2009), there seems to be a homogenization effect, i.e. loss of zonal differences, when dynamic compression is applied, as shown by increasing matrix synthesis by SZ and decreasing synthesis by DZ cells in response to compression. Dynamic tensile loading (0-10% tension, 1 Hz, 12 hrs/day) of zonal chondrocytes in fibrin gels also showed a leveling of matrix accumulation, through stimulation of SZ chondrocytes and maintenance of DZ chondrocyte levels{Vanderploeg, 2008 #1470}; additionally, MDZ bovine chondrocytes in alginate increased PRG4 expression in response to dynamic tension (0-9% tension, 0.5 Hz, 3 hrs/day) [Wong, Bone 2003] . This is undesirable from a biomimetic point of view when zonal chondrocytes are to be used; SZ and DZ cells in native cartilage have specific properties and should retain these in tissue-engineered cartilage.

Based on stresses experienced by chondrocytes *in vivo*, maintenance of the DZ phenotype might benefit from exposure to hydrostatic pressure, whereas the SZ phenotype might be promoted by shear loading, and more complex loading protocols may be needed to generate zonal differences within a construct. Indeed, a combination of compression and shear in a joint-simulator bioreactor resulted in increased expression of PRG4 by mixed chondrocytes {Grad, 2005 #712}, but only increased the PRG4 expression levels of DZ chondrocytes when separate subpopulations were used, and only those near the articulating surface were affected {Li, 2007 #2039}. This suggests that by applying specific loading conditions, one may be able to generate appropriate zonal differences in the constructs without the need for using multiple chondrocyte sub-

populations. A depth-dependent mechanical loading protocol, for instance as described by Kock *et al.* (Kock *et al.* 2011), may also be a good step forward in (re)creating the desired anisotropic structure and composition in tissue-engineered constructs.

2.3 Generation of organized constructs with zonal cell populations

Since zonal chondrocytes do not show self-organization *in vitro* (Hayes *et al.* 2007), zonal subpopulations in tissue constructs should be deposited in layers to mimic the native zonal stratification. A number of studies describe such approaches (Table 1). In such layered engineered constructs of chondrocyte subpopulations embedded in diacrylated poly(ethylene)-based materials (Kim *et al.* 2003; Sharma *et al.* 2007) or agarose hydrogels (Ng *et al.* 2009), the compartment with cells from the deep layer produces more GAGs (Kim *et al.* 2003; Sharma *et al.* 2007) and collagen (Sharma *et al.* 2007), and has higher shear and compressive strength (Sharma *et al.* 2007) than the compartment with cells from the superficial layer. Values for collagen, GAG and mechanical properties in these constructs are comparable to, or higher than, non-zonal engineered constructs consisting of full thickness chondrocytes (Ng *et al.* 2009; Sharma *et al.* 2007). Further, GAG content of the compartment with cells from the superficial layer is significantly lower when cultured in the absence of cells from the deeper layers (Ng *et al.* 2009). No differences were seen in COMP gene expression between compartments (Kim *et al.* 2003), but cultured SZ cells secrete PRG4, while DZ cells do not (Ng *et al.* 2009). In stratified scaffold-free constructs combining SZ and MZ cells, intermediate properties can be observed for thickness, GAG and collagen content, when compared to SZ and MZ only constructs. Further, in that type of construct zonal

proteoglycan PRG4 is mainly seen in parts containing SZ cells (Klein *et al.* 2003). In the only study assessing zonal chondrocytes constructs *in vivo*, scaffold-free implants in the patellofemoral groove of mini-pigs showed limited matrix production, and loss of zonal organization (Chawla *et al.* 2007).

In summary, stratification of zonal cells can lead to depth-dependent differences *in vitro*; however, the step towards *in vivo* zonal performance remains a challenge. Simple, anatomically relevant shapes with layered zonal chondrocytes have been fabricated using a molding method in a scaffold-free approach{Han, 2008 #1425}. State-of-the-art techniques such as magnetically guided 3D cell patterning and additive manufacturing (AM) may prove to be useful and versatile approaches for this application. Using the former, the manipulation of cells into multi-directional cell arrangements is possible, both *in vitro* and *in situ* (Grogan *et al.* 2012). By harnessing the capabilities of AM formerly also alluded to as Solid-Freeform Fabrication (SFF) or Rapid Prototyping (RP) living tissue of arbitrary 3D shapes can be created directly from computer-aided design (CAD) data (Fedorovich *et al.* 2012; Klein *et al.* 2009; Melchels *et al.* 2011; Schuurman *et al.* 2011) (Figure 2). Not only can patient-specific constructs be created directly from medical imaging data sets, but the complex 3D multi-tissue configurations of the native articular cartilage can be more accurately reproduced by using cell printing in an AM modus (Mechels et al 2012). Moreover, most articular cartilage defects have a very specific 3D shape and hence unique spatial distribution of the various chondrocyte phenotypes, which traditional cartilage tissue engineering techniques have great difficulty reproducing. Further, AM allows for the fabrication of constructs which have mechanical properties similar to native tissues (Cui *et al.* 2012; Schuurman *et al.* 2011).

An important feature is porosity of the constructs that can be controlled in great detail (Fedorovich *et al.* 2008), creating the opportunity to optimize diffusion of nutrients and waste products (Shipley *et al.* 2009), and thus to engineer large and hence clinically relevant constructs.

3. Future Perspectives

If zonal chondrocytes are to be used, apart from the previously mentioned issues, a number of general challenges need to be overcome in order to reach a clinical application. First, the challenge of obtaining sufficient cells needs to be addressed. Typically, only a limited amount of cells can be harvested from a patient, and these cells need to be expanded or combined with another cell source. Expansion of chondrocytes leads to dedifferentiation and loss of (zonal) phenotype. A promising approach is to combine primary chondrocytes with bone marrow-derived mesenchymal stromal cells (BMSCs). For full-thickness chondrocytes, this method leads to BMSC-induced chondrocyte proliferation (Acharya *et al.* 2012) and chondrocyte-enhanced chondrogenesis by BMSCs (Acharya *et al.* 2012; Mo *et al.* 2009; Wu *et al.* 2011). In this way, not only the quantity problem is solved, it may also bring one-stage surgery for cartilage defects a step closer to the bedside. Of course, for a zonal approach it will be necessary to investigate whether a co-culture of this kind also benefits zonal chondrocytes.

However, the arguments raised above urge open-minded consideration of the entire zonal concept. The idea that zonal neo-tissue or constructs reflecting the native structural depth-dependent differences may have advantages over homogeneous

material is valid and still stands. Yet, most of the attempts to generate zonal tissue have used cells from zonal subpopulations to achieve this, which approach is built on the premise that the cells are leading in determining zonal characteristics. This seems logical, as the cells produce the matrix and not *vice versa*. While this is true, another mechanistic option is that the cells are driven by the environmental (biomechanical and biochemical) conditions, which change with increasing depth. Load dissipation in cartilage is depth-dependent and non-linear (Koolstra *et al.* 2006); oxygen tension also changes rather dramatically with distance from the surface in an a-vascular tissue like cartilage (Zhou *et al.* 2004). In this concept, the cells are programmed according to their depth-dependent location and adapt their morphology and expression patterns driven by the biomechanical and biochemical cues that change with distance from the articular surface: they follow instead of lead. As the topographical heterogeneity in biochemical and structural extracellular matrix characteristics of articular cartilage is known to develop under the influence of biomechanical loading (Brama *et al.* 2002, 2009), such a mechanism is highly likely. If the micro-environment rather than the cells is leading in creating the zonal structure, one may wonder if strategies based on use of zonally harvested cells are not overcomplicated indeed, and potentially even inherently ineffective. An approach based on the use of a single cell source, combined with the right biochemical and/or biomechanical cues may be simpler and more effective. There are some indications that this may be the way to go. Use of specific different biomaterials in one construct, BMSCs can be directed towards differentiation into zonal chondrogenic cells (Nguyen *et al.* 2011a; Nguyen *et al.* 2011b), as an alternative for zonal chondrocytes. This method shows great promise, since it potentially could solve the problems of limited availability of zonal chondrocytes, donor site morbidity and

chondrocyte dedifferentiation. Another promising approach for tissue engineering, that might lead to the formation of zonal cell populations, is the combination of a structure with biomechanical characteristics similar to the natural situation (hence creating a similar force dissipation pattern) with cells that resemble juvenile cells (*e.g.* MSCs, but induced pluripotent stem cells (IPS) could be a viable option too)). The realization of such an approach seems feasible and it has been shown already that the combination of different biomaterials and smart scaffold design can affect the ECM deposition by influencing cell alignment (Wise *et al.* 2009). For the fabrication of such complex multiple-material structures AM techniques are useful (Fedorovich *et al.* 2012; Mironov *et al.* 2003; Schuurman *et al.* 2011). To further enhance the desired anisotropic tissue formation, a depth-dependent mechanical loading protocol could be employed. A mechanical loading regime can also be used to test whether a construct will be able to withstand the compressive loads and shear forces it will be subjected to *in vivo*. Possibly, zonal cartilage architecture could also be combined with pre-vascularized bone grafts to enhance vascularization and integration of the implant. When biomimetic and/or cell-instructive biomaterials would be used, specific cell types can be attracted and cell behavior such as proliferation and differentiation selectively promoted (Lutolf *et al.* 2005; Romano *et al.* 2012).

Such an environment-driven cell differentiation approach is from a translational perspective much less complicated and thus more likely to make it to the clinic than one involving use of various populations of zonal chondrocytes. However, the challenge of producing satisfactory cartilaginous tissue with a zonal composition *in vivo* is still great and may even be greater than it seems, since negative outcomes of attempts to do so are not likely to be published (Stern *et al.* 1997). We therefore urge research groups to also publish their negative results, avoiding unnecessary duplication of the work by others. In this way more promising approaches van be explored, thus accelerating the progress towards the ultimate goal of regeneration of fully functional cartilage.

We conclude from our literature review that the limited successes achieved so far in the restoration of the native tissue organization of articular cartilage, reflect the lack of proper understanding of the requirements for zonal cartilage. It is striking, that while the first reports about zonal differences in cartilage tissue date back more than 20 years ago (Archer *et al.* 1990; Aydelotte *et al.* 1988) and a substantial amount of *in vitro* work has been performed on full-thickness cartilage tissue engineering, only one short-term *in vivo* study relying on the zonal concept has been reported (Chawla *et al.* 2007). Hence, there is reason to believe that an overly simplistic conception of how the zonal characteristics of cartilage are generated and maintained *in vivo* has led to an overcomplicated strategy to achieve the same in artificial constructs. Alternatives for the use of zonal chondrocytes, like the use of mechanical loading regimes, instructive biomaterials and smart scaffold design that prompt cells to develop a zonal phenotype exist and should be further investigated. The use of BMSC's or IPS in this context seems especially promising. In our view, the regeneration of zonal cartilage remains an

utmost important aim for the cartilage tissue engineering community, but the way how to realize this needs careful reconsideration.

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Figures and tables

Table 1. Overview of stratified approaches using zonal chondrocyte subpopulations. SZ: superficial zone cells, MZ: middle zone cells, DZ: deep zone cells, PEGDA: poly(ethylene glycol) diacrylate, PEODA: poly(ethylene oxide) diacrylate, P0: primary cells, P1: cells expanded for 1 passage.

Cell types used	Material	Cell type	Key results	Reference
SZ, MZ, DZ	PEGDA	Immature, P0	Cell size and intensity safranin-O staining increasing with depth,	(Kim *et al.* 2003)
SZ, MZ	Scaffold-free (filter-culture)	Immature, P0	SZ/MZ constructs: matrix growth and compressive properties lie in between the values of SZ and MZ alone constructs	(Klein *et al.* 2003)
SZ, MZ	Scaffold-free (filter-culture)	Immature, P1	In vivo: stratification of cells lost	(Chawla *et al.* 2007)

SZ, DZ	PEODA	Immature, P0	Stratified constructs: lower proliferation and higher matrix synthesis DZ cells alone, and greater shear and compressive strength than SZ or DZ cells alone.	(Sharma *et al.* 2007)
SZ, MDZ	Agarose	Immature, P0	GAG, collagen and mechanical properties higher for zonal constructs than non-zonal constructs. Zonal constructs: depth-dependent mechanical properties	(Ng *et al.* 2009)

Table 2. Overview of mechanical loading protocols for zonal subpopulations (SZ: superficial zone cells, DZ: deep zone cells) and their effect on zonal behavior.

Loading type	**Culture type**	**Effect SZ**	**Effect DZ**	**Reference**
Cyclic compression	Agarose hydrogel	Decreased GAG synthesis, increased proliferation	Increased GAG synthesis	(Lee *et al.* 1998)
Cyclic compression	Cells seeded on calcium polyphosphate discs	Increased collagen and GAG synthesis	Decreased collagen and GAG synthesis	(Raizman *et al.* 2009)
Cyclic compression Tensile loading	Alginate hydrogel Fibrin hydrogel	Increased GAG synthesis	GAG synthesis unaffected, decreased collagen	{Jeon, 2012 #2094} (Vanderploeg *et al.* 2008)

Figure 1. Zonal organization in normal articular cartilage. A) Three-dimensional histology and schematic showing cell and collagen fibril organization in the superficial, middle and deep zones. Also depicted are changes in levels of oxygen, collagen cross-links (lysylpyridinoline (LP), hydroxylysylpyridinoline (HP)), and compressive modulus (H_{A0}) through the thickness of the tissue. B) Equilibrium partitioning of an ionic contrast agent (EPIC) micro-CT map of GAG distribution in human articular cartilage, comparable with C) Alcian Blue staining. D) PRG4 (Nugent-Derfus *et al.* 2007) appears to be suitable as a marker for the superficial zone, as do E) developmental endothelial locus-1 (del-1) (Pfister *et al.* 2001) and F) Notch 1 (Ustunel *et al.* 2008). G) Cartilage intermediate layer protein (CILP) (Lorenzo *et al.* 1998) is found in the interterritorial matrix of middle and deep zones, whereas H) Jagged 1 (Ustunel *et al.* 2008) is highly expressed in cells of the middle and deep zones. Reproduced and adapted from Klein *et al.* (Klein *et al.* 2009).

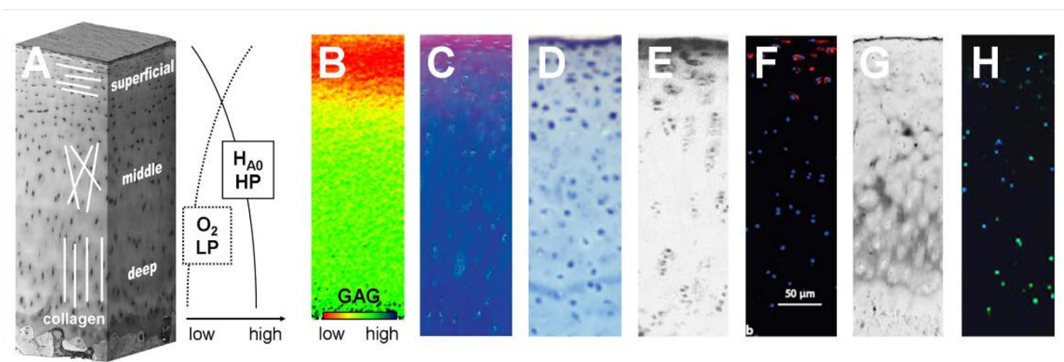

Figure 2. Possible combination of the use of additive manufacturing, smart materials, and (zonal) cells. A 3-dimensional (3D) design is loaded into the computer and converted to a 3D shape, using multiple cell types. Adapted from (Schuurman *et al.* 2011).