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The pericellular hyaluronan of articular chondrocytes

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Abstract

The story of hyaluronan in articular cartilage, pericellular hyaluronan in particular, essentially is also the story of aggrecan. Without properly tethered aggrecan, the load bearing function of cartilage is compromised. The anchorage of aggrecan to the cell surface only occurs due to the binding of aggrecan to hyaluronan—with hyaluronan tethered either to a hyaluronan synthase or by multivalent binding to CD44. In this review, details of hyaluronan synthesis are discussed including how HAS2 production of hyaluronan is necessary for normal chondrocyte development and matrix assembly, how an abundance or deficit of pericellular hyaluronan alters chondrocyte metabolism, and whether hyaluronan size matters or changes with aging or disease. The biomechanical role and matrix assembly function of hyaluronan in addition to the functions of hyaluronidases are discussed. The turnover of hyaluronan is considered including mechanisms by which its turnover, at least in part, is mediated by endocytosis by chondrocytes and regulated by aggrecan degradation. Differences between turnover and clearance of newly synthesized hyaluronan and aggrecan versus the half-life of hyaluronan remaining within the inter-territorial matrix of cartilage are discussed. The release of neutral pH-acting hyaluronidase activity remains one unanswered question concerning the loss of cartilage hyaluronan in osteoarthritis. Signaling events driven by changes in hyaluronan-chondrocyte interactions may involve a chaperone function of CD44 with other receptors/cofactors as well as the changes in hyaluronan production functioning as a metabolic rheostat.

Keywords

Hyaluronan; Cartilage; Chondrocytes; Hyaluronidase; Aggrecan

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1. Introduction

This review will focus on the role and regulation of hyaluronan (HA) in articular cartilage. Particular focus will also be given to the biology of HA closely associated with the surface of chondrocytes, namely pericellular HA. A recent review in *Matrix Biology* by Wilusz *et al.* (2014) entitled, *The structure and function of the pericellular matrix of articular cartilage* [1] is available that details the role of other extracellular matrix (ECM) macromolecules enriched within the chondrocyte pericellular matrix (PCM) such as type VI collagen and perlecan, how the PCM uniquely contributes to the mechanical properties of cartilage and the pericellular structure known as the chondron.

For the most part, the story of pericellular HA in articular cartilage is the story of aggrecan. Without aggrecan, the load bearing function of cartilage would be compromised. HA retains aggrecan within cartilage thus supporting this biomechanical function. Methods to restore aggrecan production and retention could combine for treatment of early stage osteoarthritis (OA). Although investigators have searched and speculated on the presence of aggrecan receptors, no contender for this function has been advanced. Thus, the anchorage or tethering of aggrecan to the cell surface only occurs due to the binding of aggrecan to HA and with HA tethered either to CD44 or a HA synthase.

In this review many questions concerning HA in cartilage, pericellular HA in particular, will be addressed. How is HA synthesized in cartilage and how is it degraded? What is the size of HA in cartilage, does size matter and does it change with aging or disease? What role does pericellular HA play during early chondrogenesis and later stages of cartilage organization? Does HA play primarily a biomechanical role, a role in cell signaling or both? Do changes in HA content or size affect signaling in chondrocytes and if so, is this receptor mediated? Is an abundance or deficit of pericellular HA good or bad for the physiology of chondrocytes? And lastly, what questions remain to be addressed concerning HA in cartilage? What will not be covered are results concerning the addition of pharmaceutical HA to chondrocytes, cartilage or synovial joints—a field often referred to as viscosupplementation.

2. How is HA synthesized in cartilage?

As in other cell types, chondrocyte-derived HA is synthesized by way of multimerized, multi-pass, transmembrane HA synthases (HAS) [2]. Early studies suggested that of the three mammalian genes available, *HAS2* is the predominant isoform involved in the synthesis of HA in bovine and human cartilage although all three isoforms may be present [3]. Phosphorothioate antisense oligonucleotides specific for *HAS2* blocked HA production in human chondrocytes [4]. Treatment of chondrocytes with IL-1α [5, 6] or BMP7 [7, 8] affected increases in *HAS2* mRNA with little to no effect on *HAS1* or *HAS3*. Moreover, given that no physical or developmental changes were observed in non-lethal $Has1^{-/-}$, $Has3^{-/-}$ or $Has1^{-/-}/Has3^{-/-}$ double knock-out (KO) mice, it is likely that the cartilage of these mice has little dependency on HAS1 or HAS3 for HA production [9, 10]. Conditional inactivation of *Has2* in early limb bud mesenchyme by introduction of the *Prx1-Cre* transgene results in skeletal deformities and severely shortened limbs due to abnormal and

disorganized growth plates and a decrease in aggrecan deposition [11]. In addition, increased cell density and a reduction in cell-cell spacing between chondrocytes was observed and associated with a decrease in the amount of ECM organized between chondrocytes. The organization of the ECM in the *Has2*-deficient growth plates suggests the HAS2 production of HA is necessary for the normal progression of chondrocyte maturation and more importantly, expansion of the lacunar space during hypertrophy as was observed in earlier studies [12]. Another study examined conditional inactivation of *Has2* that occurred later in cartilage development by introduction of the *Col2-Cre* transgene. This inactivation of *Has2* also resulted in limb shortening (45–50% shorter), disorganized growth plates and a paucity of cell spacing between chondrocytes [13]. However, overall aggrecan deposition within the inter-territorial matrix, was relatively unchanged in these conditional KO mice. This aggrecan retention could be due to residual HA synthesized at earlier developmental stages, contribution of *Has1* and *Has3* or, other mechanisms available to retain aggrecan within the ECM.

In more recent work, *Has2* deletion in two different rat chondrosarcoma cells lines, by way of a CRISPR/Cas9 gene editing approach, resulted in the total elimination of HA as measured by immunofluorescence (HABP staining) and HA-ELISA [14]. The *Has2* KO chondrocytes exhibit no capacity to retain aggrecan and cell-cell spacing was substantially reduced when the KO cells were grown as 3D pellet or alginate bead cultures, as compared to wildtype. The lack of HA and aggrecan assembly within the PCM did not affect the proliferative capacity of these *Has2* KO RCS cells and there was no apparent change in migratory capacity -- although both are not major features of chondrocytes and may only apply to transformed cells [15]. HA knockout animals or cells represent loss-of-function experiments; the straightforward conclusion that can be drawn from all of these data is that cell-associated HA, together with aggrecan within the PCM, are critically responsible for establishing cell-cell spacing. Both ECM secretion and stiffness, studied by atomic force microscopy, were correlated with spacing and flattening of chondrocytes during growth plate development [16] In reverse, the condensation phase of chondrogenesis is a period in development in which tight packing and reduced spacing between presumptive chondrocytes is required, so much so that the cells form gap junctions [17]. During this phase, *Has2* levels are decreased in the limb bud regions where condensation is set to occur [18]. The overexpression of *Has2* (Adeno-*Has2*) in stage 22–24 embryonic chick wing bud mesenchymal cells blocked the condensation event in micromass culture and subsequent chondrogenesis [18]. *Has2* expression and HA are both elevated in the peripheral and distal subridge mesodermal cells where enhanced cell-cell spacing prevents unwanted chondrogenesis. In summary, HAS2 is largely responsible for the synthesis of HA in cartilage, HA in cartilage is responsible for the retention of aggrecan, particularly aggrecan retention within the PCM and, PCM aggrecan and HA contribute to the establishment and maintenance of cell-cell spacing distances between chondrocytes.

2.1. The HA-aggrecan dependent PCM glycocalyx

As noted above, the story of cartilage HA is really the story of the proteoglycan aggrecan. Why do we say this? The role of HA in regulating cell spacing is a good example. When chondrocytes are examined *in vitro* in monolayer culture, they exhibit a pericellular

glycocalyx (better known as a coat) that can only be visualized on live cells through the use of a particle exclusion assay [19]. Via this method, when small particles are applied (typically fixed horse red blood cells), a pericellular zone is visualized surrounding chondrocytes that repels the particles. This pericellular zone is stable to extensive washing and centrifugation of chondrocytes (such as following their release from alginate beads [20]) but, can be dissolved within minutes following treatment with *Streptomyces* hyaluronidase suggesting that these chondrocyte gel-like coats are dependent on HA as a scaffold. As such, the HA-dependent coats observed *in vitro* provide a model to explain the mechanism as to how chondrocytes generate proper cell-cell spacing during late chondrogenesis (a common deficit observed in *Has2* knockdowns noted above) and lacunar expansion during hypertrophy. After the condensation event in the core of the limb, newly differentiated chondrocytes begin to separate from each other. It is precisely at this stage that cells re-express a pericellular coat—coats that are not present on earlier condensing cells (stage 22–24) [21].

But, is aggrecan required (or just HA alone) for pericellular coats to form? Earlier studies [22] demonstrated that ^{3}H-labeled HA could be bound to hyaluronidase-treated chondrocytes in a dose-dependent, saturating, displaceable fashion indicative of HA binding to a cell surface receptor—a receptor later identified as CD44 [23–25]. Although saturating levels of HA were present on these chondrocytes, this HA was insufficient for the establishment of a pericellular coat. The addition of purified aggrecan monomer alone was insufficient. It was only when HA and aggrecan were added together that a full re-establishment of a chondrocyte coat was achieved [19]. Moreover, exogenous, purified HA and aggrecan could be added to any cell type that was CD44^{+}, including CD44-transfected COS-7 cells, and in doing so, chondrocyte-like coats could be assembled [25–27]. These results suggested early on that HA was required for the retention of aggrecan, aggrecan was required for full coat formation and that CD44, a single-pass, transmembrane glycoprotein, could function to anchor HA-aggrecan aggregates. This concept includes the extension of HA from a random coil mushroom configuration at the cell surface to an extended filament by hyaladherin decoration [28–31]. Isolation of chick chondrocytes from embryonic tibia tarsae with purified collagenase P in 20% serum, allowed for the isolation of *freshly-freed* chondrocytes with HA-dependent coats remaining intact [20, 29] suggesting that the coats seen *in vitro* represent a structure within cartilage.

As shown in Figure 1, after 2 days in culture, bovine articular chondrocytes readily assemble pericellular coats, some with a radius equivalent to the cell diameter (Fig. 1A, D). However, the addition of purified aggrecan (bovine articular cartilage D1 fraction) to cells with an existing coat can expand this PCM to an even greater extent (Fig. 1B, E) [14, 30, 32]. Within 1–3 hours the exogenous proteoglycan assembles onto the native, endogenous HA, likely increasing the pericellular hydration and expanding the coat. No expansion by added aggrecan is observed when cells are pretreated with *Streptomyces* hyaluronidase (Fig. 1H) and pre-established coats could be dissociated via post-treatment with the hyaluronidase (Fig. 1C, F, G). The re-establishment of coats on *Has2*$^{-/-}$ rat chondrosarcoma cells required both rescue with Adeno-*Has2* and the addition of purified aggrecan even though more-than-sufficient levels of HA were being synthesized by the transgene HAS2 protein [14]. This suggests that pericellular HA, at least surrounding cells *in vitro*, is not saturated with

aggrecan. This coat expansion likely occurs on all cells exhibiting HA regardless of whether the HA is anchored via an HA synthase or to a receptor such as CD44. It has been suggested that chondrocyte microvilli and, extracellular vesicles derived from these extensions, may also contribute to HA deposition [33]. Moreover, additional macromolecules likely contribute to the assembly and maintenance of coats *in vivo*; other proteoglycans (e.g., versican [31]), link protein, IαI, TSG6, etc. [34] but, whatever the structure of the matrix array, HA serves as the underlying scaffolding filament.

2.2 HA is continually synthesized by chondrocytes and in cartilage

In classic studies using intact bovine articular cartilage explants, HA and proteoglycan exhibited continuous biosynthesis between 8 and 23 days of culture as measured by ^{3}H-glucosamine and ^{35}S-sulfate incorporation. HA represented ~1% of the total radiolabeled, newly-synthesized glycosaminoglycan; a value similar to HA percentage (1.4–1.8%) within the overall, unlabeled glycosaminoglycan measured by μg uronic acid/mg hydroxyproline [35]. Using a different approach, Maroudas *et al.* [36] examined aspartic acid racemization of the human aggrecan monomer core protein (A1D1 fraction) in fresh extracts of human articular cartilages of differing ages. Increases in the percentage D-aspartate amino acids are indicative of long-lived accumulation of a particular protein. The D/L$_{ASP}$ ratio of the aggrecan monomer did not change between age 20 and age 80 suggesting that the proteoglycan must be continuously synthesized throughout life. And, given that the percentage of HA to proteoglycan (total uronic acid) creeps upwards slowly (from 1–2% to 5–10% of the total glycosaminoglycan) over a period of 8 decades, this implies that HA is continuously synthesized as well. If HA and aggrecan biosynthesis are continuous, this would imply that HA and aggrecan must also undergo continual turnover. Subsequent, pulse-chase data on radiolabeled cartilage explants resulted in two important observations. First, the rate of aggrecan and HA turnover (half-life ~13–25 days) was strikingly similar and secondly, only 9% of the ^{3}H-label due to HA that decayed in the cartilage could be recovered in the culture media [35, 37]. Even when catabolism was enhanced by use of serum-free conditions, the turnover half-life of HA and of aggrecan were similar. This led to the early suggestions (in 1988) that in alymphatic articular cartilage, HA turnover was at least in some part, mediated by endocytosis by chondrocytes.

3. HA turnover in cartilage

HA degradation in cartilage has been confusing and controversial and several aspects remain unknown. In some studies, HA turnover occurs in close proportion to the turnover of aggrecan [6, 38–43] whereas in others, HA turnover is greater than [43, 44] or less than [36] the turnover of aggrecan. In some cases, large amounts of partially-degraded HA are released from cartilage [39–41] and others, as noted above, only a small fraction is released [35, 37]. How can we make sense of these patterns? One approach is illustrated in Figure 2. It is helpful to consider which pool of HA (and aggrecan) is being considered namely, HA within the PCM or HA within the inter-territorial ECM and secondly, whether we are discussing HA turnover in steady-state or turnover associated with altered cartilage metabolism (aging, OA, disuse, trauma). As shown, there are likely regions of overlap with overlapping mechanisms as well as circumstances in which there is no overlap.

3.1. HA turnover within the PCM

Newly-synthesized HA exhibits turnover with a half-life in the range of weeks. HA is cleared from cartilage at a rate closely timed to that of newly-synthesized proteoglycan (aggrecan) and little of the labeled HA can be recovered in the fraction released from the tissues [35, 45]. This HA turnover most likely occurs within the PCM and involves chondrocyte-mediated endocytosis. What other evidence suggests that chondrocyte-mediated endocytosis of HA occurs in cartilage? Large pericellular and intracellular accumulation of HA is observed in *Hyal1*$^{-/-}$ cartilage chondrocytes. HYAL1 is primarily an intracellular, low pH, lysosomal hyaluronidase. Recent studies using a conditional (Col2a-Cre) *Hyal2*$^{-/-}$ reported increases in HA within the PCM, increased overall HA and increased HA size [46]. HYAL2 is considered a cell surface [47], endosomal [48] and lysosomal hyaluronidase [49, 50]. In fact, many of the hyaluronidases and receptors that have been considered as critical participants in HA turnover including: HYAL1, HYAL2, HYAL3 [46, 51–54], CD44 [23, 24, 55–57], PH-20 [58], KIAA [59] and TMEM2 [60] all are associated with the chondrocytes and PCM. Lastly, Fosang *et al.* using confocal microscopy, observed the accumulation of intracellular G1-ITEGE *in situ* within cartilage chondrocytes of porcine articular cartilage [61]. G1-ITEGE and G1-DIPEN neoepitope domains represent the N-terminal G1-domain fragment of aggrecan that remains bound to HA following cleavage of the proteoglycan by an aggrecanase (ADAMTS4, ADAMTS5) or MMP (such as MMP-13). As such, HA-bound G1 fragments are often used as a surrogate for HA. We demonstrated that chondrocytes bind and internalize HA that was decorated with purified G1-ITEGE or G1-DIPEN via a mechanism dependent on CD44, with G1 and HA both delivered to low pH organelles [56, 57]. And we observed the accumulation of G1-VTEGE within intact cartilage of *Cd44*$^{-/-}$mice suggesting that local CD44-mediated endocytosis does represent a mechanism for the clearance of G1 domains after aggrecanolysis [57].

3.2. Chondrocyte-mediated endocytosis of HA

Like other cell types [50, 62–67], chondrocytes exhibit the capacity to bind and internalize HA via a mechanism dependent on CD44 [23, 24, 55, 68]. Chondrocyte CD44 is upregulated by cytokines (such as IL-1α or IL-1β) that promote a pro-catabolic phenotype including an increased (2.3-fold) accumulation of intracellular HA [6, 24]. Moreover, chondrocytes readily bind and co-internalize HA pre-decorated with a biotinylated HABP complex (aggrecan G1 domain and link protein) [55] or purified G1-ITEGE or G1-DIPEN [56]. G1-domains (including G1-ITEGE) generated by the cleavage of endogenous aggrecan of bovine articular chondrocytes treated with IL-1α or IL-1β are also internalized, an event that can blocked by transfection of a CD44-dominant negative construct (CD44Δ67 [69]) [56, 57] or CD44 siRNA [57]. Moreover, whereas IL-1β treated WT BALB/c mouse chondrocytes exhibit internalized G1-VTEGE domains, no internalization of G1-VTEGE occurred in chondrocytes or fibroblasts derived from *Cd44*$^{-/-}$ mice [57]. In sum, chondrocytes internalize HA as well as the bound remnant aggregate protein domains. This does not rule out other mechanisms for chondrocyte-mediated endocytosis of HA including the potential role of other HA receptors (LYVE-1 [70], HARE [71], PH-20 [58], HYAL2 [47, 72]) or, the possibility of phagocytosis of HAS-bound HA.

It has been proposed that HA is first cleaved extracellularly (but within the PCM), before it undergoes endocytosis [73]. Likely candidates to participate in this cleavage include HYAL2. However, in early studies with bovine articular chondrocytes supplied with high molecular mass ^{3}H-labeled HA, ~50% the internalized pool of ^{3}H-HA was made up of HA that still migrated in the void volume of a CL-2B column [23]. This not preclude mechanisms wherein HA is partially depolymerized before endocytosis but does suggest that, pre-degradation is not a requirement. However, HA size does matter. Smaller-sized lengths of HA are more efficiently internalized by cells [74]. In a recent study, sonication of fluorescein-labeled HA (Fl-HA), at 4 °C enhanced the accumulation of Fl-HA internalized within a fixed time period [32]. This enhanced accumulation was proportional to the sonication time—a process that generated progressively shorter HA chains but not small oligosaccharides. The internalized, sonicated HA chains were in the size range of 180–350 kDa.

The reverse issue concerning size also appears to be true. High molecular mass Fl-HA (~1200–1800 kDa), decorated with intact aggrecan monomer, binds to the cell surface of chondrocytes but, fails to become internalized [32, 56]. This observation provided for a hypothetical mechanism to explain the close coordination in turnover rate between newly synthesized HA and aggrecan. That is, cleavage of aggrecan is a prerequisite first step required before HA is of the sufficient size at which endocytosis is permissible. To test this hypothesis, a limited, C-terminal proteolysis of purified aggrecan monomer was used to generate a library of aggrecan monomers of progressively reduced size. Since this proteolysis proceeded from the C- to the N-terminus of intact aggrecan, all of the monomers still formed aggregates with HA. The capacity of aggrecan to restrict Fl-HA endocytosis was progressively diminished as the size of the monomer was reduced [32]. Thus, this observation suggests that the rate of local HA endocytosis in chondrocytes is governed primarily by the availability and activity of an aggrecanase or MMP within the PCM as previously suggested [75, 76]. As aggrecan becomes sufficiently degraded, a threshold size is reached which is permissive for HA internalization. It is also likely that additional depolymerization by extracellular hyaluronidases would further promote internalization. The restriction of HA (as part of an aggrecan aggregate) to internalization may also apply to other receptors in addition to CD44.

3.3. HA turnover within the inter-territorial ECM

The best work on total HA within human cartilage and how this changes with age is still the results of Holmes, Bayliss and Muir [77]. They analyzed patient knee cartilage (femoral condyles) derived from amputation or sarcoma patients, what would thus be considered normal cartilage. They demonstrated that the percentage of HA in the ECM increases with age ranging from 1–2% to 5–10% of the total uronic acid content over a period of 8 decades (ranging from 1.0 to 2.0% uronic acid/mg hydroxyproline). Interestingly, the overall total uronic acid content (predominantly due to aggrecan) does not change with age. Thus, they propose that the molar amount of PG monomers may be increasing to make up for an age-related decrease in aggrecan monomer size and, that the increase in HA content functions to retain this increase in aggrecan monomers. Marudas *et al.* [36] found that the D/L$_{ASP}$ ratio of the intact aggrecan monomer (A1D1 fraction) of a 55 year old cartilage sample, calculates

to a turnover half-life of 3.4 years; a value that was relatively constant in cartilage from individuals between 20 and 80 years old. This half-life is more than 50x longer that newly-synthesized aggrecan within the PCM. However, the D/L$_{ASP}$ ratio of free G1 domains (within the A1D6 fraction G1 still bound to HA) isolated from the same 55 year old cartilage, calculated to a half-life of 23.5 years. This fraction (A1D6) represents HA within the inter-territorial ECM that still retains remnant G1 domains (and link protein) but no intact aggrecan monomers. In other words, the half-life of HA within the inter-territorial ECM, that HA remaining following the cleavage of aggrecan, has a half-life of 23.5 years. One explanation for the difference in HA and aggrecan half-lives within this ECM is that the complete depolymerization of HA requires the HA (and bound G1) to find its way back to the PCM where it can be internalized by CD44 or degraded by local hyaluronidases. Interestingly, G1-VTEGE accumulates in the IL-1β-treated explants of *Cd44*$^{-/-}$ mouse cartilage [57].

In the Holmes *et al.* study [77], the size of HA present within the normal femoral cartilage of 16 patients, of differing ages, was examined by extraction and S1000 chromatography. This pool of HA predominately represents HA within the inter-territorial ECM. The size of this HA decreased with age; children and young adults, HA ~1000 kDa; mid-life adult tissues, HA ~750 kDa and, the HA of older adults (>60) ~500 kDa. HA degradation or depolymerization is occurring but, likely represents one or two cleavages within a single HA chain of 1000 kDa throughout 8 decades of life. Again, this may highlight a slow turnover process for HA within the more distant inter-territorial ECM. In uninjured, control *cHyal2*$^{-/-}$ mice at 9 months, knee and femoral head cartilage HA displayed a polydisperse range of sizes by S1000 chromatography (>2130 kDa to <6 kDa) [46]. Compared to WT mouse cartilage, all size ranges of HA chains >285 kDa were enhanced in the *Hyal2* KO cartilage with a major peak occurring at 460 kDa. This suggests that HYAL2 is responsible for HA turnover but again requires that HA chains must translocate back to the PCM and secondly, that a HYAL2 makes partial cleavages of HA extracellularly at a neutral pH.

3.4. HA turnover associated with altered cartilage metabolism

To mimic human OA, many models incorporate pro-inflammatory cytokines. In a study by Sztrolovics *et al.* [40] adult bovine nasal septum cartilage and human articular cartilage explants treated with IL-1β together with retinoic acid, exhibited substantial release of HA from the cartilage including release of free G1 domains and link protein. A similar release of G1 domains, link protein and HA was obtained with bovine cartilage explants with IL-1α or IL-1β together with oncostatin M [41, 78] as well as porcine cartilage explants exposed to 10 ng/ml IL-1α [39]. The size of the HA released, especially in the presence of oncostatin M, was significantly reduced [40, 41]. Under these conditions, substantial HA loss from the cartilage is closely associated with the degradation and loss of aggrecan. Even more mild treatment of human articular cartilage explants (1 ng/ml IL-1α for 2 days in the presence of 10% fetal bovine serum) resulted in substantial loss of HA (shown by HABP staining and measured by FACE quantification of HA disaccharides) that was coincident within the same zone showing loss of safranin O staining (indicative of aggrecan depletion) and reduction in chondroitin sulfate as quantified by FACE analysis [6]. A more recent proteomic study measured the release of aggrecan G1, G2 and G3 peptides into the culture medium (detected

via multiple reaction monitoring mass spectrometry) of human cartilage explants following treatment by a single impact injury followed with or without addition of proinflammatory cytokines, IL-6 and TNFα [79]. In this model, the kinetics of peptide release showed that G3 peptides were detected before G2 peptides; whereas, G2 peptide release correlated with release of soluble glycosaminoglycan. Aggrecan G1 domains were released last approximately 9 days later than the G3 peptide. Moreover, unlike the G2 and G3 peptides, a substantial proportion of the G1 domains (~70%) remained within the cartilage explants in the most injurious condition (trauma plus cytokine).

The pronounced loss of aggrecan from articular cartilage is an early critical event associated with OA [80–83]). Fig. 3A depicts normal articular cartilage with rich, red staining for safranin O (aggrecan), counterstained with fast green for protein (primarily collagen). In human OA (Fig. 3B, 3D, 3E), depletion of safranin O staining is often observed as pronounced rarified regions or zones of articular cartilage that appear to progress from the fibrillated superficial surface to the middle and then deeper zones. This much loss of aggrecan (and likely HA as noted above) must include pronounced release from the inter-territorial ECM and must occur via a mechanism that is more pronounced that the steady-state turnover of HA and aggrecan discussed above. Could local chondrocyte-mediated cell surface degradation or endocytosis mechanisms (involving CD44, HYAL2, HYAL1, KIAA or TMEM2) be responsible for this prominent loss of HA from the ECM? In some specimens, pericellular staining for safranin O is still apparent surrounding chondrocytes at the bottom of the zone with depleted safranin O staining (Fig. 3E). If, like aggrecan, HA is also degraded first within the inter-territorial matrix, would a mechanism involving a release of neutral pH-acting hyaluronidase activity be required or alternatively, a massive wave of free radical activity? Is this due to the release of a free PH-20 [58] or shed CD44/HYAL2 complexes [47]? Such questions remain unanswered concerning the loss of cartilage HA in OA.

Perhaps the issue is that we only observe OA, even experimental OA, as snapshots in time. For example, the enhanced pericellular aggrecan observed in Fig 3D could represent not the next target for degradation but instead, an early repair response phase of these chondrocytes, and indicative of newly synthesized aggrecan. On rare occasion, the staining of human OA cartilage specimens captures the loss of safranin O as shown in Fig 3B. Safranin O loss still proceeds from the superficial to deep zones. However, now the rarefication of staining could be interpreted as occurring first in the PCM matrix surrounding the chondrocytes rather than within the more distant ECM. Another rare sample is that shown in Figure 3C, an example of human ankle cartilage from a young donor with no known medical history of arthritis, wherein the articular cartilage surface and overall integrity is intact but the safranin O staining is depleted, with depletion seeming to occur first within the PCM. Could this snapshot represent an episode of routine cartilage maintenance? or, possibly early OA? A model that includes the more local, chondrocyte-mediated degradation of HA and aggrecan would be consistent with these images. In sum, whether viewed as biochemical changes or as selectively stained images as shown in Fig 3B and 3E, we could be observing different stages of OA progression or, different subtypes of OA—subtypes that utilize different mechanisms for HA depolymerization.

4. Does HA play primarily a biomechanical role, a role in cell signaling or both in cartilage?

The conditional *Has2* KO clearly shows that HA plays a profound role in cartilage development and organization. We observed similar events in our CRISPR/Cas9 *Has2* deletion in rat chondrosarcoma chondrocytes. Aggrecan is not retained to the chondrocyte cell surface without HA and aggrecan does not accumulate within the inter-territorial extracellular matrix without HA. What are some of the pathways impacted by the engagement of HA with its receptors and its endocytosis?

We and others have sought to determine whether HA in chondrocytes plays a major role in the transfer of information into chondrocytes (signal transduction). It has been suggested that the reduced stiffness of the PCM and the presence of PCM HA generate a functional microenvironment for mechanotransduction [1, 16]. Such matrix-cell transduction would occur via transmembrane receptors such as CD44 or receptors such as RHAMM. Information transfer would be changes in the status or composition related to HA that occur within the pericellular matrix—changes such as loss or shortening of aggrecan, loss of HA or loss of other HA-bound matrix hyaladherins [84]. Information transfer would also register when HA/aggrecan/link protein steady-state was reached, resulting in reduced biosynthesis or reduced catabolism as required. Alternatively, changes in HA/aggrecan/link protein might even register as altered loading or shear and thus provide mechanotransduction capability either through CD44 or indirectly, by way of canonical mechanoreceptors [85–87].

Treatment with high dose of HA oligosaccharides has been used to initiate cell signaling in chondrocytes. Canonical HA – CD44 interactions have been likened to integrin interactions with the ECM [88]. These would involve on-off switch changes induced by receptor aggregation and disaggregation; both processes linked to interactions with the underlying cytoskeleton and various adapter proteins. Unlike other cells, chondrocytes do not display profound attachment potential on HA-coated substrata (unpublished observations). Instead, it was more productive to displace the prominent HA-rich pericellular matrix from chondrocytes than to displace the chondrocytes weakly adherent to a HA-substrata. Similar to the use of RGD peptides to probe integrin interactions, one approach to interfere with HA-CD44 interactions is by the application of small HA oligosaccharides—hexasaccharides to decasaccharides (HA_6-HA_{10}). HA di- and tetrasaccharides (HA_2 and HA_4) generated from those H_6-HA_{10} oligosaccharides had no capacity to displace matrices [38, 89, 90]. Larger HA oligosaccharides (HA_{12}-HA_{32}) were also effective in PCM displacement, but not used because of the potential of these oligosaccharides to interfere with non-link-stabilized aggrecan-HA interactions [91, 92]. HA oligosaccharides have the advantage of differentiating between HA anchored to the cell surface via retention to the HA synthase (non-displaceable HA) or anchorage via binding to a receptor (displaceable HA). As such, HA oligosaccharides could displace high molecular mass HA from a cluster of receptors such as CD44 and induce a signal by the unclustering event. CD44 clustering in COS-7 transfectants with the addition of HMW HA was detected by FRET; and this clustering was decreased upon subsequent addition of HA6 – HA20 that displaced the HMW HA and resulted in CD44 unclustering [93]. True physical clustering of CD44 in chondrocytes has

not yet been definitively shown but could be inferred by changes in CD44 association with the actin cytoskeleton. A lower proportion of the total CD44 present in the plasma membrane could be solubilized in mild detergents when the receptor was associated with high molecular mass HA [94, 95]. This could be reversed by treatment of the chondrocytes with HA oligosaccharides or *Streptomyces* hyaluronidase. Importantly, this cytoskeletal anchoring of CD44 provided for both outside-in and inside-out events. Pericellular HA and aggrecan were lost from chondrocytes treated with cytochalasin or latrunculin A [94]. Unclustering of CD44, and yielding more CD44 release in mild detergent (as shown by others in HMLE cells [96]) was also obtained by transfecting chondrocytes with a CD44-dominant negative (CD44Δ67; blocks binding of HA to CD44) [69] or transfection of chondrocytes with the cytoplasmic tail domain of CD44 (CD44-ICD, blocks the binding of CD44 to Ankyrin-3 and actin cytoskeleton) [95]. Wash-out of the HA oligosaccharides and the application of high molecular mass HA favored the restoration of putatively clustered CD44. In addition, bovine articular chondrocytes transfected with pCD44HΔ67 (blocking binding of HA) lost the capacity to retain a pericellular coat [69]. Conversely, bovine chondrocytes transiently transfected with pCD44-ICD (disrupting CD44-cytoskeletal binding) also did not exhibit pericellular coats [95].

A diversity of downstream events are activated during HA-mediated cell signaling in chondrocytes. The quiescent, steady-state of chondrocytes (non-activated) is represented by HA/aggrecan/link protein aggregates anchored to the plasma membrane and cells elaborating a pericellular coat. The best representation of this *in vitro* is to allow chondrocytes to recover from enzymatic isolation in alginate beads for 1 week [7]. Upon treatment with HA oligosaccharides or *Streptomyces* hyaluronidase, cell signaling events occur rapidly including activation of p-p38 MAPK, p-ERK1/2, NF-κB and, pAKT [89, 97, 98]. Downstream of these events is the upregulation of pro-catabolic *iNOS*, NO[99], gelatinase, collagenase and aggrecanase gene products including MMP-3,-9,-13, ADAMTS4, ADAMTS5 [89, 90, 97, 98] as well as an upregulation of *HAS2*, *COL2* and *COMP* [97, 98]. Several interesting observations of this work were that unlike IL-1α or IL-1β treatment of chondrocytes, HA oligosaccharide-treatment does not result in a strong enhancement of CD44 and does not activate an AP-1 (cJun, cFos) promoter construct [24, 89]. Inhibitors of NF-κB block MMP-3 and MMP-13 expression and activity with no effect on *HAS2*, while AKT inhibitors (wortmannin and LY294002) block *HAS2* expression (and HA production) but not MMP-3 or MMP-13 [89, 98]. Treatment of chondrocytes with testicular hyaluronidase elicited similar effects (increases in MMP-1, -3, -9 with no effect on TIMP1, -2) and an increase in CD44 [100]. One explanation for these results was the possibility of bifurcation or dual activation of signaling pathways. In some studies, the addition of exogenous HA has been shown to partially block activated pro-inflammatory pathways in chondrocytes [89, 97, 101, 102].

However, beginning in 2005, work by Paul Noble's group demonstrated that HA oligosaccharides (135 kDa) could activate macrophages via TLR2/TLR4/MyD88-dependent pathways [103]. Several years later, other groups [104–107] demonstrated that TLR4 was also present on chondrocytes and participated in HA oligosaccharide-mediated activation of pro-catabolic pathways—effects that could be blocked by the addition of high molecular mass HA. The upshot of these observations was two-fold. First, it can no longer be simply

concluded that HA-oligosaccharide-initiated signaling events were mediated solely via CD44. Although HA oligosaccharides do have the capacity to displace HA and HA-aggrecan-link protein complexes retained at the chondrocyte cell surface via CD44, even on non-living chondrocytes [19, 25] and alter the detergent-extractability of CD44 [95], other receptor pathways may be activated. For example, while treatment of CD44-negative COS7 cells with HA oligosaccharides generated a 7.5 fold increase in MMP-13-luciferase promoter activity, this response was 12.5-fold after pCD44 transfection of these cells, implicating more than one receptor [89]. Care must be also taken in interpretation of hyaluronidase treatment of chondrocytes—a condition that also generates HA fragments.

The second effect of these results was a heightened focus to identify the hyaluronidase activity responsible for HA degradation in chondrocytes. If all HA degradation is via the canonical pathway, (namely via CD44 endocytosis and complete depolymerization by HYAL2, HYAL1, and exoglycosidase activities in lysosomes), no subsequent extracellular alterations in TLR4 activity would be expected. However, if extracellular HYAL2 or PH-20 were active and pronounced or, TMEM2 or KIAA degradation recycled and released large pools of HA oligosaccharides (i.e., as DAMPS [108]), local extracellular catabolism of HA could serve as a driving force for continued TLR4-mediated or CD44-mediated pro-catabolic activation of chondrocytes. Certainly, the level of HA oligosaccharides generated by local cleavage events would not likely match exogenous, experimental doses of HA oligosaccharides. Nonetheless, some investigators suggest that since cartilage does not have drainage via lymphatics or blood vessels typical of other tissues, local degradation products and fragments are likely to accumulate and exert a local effect (as DAMPs) in the lacunar space [109, 110].

Whether endogenous or exogenous HA oligosaccharides activate innate immunity pathways in cartilage remains controversial. One ongoing question is whether the potential activation of TLR4 in chondrocytes is due to the HA oligosaccharides or endotoxin lipopolysaccharide (LPS) contamination of laboratory-grade HA oligosaccharide preparations or, contamination of the hyaluronidases used to degrade endogenous hyaluronan [111–113]. While this is clearly a concern in all HA-related studies and stresses the importance of reagent purity, there remain examples wherein pharmaceutical-grade HA oligosaccharides elicit signaling responses in chondrocytes. In our studies, HA oligosaccharides generated in our laboratory were tested side-by-side with pharmaceutical-grade oligosaccharides; we observed that both preparations induced a stimulation of chondrocyte iNOS and NO release [99]. Moreover, di- and tetrasaccharides generated from the same laboratory preparation of oligosaccharides (or pharmaceutical-grade) exhibited no stimulatory activity [89, 90, 99]. The other confounding question is whether human chondrocytes express functional TLRs that are sensitive to HA oligosaccharides or LPS. While Tlr2 and Tlr4 are clearly present and active in murine cartilage [104] controversy remains as to whether TLR4 protein is expressed by human chondrocytes. TLR2 but not TLR4 is observed in human chondrocytes by some investigators even after stimulation with IL-1β and fibronectin fragments [114, 115]. However, other groups report the expression of multiple TLRs (TLR1 through TLR9) in human chondrocytes [116] as well as a prominent role for TLR4 [109, 117, 118] including the cooperative binding between TLR4 and CD44 [117]. In summary, in defining mechanisms for direct HA-mediated signaling in chondrocytes, including the effects of HA of differing

sizes, potential LPS contamination of reagents and, that signaling events may be mediated by a variety of receptors, remain important considerations.

Besides direct HA-mediated signaling events due to unclustering of CD44, other indirect signaling events may also be driven by changes in HA – CD44 interactions in chondrocytes. In many cell systems, CD44 acts as a co-receptor with other signaling receptors such as TGFβ type I and type II receptors in kidney, breast and retinal epithelial cells [119–121]. CD44 can serve as a docking protein or co-receptor to other classical signaling receptors such as c-Met [122], ErbB or other EGF-R [123–125], TGFβR1 [120], and the MIF-CD74 receptor complex [126]. We have validated the interaction of CD44 with SMAD1, crucial to the canonical BMP/BMP-R/SMAD1 signaling pathway [42]. Nuclear translocation of SMAD1/SMAD4 upon BMP7 stimulation (but not TGFβ-induced SMAD2/SMAD4 translocation [127]) was reduced in *Streptomyces* hyaluronidase-treated chondrocytes, but restored with HA add-back [128]. We speculate that the involvement of CD44 with these receptors and pathways is as a chaperone function. CD44 can become palmitate acylated at cysteine residues and translocate into lipid rafts [66]. This appears to be a precursor stage for CD44 endocytosis as well as the endocytosis of HA, bound G1 domains [55–57, 66, 129, 130] and possibly various bound receptors/cofactors. For example, CD44 was shown to co-immunoprecipitate with TGFBR2 during TNFα-induced EMT events associated with retinal pigmented epithelial cells and leading to proliferative vitreoretinopathy [119]. The authors speculate that the association with CD44 promotes clathrin-dependent internalization of clustered TGFβ receptors into endosomal compartments enriched with the Smad2 anchor SARA necessary for optimal TGFβ signaling. Moreover, the injection of TNFα into the subretinal region of the eye of *Cd44*$^{-/-}$ mice failed to affect changes in epithelial cell morphology (switch to fibroblast) or loss of N-cadherin [119]. Such CD44 chaperone partnering events may occur in chondrocytes with various receptor kinases. The difference in chondrocytes however, is that CD44 endocytosis or movement into lipid rafts is more restricted due to the composition of the ECM. We have shown that HA decorated with intact aggrecan monomers cannot be internalized whereas decoration with partially-degraded aggrecan monomers (C-terminal shortening) is permissive [32]. In such fashion, changes in the composition of the HA-rich pericellular matrix may provide for signal transduction via indirect association of CD44 with other receptors/cofactors.

5. Is an abundance of pericellular HA good or bad for the physiology of chondrocytes?

Given that HA is required for the retention of aggrecan to the plasma membrane of chondrocytes, it might be assumed that enhancing this capacity would be beneficial for cartilage function. Replacement of lost cartilage HA has been an ultimate goal and challenge for intraarticular injection of pharmaceutical HA therapies. Unfortunately, the available literature is unclear on this hypothesis. Exposure of cartilage explants to BMP7 results in a substantial upregulation of HA deposition within the ECM and PCM of the tissue [7] and in another study, BMP7 provided protection from HA oligosaccharide-induced cartilage degradation [131]. As noted above, *Hyal1*$^{-/-}$ mice exhibit pronounced pericellular and intracellular HA accumulation in chondrocytes [53, 132]. However, these same mice exhibit

signs of early OA-like degeneration including loss of cartilage proteoglycan. Human patients with HYAL1 deficiency (Mucopolysaccharidosis IX) also exhibit joint abnormalities [54]. The conditional knock-down of *Hyal2* in articular cartilage also results in enhanced accumulation of high molecular mass HA within lacunae, formation of hypertrophic-like lacunar spaces but also, an aggravation of OA-like symptoms in age-matched or medial meniscus-destabilized mice [46]. The exact mechanism for the sensitivity of these mice to the development of OA-like changes remains unknown. One might argue that mechanistic differences exist between enhancing pericellular HA via increased biosynthesis versus the knock-down of hyaluronidases. For example, HAS2 utilization of UDP-sugars for HA production serves as a metabolic rheostat, responding to (and possibly altering) the metabolic balance within cells [2]. However, no joint defects or aging-related OA-like changes have been noted in naked mole rats—the longest living rodent known with a life span greater than 30 years [133]. These rodents exhibit very pronounced deposition of HA in all tissues and, due to two Asn→Ser point mutations in *Has2*, the naked mole rat HA is 5x the molecular mass of HA present in mice (600–12,000 kDa as compared to 500–3000 kDa in mice). Two asparagines that are typically 100% conserved among mammals are replaced with serines in the naked mole-rat *Has2* gene [133]. The mole rat sequence contains two substitutions (N178S and N301S) within the cytoplasmic loop active site that are not present in other known *Has2 or HAS2* coding sequences. Overexpression of the cDNA for this naked mole-rat *Has2* in human HEK293 cells resulted in the cells secreting a high molecular mass HA similar to the one produced by mole rat cells. However, the increased HA in the naked mole rat is not due solely to changes in biosynthesis. Overall hyaluronidase activity is also reduced in all tissues of the naked mole rat—a deficit that could be reversed by overexpression of *Hyal2* [133].

Summary and conclusions

In summary, HA in association with the proteoglycan aggrecan provides for appropriate cell-cell spacing during chondrocyte development and maturation. Cartilage biomechanics requires the presence of abundant aggrecan and HA provides the principal means of retaining aggrecan within the inter-territorial matrix of cartilage as well as the retention of aggrecan to the chondrocyte cell surface. HA and aggrecan are continuously synthesized in cartilage and thus, necessitate mechanisms for their continual turnover. Multiple mechanisms likely participate in this turnover including local, receptor-mediated endocytosis of HA (and residual G1 domains of aggrecan) by chondrocytes. It is likely that in disease states such as OA, limiting the degradation of aggrecan will secondarily reduce HA turnover as well as cell signaling events that further promote and exacerbate matrix degradation.

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Abbreviations used

HA	hyaluronan
ECM	extracellular matrix
PCM	pericellular matrix
OA	osteoarthritis
HAS	hyaluronan synthase
Fl-HA	fluorescein-labeled hyaluronan

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Highlights

- Hyaluronan is synthesized in cartilage by hyaluronan synthase-2 (HAS2).
- Hyaluronan and aggrecan contribute to cell-cell spacing in cartilage.
- Hyaluronan functions to anchor aggrecan to the surface of chondrocytes.
- Hyaluronan turnover involves chondrocyte-mediated endocytosis.
- Turnover of hyaluronan may differ in the pericellular and inter-territorial extracellular matrices and during disease or steady state.

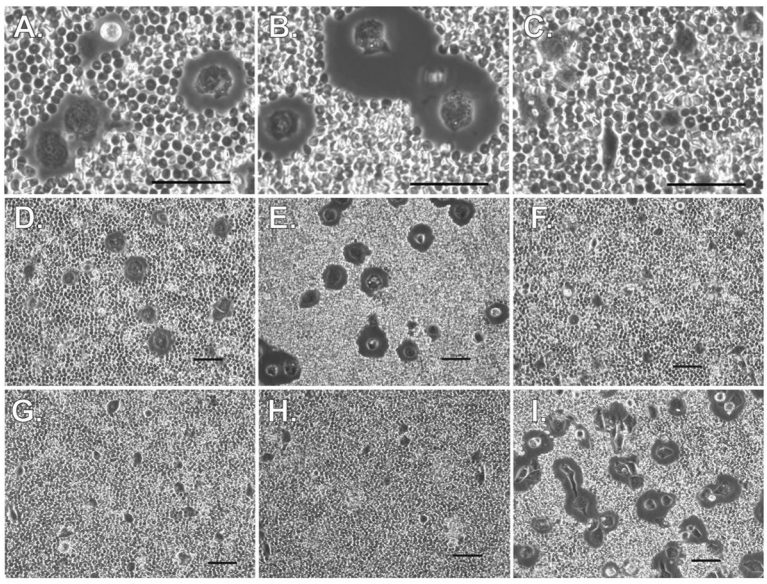

Fig. 1. Exogenous aggrecan enhances the hyaluronan-dependent chondrocyte pericellular matrix

(A, D) Bovine articular chondrocytes readily assemble pericellular coats after 2 days in monolayer culture, as revealed using the particle exclusion assay [18]. (B, E) The addition of 2 mg/ml purified aggrecan (bovine articular cartilage D1 fraction) to chondrocytes with an existing coat expands the coat within 90 minutes. (G) *Streptomyces* hyaluronidase treatment (10 units/ml medium + FBS, 60 minutes, 37 °C) removes the endogenous coats on bovine chondrocytes. (H) No expansion by added aggrecan is observed when cells are pretreated with *Streptomyces* hyaluronidase. (C, F) After coats are enhanced with exogenous aggrecan, these pre-established coats were dissociated via post-treatment with *Streptomyces* hyaluronidase (10 units/ml medium, with gentle shaking at 30 minutes). (I) Pre-enhanced coats with exogenous aggrecan persist with gentle shaking in the absence of hyaluronidase. Bars = 50 μm in each panel.

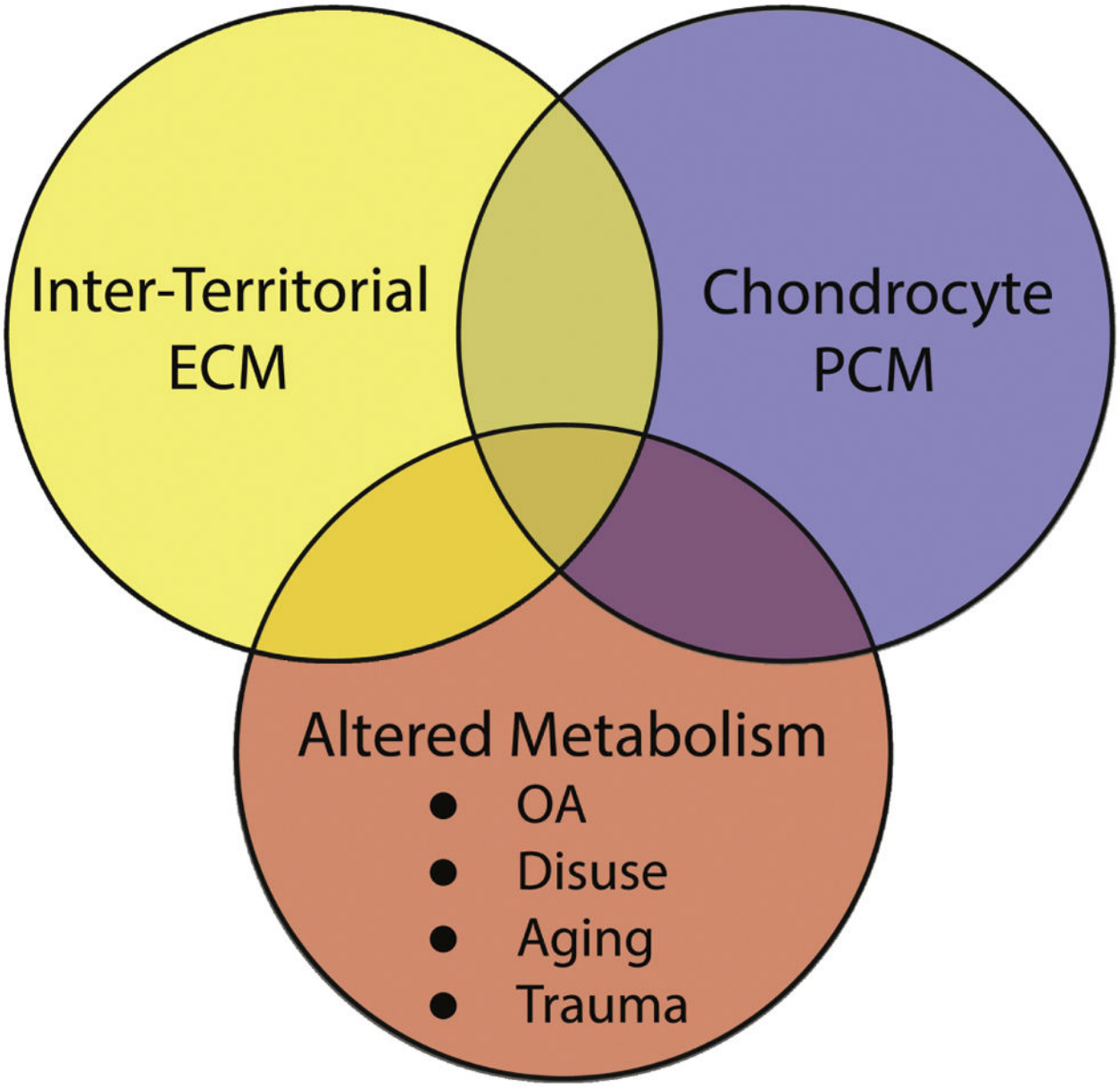

Fig. 2. Hyaluronan turnover in cartilage differs with metabolic state and regionally within the matrix

One helpful approach to consider in the matter of hyaluronan turnover is which pool of hyaluronan (and aggrecan) is being considered as well as chondrocyte metabolism. Hyaluronan turnover within the pericellular matrix (PCM) differs, for the most part, from hyaluronan turnover within the inter-territorial extracellular matrix (ECM). Secondly, hyaluronan turnover in steady-state and hyaluronan turnover associated with altered cartilage metabolism [aging, osteoarthritis (OA), disuse, or trauma] also differ. With these considerations there are likely overlapping mechanisms as well as circumstances in which there is no overlap.

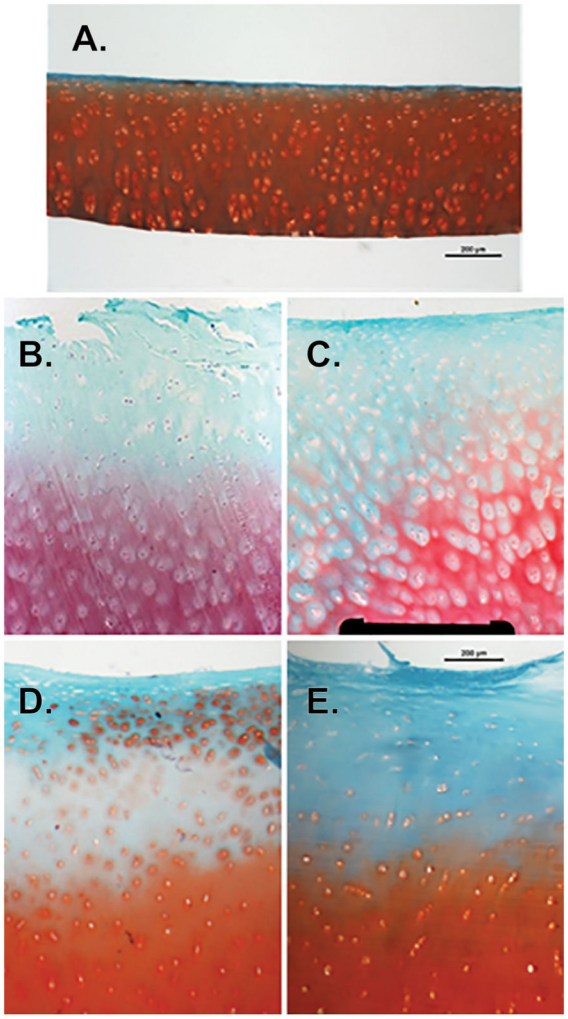

Fig. 3. Loss of aggrecan is variable in human osteoarthritic cartilage tissues

Histological images of articular cartilage stained with safranin O/fast green are shown. (A) Normal bovine articular cartilage. (B) Human osteoarthritic cartilage with fibrillated articular surface and rarefication of staining of the pericellular matrix throughout all zones. (C) Human ankle cartilage, from a 39 year old female donor with no known medical history of arthritis, with an intact articular surface but depletion of pericellular safranin O staining throughout all zones. (D) Human osteoarthritic cartilage showing the loss of safranin O straining from the inter-territorial matrix from the surface to the middle zone, but enhanced pericellular safranin O staining throughout the middle zones. (E) Human osteoarthritic cartilage with the loss of safranin O staining progressing as a wave from the articular surface but persistent pericellular safranin O staining surrounding chondrocytes at the bottom of the safranin O depleted zone.